WHEAT

Physiological Breeding I: Interdisciplinary Approaches to Improve Crop Adaptation

Matthew Reynolds, Alistair Pask
and Debra Mullan (Eds.)

CIMMYT MR
International Maize and Wheat Improvement Center

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Foreword

"Know where to find the information and how to use it - that's the secret of success" - **Albert Einstein**

Most introductions addressing cereal breeding begin with the Green Revolution. This one is no exception. While this Foreword is certainly not an attempt to write the history of crop physiology and breeding for abiotic stress or give credits, a few landmarks should be noticed on the long road arriving at this important publication. Since the new generation of researchers is not in the habit of reading anything older than three years, I am obliged to provide a brief perspective, having age to my advantage. Looking back at the road will point you to the way ahead.

The Green Revolution which consisted of a large increase in cereal grain yield took place in wheat and rice towards the mid-Twentieth Century. It was driven by wheat and rice breeders who sought to reduce plant height in order to reduce lodging and thus also allow for increased nitrogen fertilization. The "Green Revolution" in sorghum (which was not defined as such when it took place) was also driven by a reduction in plant height and took place several decades earlier. It was not directed at increasing yield but rather towards achieving a "combine height" dwarf sorghum that could be harvested mechanically. The bonus of these modifications was an increase in grain yield potential.

Breeding and physiology

The Green Revolution in cereals promoted optimism about the capacity of plant breeding to continue increasing yield and it drove plant physiologists to understand the physiological basis of yield and its improvement. As yield is attained by a plant population grown in the field, we have both crop physiology and yield physiology. An additional impetus for crop physiology research at that time was driven by the fascination with and the need to understand heterosis in maize and sorghum.

The great development in crop and yield physiology in the 1960s and 1970s was landmarked by books and monographs published by research groups spanning from the US, the UK, the Netherlands, Russia, India and Australia. My personal favorite during my student years was 'The Growth of Cereals and Grasses' (Milthorpe and Ivins, 1965) which was produced by the Easter School of Agricultural Science at the University of Nottingham, UK. This publication opened the door to cereal crop physiology as we know it today.

Funding for such research followed suit with the purpose of continuing the increase in genetic yield potential of cereals while stabilizing the achievement through genetic resistance to abiotic stress. This was initially most pronounced in Australia for wheat and in Nebraska and Texas for sorghum, not withstanding other cases. Lloyd T. Evans, who was Chief of the Division of Plant Industry, CSIRO, Canberra in 1971–1978 represents very well the support of crop physiology research towards wheat improvement by the many outstanding wheat scientists working there at the time (Evans, 1975).

The support by The Rockefeller Foundation of an integrated sorghum physiology and breeding research group at the University of Nebraska in the very early 1970s was probably among the first real and significant contribution of crop physiology to sorghum and maize breeding in the US. At about the same time, crop physiology and breeding research at Texas A&M University discovered the genetic and physiological basis to photoperiod and temperature effect on flowering in sorghum. It followed earlier work of J.R. Quinby who laid the basis for sorghum genetics and sorghum hybrids. This research opened the way for their sorghum conversion program at Lubbock, Texas. That program converted tropical sorghum from Africa and Asia into temperate types which were then used in hybrid sorghum breeding to achieve a higher level of yield and grain quality. These materials also provided genes for osmotic adjustment and non-senescence as major mechanisms of drought resistance which were later incorporated into hybrid sorghum on a global scale.

The integration of crop physiology into plant breeding was then adopted in the breeding programs of most Consultative Group on International Agricultural Research (CGIAR) institutions, earlier or later as the case was. The 'Sorghum in the Seventies' Conference in Hyderabad India, which just preceded the establishment of

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in 1972, had crop physiology and plant stress issues as an important part of the program and discussions towards formulating ICRISAT was the mandate. Teams of breeders and physiologists became commonplace in addition to the phenomenon of physiologists working as breeders and breeders occupied with physiology.

The physiological basis of the Green Revolution in the cereals was identified very early as an increase in harvest index from around 20–30% to about 40–50%, depending on the crop and the case. The yield components involved in this increase were also identified, with grain number per inflorescence as the primary one. Unfortunately, this led to failing attempts in direct breeding for increased kernel number, such as breeding uniculm wheat with a gigas ear or multiple spikelets in sorghum. Crop physiology then led breeders to understand that yield formation in cereals is derived from an intricate balance between yield components' development, source to sink communication, crop assimilation and assimilate transport – linked to crop phenology and plant architecture.

In the course of these developments, and as reflected in several chapters of this book, crop physiologists developed the concept of use-efficiency regarding how the plant uses its essential resources such as irradiance, water, or nutrients. Thus we have radiation use efficiency (RUE), water use efficiency (WUE), nitrogen use efficiency (NUE) or phosphorus use efficiency (PUE). It is therefore often assumed that breeding for increased efficiency can lead to greater productivity. Efficiency is an important component of any production system. However, when crops are considered, one should always remember that greater efficiency is a ratio which can be increased by either greater production for a given input or by reduced input for the same production.

This book addresses very well the current serious challenge facing agricultural research where crop improvement is required to address two hard tasks: (1) a greater rate of increase in yield potential as compared with present rates, and (2) the support of this increase by developing abiotic and biotic stress resistance. Furthermore, these challenges are to be met on the background of another "Green Revolution", namely fewer inputs and reduced chemical use.

We now understand that where breeding for higher yield potential is concerned, crop architecture, harvest index, phenology, and development within the bounds of a given season and crop management system, have all been optimized, or nearly so in modern cereal production systems. Consequently any serious improvement in cereal yield potential beyond the common average present crawl of 0.5–1.0% per annum must come from a genetic–physiological intervention in photosystem biochemistry and function. This is where molecular plant biology might finally achieve its glory in plant breeding. We are already aware of ongoing, exciting research exploring the way to modify C3 plants such as rice into a C4 metabolism. Another example is the "20:20" project announced for wheat by Rothamsted Research in the UK in 2011 where a yield target of 20 t ha^{-1} to be achieved in 20 years was set. Such ambitious projects might be driven mainly by innovations in molecular biology using advanced genomics methods. However, past experience indicates that sometimes such projects can drift into the pure molecular and genomic domain, losing their original goal to impact food production. If such formidable projects are to be seriously directed at delivery, I would audaciously suggest that they be led by breeders and crop physiologists who can navigate the project towards the designated port rather than get lost on an island, even a beautiful one.

Stress, drought and heat resistance

We recognize cereal landraces as genetic sources for abiotic stress resistance and we are using them for this purpose. These are the simple products of farmers who repeatedly selected seed that survived historical drought years in their fields. No science was involved, only a very long time and a determination to provide for their own livelihood. These landraces attend to the fact that abiotic stress resistance has been here for a very long time. We are now only trying to improve it more effectively.

The first few breeders of the scientific agriculture era who tried to address drought resistance were for example Robert Gaus from Colorado with wheat and M.T. Jenkins from Iowa with maize, both working during the early-Twentieth Century. One of the first dissections of drought resistance in terms of crop physiology was made by J.H. Martin (1930) from the Office of Cereal Crops and Diseases, USDA, Washington D.C.

These and other breeders at that time had very little physiology in support of their work. Plant abiotic stress was commonly regarded simplistically in wheat breeding programs as an assembly of non-descript problems which reduce yield in certain environments and years. It was phenotyped as yield reduction in certain years or locations. L.P. Reitz who was the senior author of the wheat breeding "bible" at the time (Reitz and Quisenberry, 1967) stated with a sense of acceptance that "Breeders worship the yield column in their field-books". The weighing balance was the most important phenotyping tool. This is not to discount the fact that productive and stress resistant cereal cultivars were still developed.

During the early-Twentieth Century and onward many field crop breeders adopted the hypothesis that high yield potential is the solution for sustained yield also in all stress environments. Genotype × environment interaction was regarded as a nuisance. As the cause for environmental variation in breeding materials was nondescript, quantitative genetics of yield and statistical analysis of field experiments over years and locations became the most important tools in breeding for dryland crops. The increase in number of field tests over locations and years in order to identify the high yielding genotype that will perform best in all environments became a very demanding activity in dryland crop breeding. It is therefore well understood why plant breeding was often described as a "numbers game". Quantity somehow turned into quality – at a great cost. It was only later that plant breeders began enquiring about the possible reasons for genotype by environment interaction and together with physiologists sought for solutions to ameliorate the genotype with respect to a specific problem environment. Kenneth J. Frey from Iowa State University who worked mainly with oats was very influential in proving that stress environments may require specific cultivars and that one cultivar for all seasons is a rarity. I believe that his work and publications brought about a paradigm shift in plant breeding at that time. We now accept that high yield potential has a positive impact on yield under stress – but to a limit.

Probably the first realistic attempt at a formal application of plant physiology to breeding for drought resistance was published by Ashton (1948), where various methods, some of which are unrealistic today, were detailed. The quantum leap in addressing abiotic stress in physiology and breeding came when Jacob Levitt published his first book (Levitt, 1972) where he compiled the available science on the subject and suggested the first logical definitions of, and the available methods for, measuring stress and stress resistance in plants. Many additional books and reviews followed. The expanding research on plant abiotic stress and resistance included important landmark conferences such as the one organized by the Boyce Thompson Institute in 1977 (Mussell and Staples, 1979) which brought together the international expertise on the subject at the time. Healthy debates were carried out on drought resistance and its improvement during that period. The value of osmotic adjustment in drought resistance is remembered as a notable one, especially after its initial discovery towards wheat breeding. This book indicates that we are now approaching a wide consensus about breeding for abiotic stress resistance in wheat and other crops.

Final word

This book has grown and matured after the previous one which was also produced under the auspices of CIMMYT (Reynolds *et al.,* 2001). Despite the huge progress seen now in this new text, the previous publication is still very relevant to wheat breeders and should be kept on the shelf.

In my own book on this subject (Blum, 2011) I pointed out that many breeders expressed serious loss at how to integrate drought resistance breeding into their program. Most were not certain about the desirable ideotype for their target environment, the protocols for drought phenotyping and the selection methods to use. In short, many felt unqualified to deal with breeding for drought resistance under water-limited environments. It is also evident that despite the huge advance in plant genomics and molecular marker technology, most breeders still work with the whole plant and mostly in the field. This book is therefore an extremely valuable contribution towards contemporary wheat breeding and very likely other cereals.

At the same time this book is also valuable for molecular biologists who sometimes stumble upon incorrect methods of phenotyping stress resistance. This text should clarify the correct approach to testing modified genotypes through the pot and into the field. This book is therefore a manual for all who work towards crop improvement.

When physiological methods are adapted towards plant breeding it is important to understand that sometimes they can be regarded by physiologists as imperfect. For example, use of the pressure chamber to assess leaf water potential can be biased by the rate of osmotic adjustment in the specific leaf sample. Most users of the method in the field do not regard this point. Canopy temperature as an estimate of plant water status can be biased by canopy architecture when different genotypes are compared. We rarely take this into account. However, the critical physiologist should recognize that the outmost consideration in selection work within a large plant population is the ease and speed of the protocol – in addition to accuracy. The breeder is mainly interested in reducing their population towards the desirable genotype at a reasonable probability and cost, even if the method is viewed as imperfect by the perfect physiologist. Thus, for example, while genotypic variations in wheat canopy temperature as measured by the infrared thermometer due to canopy architecture might be around 1°C, the variation due to drought stress at midday can reach 5°C and up.

Finally, the importance of this publication is not only in the detailed explanation of the essential physiology and methodology towards wheat breeding but in that it links the physiology to a possible ideotype and then connects with the methods required for its selection. This is at the heart of the breeder's dilemma in approaching plant breeding for specific environments.

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Preface

This two-part manual on **Physiological Breeding** has been developed in response to demand from colleagues worldwide in fields of crop research ranging from practical breeding to gene discovery. The common thread is the need for reliable phenotyping methods, which can be applied in the following areas:

- Characterizing potential parents to permit more strategic crossing.
- Screening early-generation progeny to enrich populations for desirable alleles.
- Exploring genetic resources for valuable physiological traits to expand the gene pools commonly used in crop breeding.
- Designing and phenotyping large experimental populations to facilitate gene discovery.
- Implementing experimental control in mechanistic studies (e.g., for –omics platforms).
- Designing phenomics platforms.

These volumes –**Physiological Breeding I and II**– have been compiled with such outputs in mind and to provide practical information for breeders and other crop researchers seeking to apply tried and tested phenotyping approaches in their own programs. The manuals set out to describe criteria for choice of phenotyping methods in the context of the environmental factors to which crops must adapt, and the most appropriate tools available. They build on knowledge and methods presented in the earlier CIMMYT book, **Application of Physiology in Wheat Breeding**.

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Introduction

Introduction

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Background to phenotyping approaches

Plant improvement has relied heavily on modifying the phenotype of crops and will continue to do so, at least until a much more comprehensive understanding is achieved of the genetic basis of adaptation among elite cultivars. A very successful intervention has been to modify phenological patterns of crops to avoid stress (Ludlow and Muchow, 1990). Another is to minimize the occurrence of stress through the development of a good root system that permits water to be accessed deeper in the soil when drought occurs (e.g., Lopes and Reynolds, 2010) and allows transpiration rates that better match evaporative demand under high temperatures (Amani *et al.,* 1996). In environments where 'extra' water is not available, stress-adaptive strategies include a range of traits that reduce radiation load—wax, pigment composition, leaf angle and rolling—while increased transpiration efficiency permits available water to be used more effectively (Richards, 2006). Physiological breeding has been showing increasing impact in Australia (Richards, 2006; Rebetzke *et al.,* 2009) as well as in CIMMYT's maize and wheat breeding programs. For example, selecting for reduced anthesis-silking interval in tropical maize has significantly boosted yields under drought (Bänziger, 2006). In wheat, a new generation of drought adapted lines developed by combining stress adaptive traits have been released as part of CIMMYT's 27th Semi-Arid Wheat Screening Nursery in 2010. The use of efficient screens has allowed elite genetic resources to be identified in large collections of landraces, for use in strategic crossing (Reynolds *et al.,* 2009). Fine-tuning of phenotyping approaches has also facilitated gene discovery, firstly through developing experimental populations in which phenology is controlled, as well as through implementation of rapid screens (e.g., measuring canopy temperature) that permit precision phenotyping of large numbers of genotypes within a time frame that does not confound measurement with environmental fluxes (Pinto *et al.,* 2010).

Choice of phenotyping protocol

The choice of the phenotyping protocol employed depends on three main interactive factors: target environment (and hence specific adaptive traits), scale of operation, and the degree of precision with which traits need to be estimated (Figure 1). There will always be trade-offs between these factors. For example, target traits requiring a lot of resources to measure cannot easily be applied on a large scale, whereas high-throughput approaches may be less precise, either in terms of accuracy or the fact that they result from expression of multiple alleles.

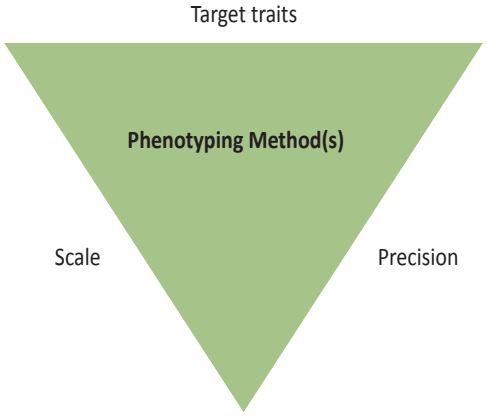

Figure 1. Factors determining the choice of phenotyping method: (1) the target environment and hence the requisite adaptive traits; (2) the quantity of genotypes to screen (may vary from dozens to tens of thousands); and (3) the required degree of precision.

Physiological Breeding

This first book lays out the theoretical basis for phenotyping as well as briefly addressing molecular breeding and crop management —two supplementary issues with implications for phenotyping applications and expression and impact of physiological traits, respectively—. Chapters are briefly outlined below under their respective section subheadings:

Section 1: Improving yield and other target traits

Improving the genetic potential of crops depends on introducing the right adaptive traits into broadly-adapted, high-yielding agronomic backgrounds. **Mega-environment breeding** (Braun and Payne) establishes the global context for such efforts. Both national and international programs recognize that breeding is more efficient if aimed at specific target zones, typically defined by water availability, ambient temperature, latitude, cropping system and biotic stress factors.[1]

Application of crop physiology in breeding for heat and drought stress (Reynolds *et al.*) addresses selected generic target traits that help crops adapt to heat and drought stress, the world's two most important abiotic stresses, and provides case studies of successful applications of physiological approaches. Breeding for **Nitrogen and phosphorous use efficiency** (Ortiz Monasterio) is becoming ever more important as the cost and availability of nutrients limit productivity in developing countries, while their inappropriate use in some cropping systems causes environmental damage. **Opportunities to improve genetic wheat yield potential** (Reynolds *et al.*) has implications for both of the preceding topics, in that genetic yield potential is associated with increased nutrient use efficiency and adaptation to abiotic stress. Improving yield potential is also important in its own right, as 70% of wheat produced in the developing world is cultivated in relatively favorable environments (Reynolds *et al.,* 2011). To achieve continued genetic progress in any environment, **Searching genetic resources for useful variation in physiological traits** (Payne *et al.*) will be necessary and the strategies available are described along with examples of successful applications.

Section 2: Phenotyping

This section describes the theoretical basis for applying diverse phenotyping tools. **Canopy temperature and water relations traits** (Cossani *et al.*) address some of the tools applied most successfully in breeding wheat for stress adaptation in Australia and by CIMMYT (Rebetzke *et al.,* 2009; Reynolds *et al.,* 2009). When measured in the right context, stomatal aperature-related traits (such as canopy temperature, stomatal conductance, and carbon isotope discrimination) can allow an efficient estimate of carbon fixation rate, making them proxies for the direct measurement of photosynthesis. So for example, under water limited conditions these traits provide estimates of rooting capacity or transpiration efficiency, while under favorable environments they are associated with limitations to yield potential such as radiation use efficiency or sink strength. **Spectral radiometry** (Mullan) is the emerging technology with probably the greatest potential for high-throughput application because it encompasses so many growth related traits, including indices for estimating crop yield, biomass, hydration status, N status, canopy temperature, photosynthetic capacity, and a range of pigments associated with photosynthesis (Babar *et al.,* 2006; Gutierrez-Rodriguez *et al.,* 2010).

Gas exchange and chlorophyll fluorescence (Lopes *et al.*) are direct approaches for measuring photosynthesis; the latter has potential for high-throughput application, while the former has the benefit of precision. Both can be applied to estimate photosynthetic rate of individual plants and specific plant organs, permitting, for example, the contribution of spikes to canopy photosynthesis to be estimated. **Strategies to identify genetic diversity in root traits** (Herrera *et al.*) considers the role that roots may play in determining crop productivity, an area that traditionally has not been well explored. The focus is on genotypic variation in root traits for increasing drought adaptation and nutrient uptake. Both established and emerging methods of root screening are considered and how they may be applied in high-throughput approaches. The chapter **Wheat development: its role in phenotyping and improving crop adaptation** (Slafer) not only gives a comprehensive account of how to distinguish the key developmental stages of wheat—crucial for correct

[1] **Biotic stresses fall outside of the scope of this manual.**

interpretation of the expression of most physiological traits—but also explains the theoretical basis for modifying wheat development to improve adaptation and yield potential.

Phenotyping in controlled environments (Saint Pierre) is often a controversial subject because of doubts about the extent to which results can be extrapolated to field conditions; this chapter attempts to provide a guide to the "pros and cons" of working in controlled versus field environments. Finally, **Field experimental designs in agricultural crops** (Crossa) offers guidelines on statistically-efficient designs that increase the power of resolution between treatments.

Section 3: Molecular markers and their application

Many physiological traits are extremely challenging or resource-use intensive to phenotype, good examples being root characteristics or spike photosynthesis. **Marker systems used in breeding** (Dreisigacker) and **Marker assisted selection** (Bonnett) explain options for developing and applying molecular markers with the long term view of complementing physiological breeding with molecular tools.

Section 4: Providing a basis for the development of sustainable cropping systems

Costly investments in conventional, physiological, or molecular breeding will produce the best results in fields that are optimally managed, allowing the full genetic potential of a genotype to be expressed. The need for adequate water and nutrients and to control biotic stresses is self evident. However, one of the most reliable and input-use-efficient ways of achieving that is through applying **The principles of conservation agriculture** (Sayre and Govaerts), outlined in the final chapter.

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WHEAT

Improving yield and other target traits

Chapter 1: Mega-Environment breeding

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Abstract

Plant breeding, using the combined potential of conventional, physiological, molecular and genetically modified technologies will provide cultivars with greatly enhanced nutrient and water use efficiency, enhanced tolerance to heat and drought, resistance to diseases and appropriate end-use and nutritional quality and possibly most importantly, greater ability to cope with the increasing extremes in temperature and precipitation occurring at one location over years. Modern crop cultivars developed by seed companies, international crop research centres, and national breeding programs often exhibit very wide geographical adaptation, as well as broad adaptation to the range of environmental and management conditions that occur within and between a target population of environments, or mega-environments. To identify such cultivars, multi-location testing remains the most efficient system. International evaluation networks based on exchange of and free access to germplasm and multi-location testing are therefore a cornerstone in the strategies and efforts to develop wheat germplasm that is adapted to the increasingly variable growing conditions encountered due to global climate change. Information from such trials must be combined with information from managed-stress trials. Wide performance adaptation is essential to respond to global climate change, to the vagaries of spatial heterogeneity within farmers' fields and their production input management efficacies, and from unpredictable temporal climatic seasonal variability.

Introduction

More than one billion people have insufficient food to sustain life, and food supply will need to double by 2050 to meet this demand. Agricultural genetics is one of the components of the solution to meet this challenge (Nature Genetics, 2009). The most serious challenges that economies and societies will face over the next decades include providing food and the water needed for food production, to a world that will see its population increase by a third in the face of mounting environmental stresses, worsened by the consequences of global climate change.

The challenge of increasing food production in the face of climate change will be greatest for the production of the staple grain crops that form the basis of diets the world over. Wheat, maize and rice are the three major staples, together covering 40% of the global crop land of 1.4 billion ha (FAO-STAT, 2009). Together they provide 37% of all protein, and 44% of all calories for human consumption (Table 1.1). Each crop provides more than 50% of the daily caloric uptake in regions with high consumption, e.g., North Africa and Central Asia for wheat, Sub-Saharan African countries and Meso-American countries for maize, and southern and eastern Asian countries for rice, and especially among the poorest people in these regions. Wheat is, with 220 million ha, the most widely grown crop. Global average yield of wheat is 3 t ha^{-1} and more than 100 countries produce more than 10,000 t. Wheat shows the widest geographical distribution and it is grown from the equator to 67°N in Scandinavia to 45°S in Argentina, Chile and New Zealand (Trethowan *et al.*, 2005).

Plant breeding, using the combined potential of conventional, molecular and genetically modified technologies will provide cultivars with greatly enhanced nutrient and water use efficiency, enhanced tolerance to heat and drought, resistance to diseases and appropriate end-use and nutritional quality and possibly most importantly, increased ability to cope with unpredictable extremes in temperature and

Table 1.1. Percentage of calories and protein in the human diet obtained from wheat, maize and rice globally and in the developing world. Source: FAO STAT, 2009.

Region	Calories (%)	Protein (%)
Wheat - World	19	20
Wheat - World Developing Countries	17	19
Maize - World	5	4
Maize - World Developing Countries	6	5
Rice - World	20	13
Rice - World Developing Countries	25	18
Total from wheat, rice and maize - World	37	44
Total from wheat, rice and maize - World Developing Countries	48	42

precipitation across regions and over years. The wide range of environments in which wheat is grown indicates that the genetic variability exists to cope with the large and rapid climate shifts we are facing, but more integrated and collaborative approaches to crop variety evaluation and the exchange of seed and information will be required to avoid rapid declines in production in severely-affected regions.

Multi-environment trials: tools for assessing crop adaptation

Modern wheat cultivars often exhibit very wide geographical adaptation, as well as broad adaptation to a range of environmental and management conditions. Mega-varieties have existed since wheat breeding started. Kharkov and Kubanka occupied one third of the USA wheat area after introduction in the early 1900s. Cultivars that spearheaded the Green Revolution like Siete Cerros (also named Mexipak and Kalyansona) were grown on millions of hectares from North Africa to South Asia. Selections from the International Maize and Wheat Improvement Center (CIMMYT) cross Veery were released in more than 40 countries (Skovmand *et al.,* 1997). The Russian winter wheat Bezostaya dominated in Eastern Europe and West Asia. This breadth of adaptation has been achieved in different ways by different breeding programs, but the most important tool has been the extensive field testing of experimental breeding lines in many environments during the selection process.

Compared to maize and rice, the wider natural adaptation of wheat can be attributed to the combination of multiple alleles of photoperiod and chilling (or "vernalization") sensitive genes that determine the crop's agro-ecological productivity from high latitudes to equatorial highlands. Spring wheats developed by CIMMYT, and its predecessor organizations, that made impacts since the Green Revolution, were photoperiod-insensitive, a pre-requisite for geographic wide adaptation. The breeding system used to develop such germplasm consisted of shuttling alternating generations of wheat between two contrasting north to south environments in Mexico – the Yaqui Valley (Ciudad Obregon, Sonora, Mexico) where days are short during the ''winter cycle'' and where photo-insensitivity is required for earlier flowering to avoid terminal heat stresses, and Toluca (State of Mexico, Mexico) where there are longer days and cooler nights. This shuttle was the foundation of the success of what we know today as the Green Revolution wheats, whose main output was a completely new kind of wheat: semidwarf, high yielding, insensitive to photoperiod, and disease-resistant (Trethowan *et al.*, 2007).

The second important component for success is the multi-environment testing of lines selected under the shuttle scheme. Every year, several hundred new wheat lines are sent to around 200 co-operators in more than 50 countries, who evaluate the material and share the results with the international wheat community. Without this International Wheat Improvement Network (IWIN), in which basically every major wheat program worldwide participates, and which is based on germplasm and information exchange between CIMMYT and co-operators – the International Centre for Agriculture in Dry Areas (ICARDA) uses a similar system – it is unlikely that wheat developed in Mexico would have had a global impact on wheat improvement. Extensive reviews of the impact from CIMMYT wheat germplasm have been conducted by Reynolds and Borlaug (2006), and by Lantican *et al.* (2005). The information on the performance of the wheat lines in International Nurseries obtained through IWIN is paramount for the crossing plan at CIMMYT. Using parents that performed well across a wide range of environments allowed the frequency of desirable alleles in CIMMYT germplasm to be increased and this is the basis for the high and stable yields.

Wheat mega-environments and the impact of global climate change

CIMMYT develops improved wheat germplasm for use in developing and emerging countries, which grow wheat on about 110 m ha (Lantican *et al.*, 2005). To address the needs of these diverse wheat growing areas, CIMMYT uses the concept of mega-environments (MEs) (Rajaram *et al.,* 1994) to target germplasm development. A ME is defined as a broad, not necessarily contiguous area, occurring in more than one country and frequently transcontinental, defined by similar biotic and abiotic stresses, cropping system requirements, consumer preferences, and, for convenience, by a volume of production. The MEs to which wheat breeding stations participating in IWIN are assigned to are shown in Figure 1.1 (Hodson and White, 2007a). Germplasm generated for a given ME is useful throughout it, accommodating major stresses, although it does not necessarily show good adaptation to all significant secondary stresses. CIMMYT's global wheat were originally based primarily on moisture regime (irrigated versus rainfed) and growth habit and related to this temperature (spring versus facultative versus winter). The wheat area in

developing countries was assigned to twelve MEs, of which MEs 1–6 are classified as spring wheat environments, MEs 7–9 as facultative and MEs 10–12 as winter wheat environments. Since every ME corresponds to a unique combination of these parameters, each one tends to be associated with a characteristic set of abiotic and biotic stresses (Braun *et al.*, 1996).

Hodson and White (2007a) expanded the criteria to classify wheat MEs by introducing additional geospatial data and discussed the impacts of global climate change on wheat (Hodson and White, 2007b). Table 1.2 summarizes the expected impact of climate change on the various MEs. The greatest impact is expected in MEs 1–5, which include subtropical to tropical spring wheat regions. An estimated 9 m ha of wheat in these regions currently experience yield losses due to heat stresses (Lillemo *et al.*, 2005). Typically heat-stressed environments are classified as ME5, with subdivisions for predominantly humid or dry conditions (ME5A and ME5B, respectively). Wheat regions already at the limit for heat tolerance, for example in the Eastern Gangetic Plains of Nepal, India and Bangladesh, are most likely to suffer and may see substantial area reductions. Similarly, under warming, large areas of ME1 will transition to ME5. Positive impacts for ME1, however, are anticipated from CO_2-driven increases in productivity, accompanied by increased water use efficiency.

High elevation, high rainfall environments (ME2A) will experience reductions in area as the elevation band providing suitable temperatures for wheat is displaced upwards. An agroclimatic study on Ethiopia (White *et al.*, 2001) concluded that the current wheat area is largely delimited by high temperature and that warming would greatly reduce the area suitable for wheat. If heat tolerance of currently grown cultivars could be enhanced by 2°C, the wheat area in the periphery of the highlands could be nearly doubled. For the acid soil area in Brazil (ME3), raising temperature will further increase the stress similar to ME5. The most severe negative impact from global climate change is expected for ME4. Drought and heat are often associated, and this combination of warming and water deficits may result in low rainfall ME4 areas becoming unsuitable for wheat production. For temperature increases up to 2°C this trend may be partially offset by CO_2-driven increases in productivity and water use efficiency.

Cool high-latitude spring wheat areas above 45°N in ME6 of Kazakhstan, Siberia, China, USA and Canada may benefit from the affects of global climate change. Warmer temperatures should allow earlier sowing and reduce chances of late-season frost. Some areas may convert to more productive winter wheats (MEs 10–12) as risk of cold induced winter-kill declines. This is already happening in Russia, where in traditional spring wheat areas today, more winter than spring wheat is grown (A.I. Morgounov, Turkey, 2009, personal communication). An expansion into areas further north is also likely (Ortiz *et al.*, 2008). Due to the low temperature throughout ME6, beneficial effects of CO_2 on productivity and water use efficiency are likely.

Regions where facultative wheat (MEs 7–9), which are intermediate to spring and winter wheats, predominate should become more suitable for fall- to winter-sown spring wheats as risk of cold damage decreases. Some ME7 areas will grow cultivars adapted to ME1. The effect on yield potential in these environments is more uncertain, but since the growing season will be shortened, this may open new options for crop diversification.

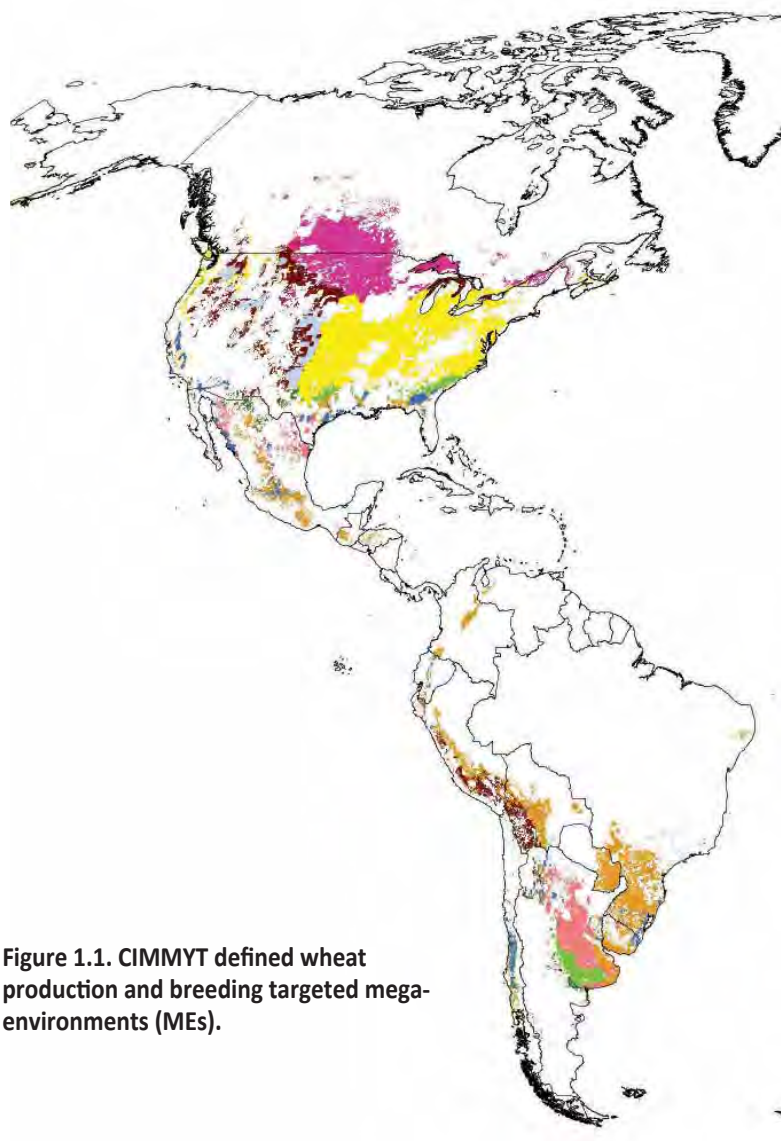

Figure 1.1. CIMMYT defined wheat production and breeding targeted mega-environments (MEs).

A disadvantage of the static definition of ME is that it does not take into account the fact that MEs tend to shift from year to year and fluctuate in weather patterns. In particular this is important for locations in ME2 (high rainfall spring wheat) and ME4 (rainfed spring wheat low rainfall) but also ME1 (irrigated) and ME5 (irrigated high temperature). The frequency with which ME2 or ME4 conditions are experienced varies between locations. Climate change may bring an increased intensity and frequency of storms, drought and flooding, weather extremes, altered hydrological cycles, and precipitation (Ortiz *et al.*, 2008). Such climate vulnerability will threaten the sustainability of farming systems, particularly in the developing world. Widely adapted, stress tolerant cultivars, coupled with sustainable crop and natural resource management will provide means for farmers to cope with climate change and benefit consumers worldwide.

Widely adapted cultivars: more important than ever to buffer temporal climatic variability

The impact of CIMMYT's wheat breeding on international collaborative wheat improvement has been discussed by Reynolds and Borlaug (2006). CIMMYT's wheat breeding philosophy and methodology embraces three important principals: the development of germplasm with high and stable yield across a wide range of environments. The concept of wide adaptation has been criticized, with local or specific adaptation advocated. However, we believe that wide adaptation to a broad range of environments becomes increasingly important to develop cultivars that can cope with the climate extremes that occur at one location over years, or with variation within farmers' fields. For example,

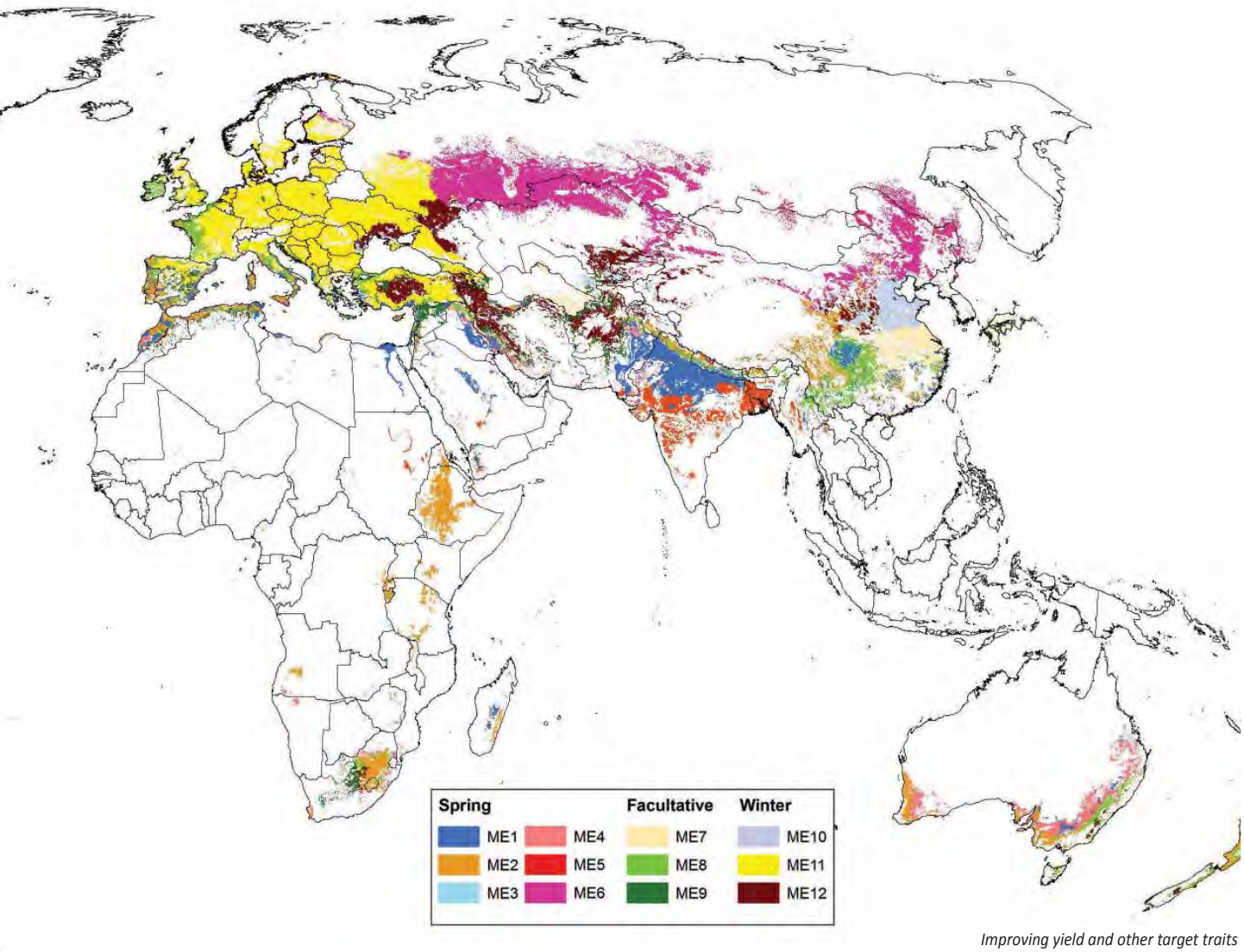

Table 1.2. Classification of mega-environments (MEs) used by the CIMMYT Wheat Program using qualitaive (ME1–12) and geospatial criteria (ME1–6).

ME	Latitude	Wheat area (m ha)	Criteria	Temperature regime	Sowing time	Major biotic and abiotic stresses	Representative locations/regions	Change in ME due to climate change and consequences for germplasm development N=negative; P=positive; U=unknown adapted from Hodson and White, 2007b)
"Spring" Wheat								
1	<40°	32.0	Low rainfall irrigated, coolest quarter (3 consecutive months) mean min temp. >3°C <11°C.	Temperate	Autumn	Resistance to lodging, SR, LR, YR, KB, *Alternaria* spp.	Yaqui Valley, Mexico; Indus Valley, Pakistan; Gangetic Valley, India; Nile Valley, Egypt.	N–Rising temperatures result in large areas evolving to ME5. N–Reduced precipitation in subtropical regions restricts irrigation; supplementary irrigation results in temporary drought periods requiring germplasm with high yield and tolerance to drought (adapted to ME1 and ME4). P–Reduced irrigation due to impact of elevated CO_2 on water use efficiency. N-Increased insect problems.
2A	<40°	4.0	High rainfall in summer; wettest quarter mean min temp >3°C <16°C, wettest quarter (3 consecutive wettest months) precipitation > 250 mm; elevation 1400 m.	Temperate	Autumn	As for ME1 + resistance to LR, YR, *Septoria* spp., PM, RDC, BYDV, sprouting.	Highlands East Africa and Mexico, Andes.	N–Rising temperatures result in some areas evolving to ME5. N–Reduced precipitation results in areas evolving to ME4.
2B	<40°	3.0	High rainfall winter rain; coolest quarter mean min temp. >3°C <16°C; elevation 1400m	Temperate	Autumn	As for ME1 + resistance to LR, YR, *Septoria* spp., PM, RDC, BYDV, sprouting.	Mediterranean Coast, Caspian Sea.	U–Changes in precipitation patterns in areas will have variable effects. N–Frequency of climate extremes over years increase requiring germplasm with high yield potential, wide spectrum of disease resistance and tolerance to drought.
3	<40°	1.7	High rainfall acid soil; climate as in ME2 and pH <5.2.	Temperate	Autumn	As for ME2 + acid soil tolerance.	Passo Fundo, Brazil.	N–Rising temperatures result in large areas evolving to ME5. U–Changes in precipitation patterns in areas will have variable effects.

Table 1.2. Classification of mega-environments continued...

ME	Latitude	Wheat area (m ha)	Criteria	Temperature regime	Sowing time	Major biotic and abiotic stresses	Representative locations/regions	Change in ME due to climate change and consequences for germplasm development N=negative; P=positive; U=unknown adapted from Hodson and White, 2007b)
"Spring" Wheat								
4A	<40°	10.0	Low rainfall, winter rainfall dominant; coolest quarter mean min temp >3°C <11°C; wettest quarter precipitation >100 mm <400 mm.	Temperate	Autumn	Resistance to drought, *Septoria* spp., YR, LR, SR, RDC, Hessian fly, Sawfly.	Settat, Morocco; Aleppo, Syria; Diyarbakir, Turkey	N–Rising temperatures exacerbates water deficits, either further reducing yields or making production uneconomical. P–Reduced water deficits through impact of elevated CO_2 on water use efficiency.
4B	<40°	5.8	Low rainfall, summer rainfall dominant; coolest quarter mean min temp >3°C <11°C; wettest quarter precipitation >200 mm <500 mm.	Temperate	Autumn	Resistance to, drought *Septoria* spp., LR, SR, *Fusarium* spp.	Marcos Juarez, Argentina.	N–Changes in precipitation patterns likely to increase drought risk.
4C	<40°	5.8	Mostly residual moisture; coolest quarter mean min temp >3°C <16°C; wettest quarter precipitation >100 mm <400 mm.	Hot	Autumn	Resistance to drought, and heat in seedling stage, SR.	Indore, India.	U–Changes in precipitation patterns in areas will have variable effects.
5A	<40°	3.9	High rainfall/ irrigated, humid; coolest quarter mean min temp >11°C <16°C.	Hot	Autumn	Tolerance to heat, Helmintho-*sporium* spp., *Fusarium* spp., sprouting; in Brazil Bolivia and Paraguay wheat blast.	Eastern Gangetic Plains in Nepal, India, Bangladesh; Londrina, Brazil.	N–Rising temperatures result in large areas becoming unsuitable for wheat; cropping systems and agronomy practices allowing early sowing of wheat paramount. U–Elevated CO_2 may increase water use efficiency, but the same mechanism implies increased canopy temperature, which likely would exacerbate heat stress.
5B	<40°	3.2	Irrigated, low humidity; coolest quarter mean min temp >11°C <16°C.	Hot	Autumn	Resistance to heat and SR, LR.	Gezira, Sudan; Kano, Nigeria.	N–Rising temperatures result in large areas becoming unsuitable for wheat. N–Increasing biotic stress. U–Elevated CO_2 may increase water use efficiency, but the same mechanism implies increased canopy temperature, which likely would exacerbate heat stress.

Table 1.2. Classification of mega-environments continued...

ME	Latitude	Wheat area (m ha)	Criteria	Temperature regime	Sowing time	Major biotic and abiotic stresses	Representative locations/regions	Change in ME due to climate change and consequences for germplasm development N=negative; P=positive; U=unknown adapted from Hodson and White, 2007b)
"Spring" Wheat								
6	>40°	11.0	Moderate rainfall/ summer dominant; high latitude quarter 45°N; coolest mean min temp <–13°C; warmest quarter mean min temp> 9°C.	Temperate	Spring	Resistance to drought, SR, LR, Tan spot, Scab, photoperiod sensitivity.	Kazakhstan; Siberia; Harbin, China.	P–Rising temperatures allow wheat production in higher latitudes - wheat area expansion likely. P–Lengthen growing season permits marginal areas to become productive. P–Reduced risk of winter-kill allows conversion to more productive winter wheat.
Facultative Wheat								
7A	<40°	6.0	Irrigated.	Moderate cold	Autumn	Rapid grain fill, resistance to cold, YR, LR, PM, BYD, Bunt, LS.	Henan, China.	U–Reduced cold stress allows growing fall sown spring wheat, possibly reducing yield potential but shortening growing cropping systems. P–Reduced irrigation due to impact of elevated CO_2 on water use efficiency.
7B	<40°	3.0	Irrigated, often only supplementary irrigation.	Moderate cold	Autumn	YR, Bunt, LR, SR, LS.	Turkey; Iran; Central Asia; Afghanistan.	U–Reduced cold stress allows growing fall sown spring wheat, possibly reducing yield potential but shortening growing season offering more options for diversifying cropping systems. P–Reduced irrigation due to impact of elevated CO_2 on water use efficiency. N–Supplementary irrigation with temporary exposure to drought requires germplasm adapted to ME7 and ME9 adaptation to ME.
8A	<40°	0.2	More than 600 mm rainfall, medium cold, photosensitive.	Moderate cold	Autumn	YR, *Septoria* spp., PM, *Fusarium*, RDC.	Chillan, Chile	U–Reduced cold stress allows growing spring wheat, possibly reducing yield potential but shortening growing season. U–Increasing biotic stress.

Table 1.2. Classification of mega-environments continued...

ME	Latitude	Wheat area (m ha)	Criteria	Temperature regime	Sowing time	Major biotic and abiotic stresses	Representative locations/regions	Change in ME due to climate change and consequences for germplasm development N=negative; P=positive; U=unknown adapted from Hodson and White, 2007b)
8B	<40°	0.5	More than 600 mm rainfall.	Moderate cold	Autumn	YR, Bunt, LR, RDC, PM.	Transitional zones and Trace, Turkey.	U–Changes in precipitation patterns in areas will have variable effects. N–Frequency of climate extremes over years increase requiring germplasm with high yield potential, wide spectrum of disease resistance and tolerance to drought.
9	<40°	6.8	Low rainfall <400 mm, winter/spring rainfall dominant.	Moderate cold	Autumn	Resistance to drought, cold, heat at grain fill, YR, Bunt, LR, SR.	West and Central Asia; North Africa (mainly non-dwarf cultivars grown).	U–Reduced cold stress allows growing spring wheat, possibly reducing yield potential but shortening growing season. U–Changes in precipitation patterns in areas will have variable effects. P–Reduced water deficits through impact of elevated CO_2 on water use efficiency. N–Rising temperatures exacerbates water deficits, either further reducing yields or making production uneconomical.
"Winter" Wheat								
10A	<40°	4.6	Irrigated.	Severe cold	Autumn	Resistance to winter-kill, YR, LR, PM, BYD.	Beijing, China.	P–Warmer winters reduce severity of winter-kill, increasing yields. N–Warmer spring and summer hasten grain-filling. P–Reduced irrigation due to impact of elevated CO_2 on water use efficiency.
10B	<40°	1.6	Often supplementary irrigation.	Severe cold	Autumn	Resistance to winterkill, YR, BYD, Bunt, Smut, RDC, Nematodes.	Turkey; Iran; Central Asia.	P–Warmer winters reduce severity of winter-kill, increasing yields. N–Warmer spring and summer hasten grain-filling. P–Reduced irrigation due to impact of elevated CO_2 on water use efficiency.

Table 1.2. Classification of mega-environments continued...

ME	Latitude	Wheat area (m ha)	Criteria	Temperature regime	Sowing time	Major biotic and abiotic stresses	Representative locations/regions	Change in ME due to climate change and consequences for germplasm development N=negative; P=positive; U=unknown adapted from Hodson and White, 2007b)
11A	>40°	Area in LDC insignifi-cant.	High rainfall/ irrigated, long season.	Severe cold	Autumn	Resistance to *Septoria* spp., *Fusarium* spp., YR, LR, PM, RDC, BYD.	Central and Western Europe; NW USA.	P–Warmer winters reduce severity of winter-kill.
11B	<40°	Area in LDC insignifi-cant.	High rainfall/ irrigated, short season.	Severe cold	Autumn	Resistance to LR, SR, PM, *Fusarium*, *Septoria*, BYD, winter-kill, sprouting.	SE Europe, North Korea, China.	P–Warmer winters reduce severity of winter-kill.
12	<40°	7.9	Low rainfall between 300–450 mm.	Severe cold	Autumn	Resistance to winter-kill, drought, heat during grain-fill, YR, bunts, Nematodes, RDC, Zinc deficiency, in Turkey and Iran mainly non-dwarf varieties grown.	Ankara, Turkey; West and Central Asia; China.	P–Warmer winters reduce severity of winter-kill. P–Reduced water deficits through impact of elevated CO_2 on water use efficiency. N–Increased frequency of years with severe drought. N–increased insect problems.

Moisture regime refers to rainfall just before and during the crop cycle. High = >500 mm; Low = <500 mm
Temperature regime: Hot = mean temperature of the coolest month >17.5°C; Cold = <5.0°C
Biotic stresses: LR=leaf rust, SR=stem rust, YR=yellow (stripe) rust, PM=powdery mildew, BYD=barley yellow dwarf, LS=Ustilago tritici, KB=Karnal bunt, RDC=root disease complex. LDC=less developed countries.

wheat production in North Africa often fluctuates year to year between drought-prone drylands (ME4) and higher rainfall (ME2) environmental seasons (D. Hodson, Mexico, 2007, personal communication).

The international multi-environment nursery system is the best mechanism to identify and release spatially widely adapted wheat cultivars (Rajaram and Ceccarelli, 1998). CIMMYT's wheat breeding program emphasizes the development of wheat cultivars with stable yields over a wide range of environments. Such cultivars, identified through testing by national agricultural research systems (NARS) partners in the IWIN, form the genetic basis to further enhance tolerance to heat and drought stress. The resolution of this spatial adaptation can be expressed amongst geographically distinct countries and continents to performance stability across a region, or within a more local perspective within a farmer's heterogeneous field. In most cases, widely adapted germplasm is not only input responsive, but also input efficient (Braun *et al.*, 1996; Manske *et al.*, 2000). Such performance stability can also be expressed temporally, between years.

Climate change will cause major changes in soil microbial systems and occurrence and distribution of weeds, insects, and diseases (Easterling *et al.*, 2007). Yield losses from pest and diseases are an estimated 28% for wheat, 31% for maize and 37% for rice and losses could be as high as 50%, 67% and 77% respectively, without effective plant protection (Oerke, 2006). It is likely that more epidemics will occur in the future when diseases and pests spread to areas where they were previously not important. Testing elite lines in hot spots for a given disease is an effective way to identify resistant germplasm. This is exemplified by the approach used to develop wheat lines resistant to wheat stem rust race Ug99. Most wheat cultivars currently grown worldwide are susceptible to this race. Countries where stem rust is a potential threat for wheat production have sent more than 40,000 accessions for evaluation in Kenya and resistant accessions are now multiplied. Screening at hot spots for specific diseases, such as North Africa for leaf rust and *Septoria tritici* in durum, Ecuador and West Asia for yellow rust, Southern Cone in Latin America for a complex of diseases including *Fusarium* head scab, leaf rust, and *Septoria tritici* mildew in bread wheat, *Fusarium* head scab in China and Spot blotch in the Eastern Gangetic Plains are paramount to develop widely adapted germplasm buffered against the major biotic stresses. Pre-emptive breeding, i.e., developing wheat cultivars that are resistant to a disease that currently is not present in a wheat growing zone but could be introduced is an important strategy to assure food security.

More than 80% of all fresh water is used for agriculture, and about 90% of all irrigated wheat is grown in less developed countries (Brown, 2004). The risk to wheat being exposed to temporary or partial drought during its growing cycle is consequently increasing. As the frequency of extremes in precipitation will increase at given locations, the wheat production environment of these location will fluctuate between ME4 (dryland) and ME2 (high rainfall). Because the expected climate of a location is unknown at the time of sowing, farmers need cultivars that are input responsive and productive across a range of production environments. Cultivars must be developed that can exploit available moisture in wetter years combined with drought tolerance for years which lack optimum levels of precipitation.

CIMMYT develops wheat germplasm that combines high yield potential under favourable conditions, with tolerance to less favourable drought or water-limiting environments. Many CIMMYT derived cultivars have been released for irrigated, rainfed and drought-prone environments including those based on Pavon 79, Seri 82 and Attila (Skovmand *et al.*, 1997). Evidence for the success was provided by Blum (2005), who, in his review on breeding for drought tolerance concluded that it is possible –within biological limits– to combine drought resistance and yield potential if selection is designed to recombine a high yield potential genotype with relevant dehydration-avoidance factors that are not associated with lower yield potential, e.g., osmotic adjustment.

The main elements of global climate change; increasing temperature and CO_2 concentration, drought, and changes in disease occurrence and soil-microbial will affect wheat areas worldwide. The effect of global climate change on wheat will vary by region. In general, wheat production in high latitudes will initially benefit from temperature increases, while in low latitudes wheat yields will decrease with increasing temperature (Table 1.3). The most severely affected areas will be the low-land areas in Asia, with China, India, Bangladesh, Nepal, Iran, Egypt, Sudan, Brazil and Paraguay. North African countries will face yield reductions from extended periods of drought. For less developed countries, the main challenge for wheat breeders at this stage is selecting genotypes able to tolerate heat stress and water deficits.

The future of crop MEs as a breeding tool

A limitation of the ME concept is its stochastic nature, whereas in reality a given location will vary temporally from year to year, and spatially within farmers' fields and locally. The combination of water and temperature defines the occurrence of biotic and abiotic stresses and the ME concept was very useful in defining germplasm that has a specific combination of traits required within a given ME.

To better target germplasm development in the future, the ME will need to be refined to address different needs of the various production systems. GIS and remote sensing are powerful tools to classify environments with bio-physical parameters (Hodson and White 2007a; Lobell and Ortiz-Monasterio, 2007) and to estimate probability ranges for precipitation and use soil parameters e.g., micro-nutrient deficiencies or toxicities and pH to characterize environments.

Cooper and Fox (1996) suggested using probe genotypes as an indirect approach to characterize environments. Though a limitation of this approach is the dependency on suitable contrasting genotypes and that using contrasting genotypes for different traits may lead to varying environmental characterization, Mathews *et al.* (2004) used pairs of two contrasting genotypes, ideally iso-lines, for 14 adaptation-relevant traits and identified environment specific factors that contribute to environmental classification.

Combining remote sensing with modelling further enhances the options to classify environments. Lobell and Ortiz-Monasterio (2006a; 2006b; 2007) used modelling and remote sensing to estimate grain yields and measure the effect of night and day temperature on yield. Sutherst *et al.* (2000) applied models to estimate the vulnerability of a given environment for pests and diseases.

It has been suggested to classify environments based on methods described in the previous paragraphs, including major biotic and abiotic constraints, as well as other traits important for adaptation and adoption by farmers (W.H. Pfeiffer, Colombia, 2009, personal communication). Considering available genetic variability and heritability for each of these traits and availability of markers, the probability and success rate to find solutions through breeding interventions can be calculated. This classification will also show in which environments greatest progress to raise productivity will come from agronomic or genotype by management interventions in cases where there is no or insufficient genetic variability for traits of interest. An index can be developed for important production systems and considering these factors, which eventually will allow setting priorities and allocate resources based on where the likelihood for successful intervention is highest.

The ME concept has been proven very successful in characterizing major wheat growing areas and defining germplasm pools that possess the combination of traits related to general adaptation (phenology), tolerance or resistance to the prevailing biotic and abiotic stresses and end-use quality characteristics. Since year to year climatic conditions are projected to become more variable due to climate change (IPCC, 2007), widely adapted cultivars will be crucial to buffer unpredictable climate stresses such as drought, heat and cold, while being input responsive in years with agro-ecological conditions favourable to crop productivity. To identify such cultivars, multi-location testing remains the most efficient system since it allows substitution of temporal by spatial variation. MEs are defined across continents (Figure 1.1), and therefore regional and annual fluctuations in occurrence of abiotic and biotic stresses cancel each other out. In one year elite lines can be evaluated in a multitude of different environments and those best buffered against the highly variable stresses will be selected for parents in crossing programs and as potential cultivars for further testing.

Table 1.3. Average sensitivity of wheat yield to temperature increase. Sites were assigned as either low-latitude or mid- to high-latitude and the experiments were classified as either with (+) or without (–) adaptation measures to compensate for temperature increases. Adaptation measures in these studies were changes in sowing date, changes in cultivar, and shifts from rain-fed to irrigated conditions. Studies span a range of precipitation changes and CO_2 concentrations, and obviously vary in how they represent future changes in climate variability (see Easterling *et al.*, 2007 for a complete list of references).

	Mid- to high-latitude sites			Low latitude sites		
	Temperature increase (°C)					
Adaptation measures	1–2	2–3	3–5	1–2	2–3	3–5
+	20	18	5	7	–14	–25
–	5	5	–18	–4	–16	–40
Difference	15	13	23	11	2	15

International evaluation networks based on exchange of and free access to germplasm and multi-location testing are therefore a cornerstone in the strategies and efforts to develop wheat, rice and maize germplasm that is adapted to the increasingly variable growing conditions encountered due to global climate change.

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Chapter 2: Application of physiology in breeding for heat and drought stress

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Abstract

Conceptual models of desirable trait profiles are used in wheat breeding to accumulate complementary physiological traits (PTs). The principal steps in PT breeding include characterization of potential parents for adaptive mechanisms, strategic crossing among parents that encompass as many target traits as possible, and early generation selection (EGS) of bulks for canopy temperature (CT). Other EGS techniques that are amenable to high-throughput include measurement of spectral reflectance indices and stomatal aperture-related traits. Exotic parents can be used to introduce new allelic diversity –including landraces and products of inter-specific hybridization– and both approaches have been employed to introduce stress-adaptive traits into CIMMYT germplasm. PT expression, even of un-adapted and exotic germplasm, can be used as a basis for selecting promising genotypes for use in germplasm development. Discovering the genetic basis of PTs is highly complex because putative quantitative trait loci (QTLs) may interact with environment and genetic background, including genes of major effect. Detection of QTLs is improved in mapping populations where flowering time is controlled, and new mapping populations can be designed by screening potential parents that do not contrast in *Rht, Ppd* or *Vrn* alleles. Association mapping can be employed for gene discovery using exclusively agronomically improved material.

Introduction

Although research in plant physiology encompasses all growth phenomena of healthy plants, only traits that have a likely economic impact and which show significant genetic variation can be considered for improvement in the context of plant breeding. Many such traits are expressed at the whole plant or organ level (Araus *et al.,* 2002; Slafer *et al.,* 2005; Richards, 2006), while advances in biotechnology have facilitated the identification of promising traits at the cellular and metabolic levels (Chaves *et al.,* 2003; Umezawa *et al.,* 2006; Barnabus *et al.,* 2008).

Although breeding for high and stable yield potential achieves significant impacts in most spring wheat environments, including marginal situations (Lantican *et al.,* 2003), CIMMYT targets germplasm development towards a number of discrete mega-environments that include heat and drought stress (Braun *et al.,* 2010). These abiotic stress factors represent two of the greatest challenges for adapting crops to future climate scenarios and for which physiological approaches are expected to complement conventional breeding approaches (Reynolds *et al.,* 2010).

Research in plant physiology can be focused to achieve short and long term impacts in crop improvement. Direct, shorter-term interventions in breeding include: (i) characterization of parental material for use in strategic trait-based crossing; (ii) early generation selection using high-throughput screening techniques that shift gene frequencies in subsequent generations in favor of adaptive traits before yield testing is economically feasible; and (iii) identification of useful physiological traits among genetic resources (which may show poor overall agronomic performance). In the longer term, physiological research can be focused to improve understanding of the physiological and genetic basis of adaptation to environment, including: (i) development of models of physiological trait expression; (ii) dissection of genotype × environment interaction of trait expression; and (iii) genetic studies that, through adequate experimental control, permit molecular markers to be identified with precision, facilitating their implementation in molecular breeding.

Trait-based crossing

The objective of trait-based crossing is to accumulate traits that will be complementary for a given target environment. To ensure a practical outcome of this approach, traits should be well defined in terms of: (i) the stage of crop development at which they are pertinent; (ii) the specific attributes of the target environment for which they are adaptive; and (iii) their potential contribution to yield over a range of crop

cycles. Conceptual models are developed using these criteria. For example, under water-limited situations, traits that improve water uptake, water use efficiency and partitioning to yield are likely to work synergistically to maximize productivity in the target environment (Figure 2.1). Trait accumulation in a practical breeding context involves the following main interventions:

- Potential parental lines are characterized for all traits that may contribute to genetic gains in target environments. Traits likely to show significant genotype by environment (G×E) interaction within the target region are characterized in an appropriate range of selection environments to establish robust expression.
- Crosses are designed such that traits expressed by respective parents encompass as many of the target-traits as possible. In addition to adapting to abiotic factors, strategic crossing must take into account the need for genetic resistance to a range of biotic stresses, quality parameters and phenology. Top crosses may be used to facilitate the accumulation of traits.
- Selection in F_2 considers simply inherited traits such as disease resistance, plant height and phenology, expressed in well-watered conditions.
- Early generation bulks are screened for canopy temperature (CT); families with warm canopies –compared to checks– are mainly discarded.

The result of investment in physiological trait (PT) based crossing has generated advanced lines distributed by the International Maize and Wheat Improvement Center (CIMMYT) as part of the 27th Semi Arid Wheat Screening Nursery –SAWSN– based on their consistently superior performance in breeder drought trials (Reynolds *et al.,* 2009). The underlying assumption for the PT strategy is that crosses between parents with different but potentially complementary PT expression will realize cumulative gene action in selected progeny –with the important caveat that a trait may show interaction with genetic background and environment (Reynolds and Tuberosa, 2008).

Case study 1:

Having developed PT advanced lines, it was possible to test this hypothesis in a controlled experiment by growing a group of PT lines and evaluating their trait expression

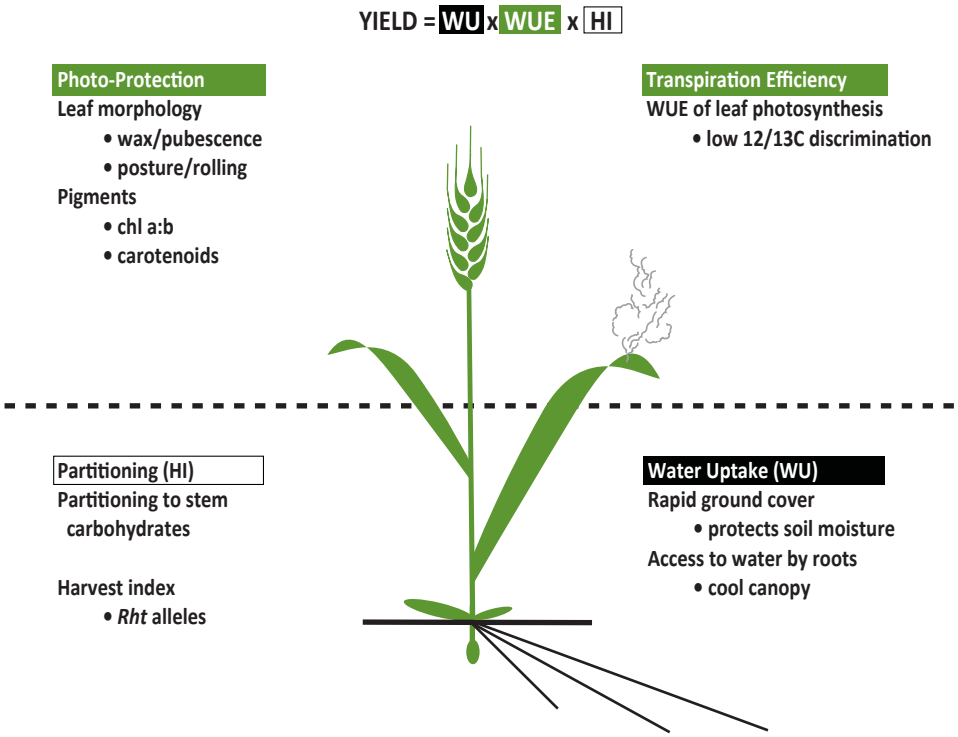

Figure 2.1. General conceptual model of physiological characteristics used in strategic crossing by CIMMYT to combine complementary drought-adaptive traits in wheat breeding. Where: HI = harvest index; WU = water uptake; and, WUE = water use efficiency. Adapted from Reynolds *et al.* (2009).

side by side with their parents from: (i) a three way cross including a drought adapted Mexican landrace, and (ii) an elite by elite cross. The selected progeny from the three way cross (WBLL4/OAX93.24.35/WBLL1) showed 5.6% better yield under drought and 5.5% better biomass than the best parent. The first parent, WBLL4, brought high biomass, transpiration efficiency –as indicated by the relatively low value of carbon isotope discrimination (CID; Condon *et al.,* 2004)– and high leaf chlorophyll concentration, a trait associated with delayed senescence or stay-green in sorghum (Borrell *et al.,* 2000). The second parent, the landrace OAX93.24.35, brought low CT associated with ability to extract water from deeper in the soil profile and high stem carbohydrates at anthesis –available for remobilization to kernels during grain-filling (Rebetzke *et al.,* 2008a). The third parent, WEEBILL1, brought high early vigor, associated with reduced water losses from the soil (Richards, 2006), low CT, associated with deep water extraction, and high leaf wax, associated with photo-protection. The data showed that the progeny from the three way cross between these lines has inherited all these characteristics, except high chlorophyll. This line also showed good yield in drought trials in north-west Mexico during three consecutive years, reasonable yield potential, and has been selected to enter the 17th Semi Arid Wheat Yield Trial (SAWYT). In the case of the elite by elite cross, Weebil1 was also used as a parent and, therefore, contributed the same traits already described, while Sokoll contributed to high biomass, low CID, and high chlorophyll. The three resulting selected progeny expressed all traits well (except high chlorophyll) and sometimes better than the best parent (i.e., water extraction and stem carbohydrates); they also showed similar yield and higher biomass than the best parent (+13%). All three sister lines were selected to enter the 27th SAWSN. Both results confirm the hypothesis that PT crossing can result in cumulative gene action in selected progeny resulting in increased yield under drought (Reynolds *et al.,* 2005, 2007a; Reynolds and Tuberosa, 2008).

Characterization of candidate parents with the view to better targeted crossing should have the highest priority in terms of physiological interventions in breeding for a number of reasons. Firstly, while a significant investment in trait measurement is needed, the information can be used for many cycles of crossing after the initial characterization has been made. Furthermore, because the number of lines in a crossing block are relatively small (typically a hundred or so per target environment) detailed characterization is possible even for relatively time-consuming traits such as soil moisture depletion or stem carbohydrates. However, physiological characterization among early generation progeny, which may number in the hundreds or even thousands for every cross, is a task requiring high-throughput screens that permit selection to be made on a similar time-frame as visual selection.

Early generation selection

Maintaining large populations of segregating lines in early generations is one of the most resource intensive aspects of plant breeding. Techniques that discriminate among lines for PTs can increase efficiency at this stage either by permitting inferior materials to be discarded and/or increasing the frequency of genes associated with superior expression of useful traits. It is hard to beat the efficiency of a well-trained human eye in terms of its ability to integrate the many morphological traits that characterize an agronomically useful plant. Nonetheless, certain physiological approaches can extend that range, for example into the infrared in the case of CT, or by increasing its resolution in the case of spectral reflectance, and can be adapted to high throughput screening platforms with similar efficiency as visual selection (Figure 2.2).

Canopy temperature

Use of infrared imaging to quantify differences in the CT of wheat genotypes under drought was first reported by Blum and co-workers in 1982 and has also been shown to be an excellent predictor of yield in hot, irrigated environments (Reynolds *et al.,* 1994). The trait was shown to explain approximately 60% of yield variation in random inbred lines (RILs) under drought stress and is applied as a selection tool by breeders working in heat and drought-stressed environments (Trethowan and Reynolds, 2007). Canopy temperature is the ideal physiological selection trait in many ways since measurement is quick, simple and inexpensive (See Cossani *et al.*, this Volume). It is also integrative, scoring many leaves at once, thus reducing error associated with leaf-to-leaf variation. The main downside is that the measurement is quite sensitive to the environment, requiring relatively cloud-free, windless days to obtain reliable data.

Case study 2:
At CIMMYT, CT is evaluated in breeders' F4 populations under drought during the late vegetative stage and again during grain-filling; bulks which are consistently cooler are selected assuming they meet visual selection criteria. Since CT has been shown to be well associated

with ability to extract water under drought (Lopes and Reynolds, 2010), selection for CT is most probably increasing gene frequencies for root-related traits in environments where water is available at depth. In hot environments cooler canopies were associated with yield among random lines (Figure 2.3) as well as providing a powerful tool for selecting advanced lines for performance at a number of heat-stressed target environments. For example, when comparing the association of yield in target environments (in Sudan, India and Bangladesh) with yield and CT measured in the selection environment (NW Mexico), it was found that both traits explained approximately equal amounts of variation in the yield of 60 advanced lines, about 40% (Reynolds *et al.,* 2001). However, CT was measured on plots of 2 m^2 instead of yield plots of 8 m^2, in about 10 seconds compared with about a minute to harvest and weigh a yield plot, and with an instrument that costs less than US$200 compared with the expense of a small plot harvester. Although it is not being suggested that CT should replace yield estimates in a breeding program, it illustrates the point that indirect selection criteria,

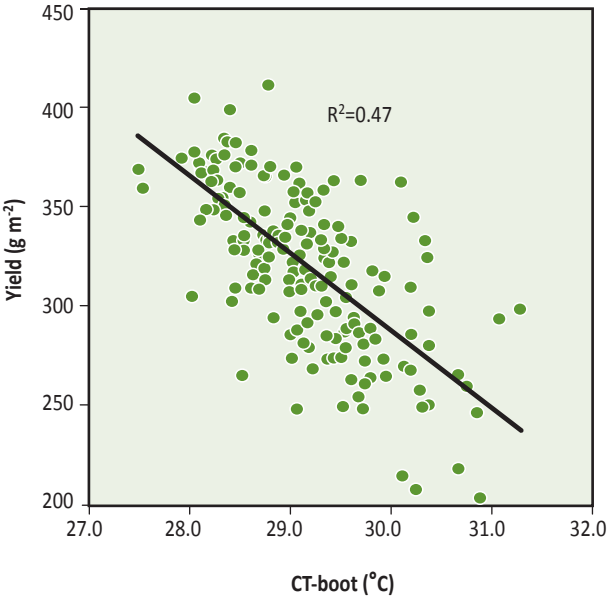

Figure 2.3. Association between yield and canopy temperature for Seri/Babax sister lines in a hot, irrigated environment, NW Mexico. CT-boot refers to canopy temperture taken during booting.

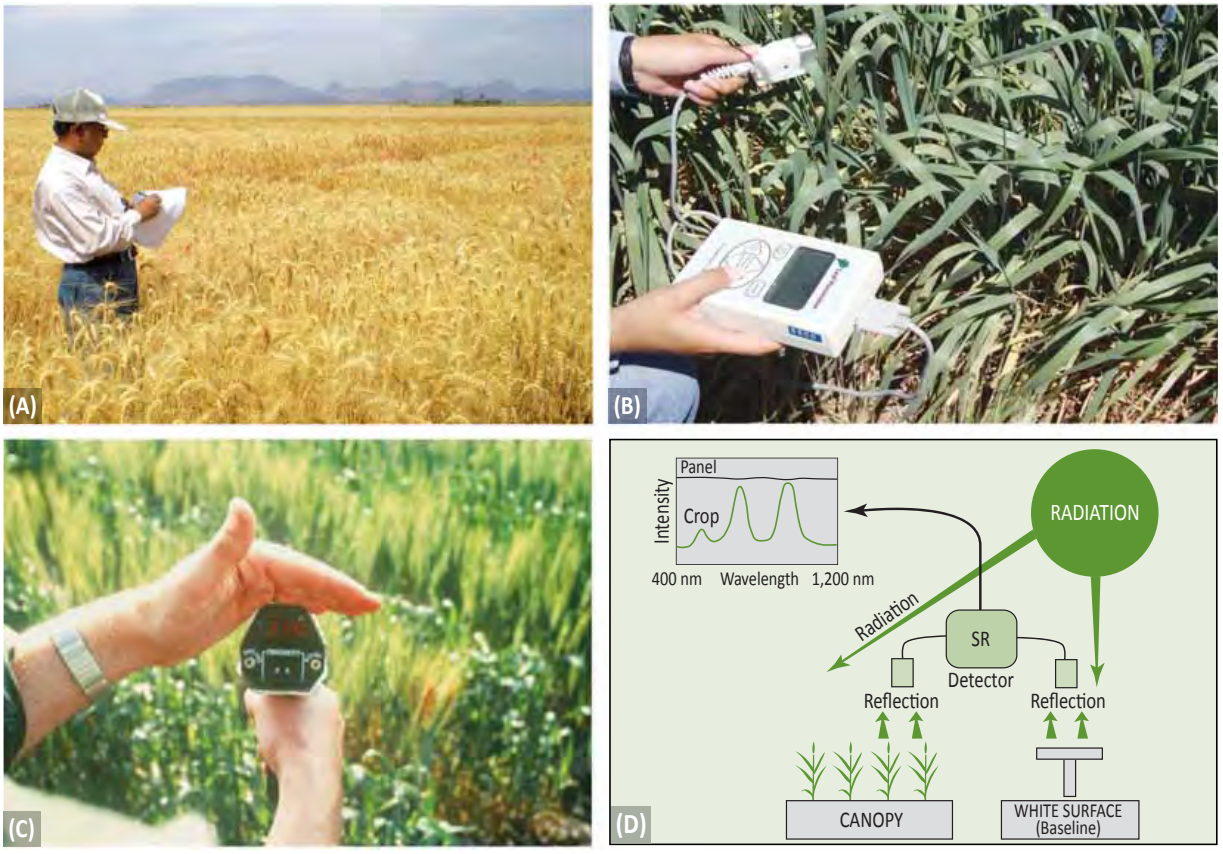

Figure 2.2. Early generation screening approaches: (A) visual selection, (B) leaf porometry, (C) canopy temperature, and (D) spectral reflectance.

like CT, have a role to play in improving the efficiency of selection. Furthermore, when comparing the merits of selecting for yield using either visual criteria or CT combined with visual criteria, it was found that the latter identified the very highest yielding genotypes (Figure 2.4) demonstrating the synergy of a combined approach.

Canopy temperature is essentially a diagnostic trait, being associated with yield in a range of conditions it is indicative of the relative fitness of a genotype to the environment. Integrative traits like CT may combine several important physiological and possibly disease reaction mechanisms (Araus *et al.,* 2002). When CT is measured on genetically diverse material in early generations, for example, under hot irrigated conditions, cooler canopies would be found in lines which combine a number of the traits considered important. These might include: (i) a root system that can match evaporative demand at high vapor pressure deficit, (ii) high intrinsic radiation-use efficiency (RUE), and (iii) photo-protective mechanisms that maintain RUE and green area throughout the growth cycle. By measuring CT strategically, for example, at different phenological stages and times of the day, genotypes that are deficient in any of those three areas could be detected and eliminated while genotypes showing consistently cool canopies would be advanced to the next generation, assuming they are otherwise agronomically acceptable.

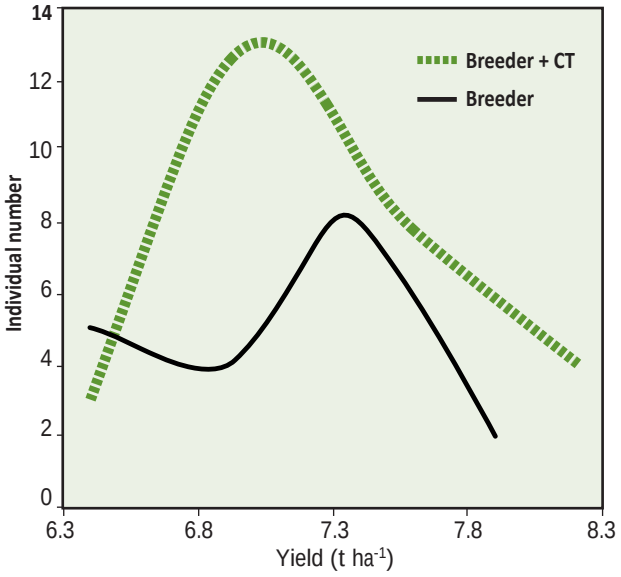

Figure 2.4. Yield of breeding lines selected either visually or through a combination of visual ('Breeder') and physiological (canopy temperature; CT) criteria, NW Mexico (van Ginkel *et al*., 2008).

Similarly, under drought, while a cool CT may be related directly to genetic potential for the capacity of roots to explore soil moisture in one environment, in another situation where, for example, micro-element deficiency or soil-borne disease are affecting root growth, cooler genotypes would be found only for those lines that contain the relevant genes.

Spectral reflectance

While measurement of CT effectively extends the range of breeders' 'vision' into the infrared, spectral radiometry can detect with high resolution an even greater range of light reflected from the canopy (in the range of 400–1100 nm for the basic radiometer). Dozens of indices have been calculated using different wavelengths that relate to a range of traits (see Araus *et al.,* 2001) including green area index (Tucker and Sellers, 1986), pigment composition of leaves (Chappelle *et al.,* 1992) and the water index that corresponds to a wavelength around 950 nm strongly absorbed by water (Peñuelas *et al.,* 1993). While some indices are sensitive to traits like ground cover that can also be estimated visually, radiometry provides a convenient way of standardizing these estimates. Other indices such as those related to pigment composition are too specific to be estimated visually given the limited resolution of the human eye to wavelength, while still others are outside the visible range. Several workers have found associations between spectral indices and yield of genotypes in a range of moisture-stressed (Aparicio *et al.,* 2000; Royo *et al.,* 2003; Guttierez-Rodriguez *et al.,* 2004; Babar *et al.,* 2006a) and irrigated environments (Babar *et al.,* 2006b). See also Mullan (this Volume).

Case study 3:

A derivative of the water index was recently shown to be a good predictor of relative in-season biomass when comparing genotypes under high yield conditions (Babar *et al.,* 2006c). This opens up the opportunity for breeders to readily select among breeding materials for lines with relatively high biomass at key growth stages; a trait which formerly would have been too resource intensive even to consider. The water index, NWI-3, has been shown to be well associated not only with performance under drought but also genotypic differences of a number of water relations related parameters including leaf water potential (ψ_{leaf}) and CT, soil moisture extraction and even root depth (Gutierrez *et al.,* 2010).

Identification of new genetic resources

Genetic improvement in complex traits has been achieved mainly by making crosses among improved lines and selecting for progeny that express higher trait value as a result of accumulation of favorable alleles, i.e., transgressive segregation. However, the more closely-related parental lines become, the less opportunity exists for combining of new alleles. The use of exotic parents facilitates greater allelic diversity from which new, favorable alleles and allelic combinations may be identified and selected. Different gene pools have been defined depending on the difficulty of employing them in breeding (Skovmand *et al.,* 2001; Trethowan and Mujeeb-Kazi, 2008). The easiest to use are those from the primary gene pool represented by germplasm that share a common genome but which have become isolated from mainstream gene pools such as landraces.

Primary gene pool

While sources of pest and disease resistance from landraces have been used quite extensively in breeding (Cox, 1991), until quite recently such material has not been systematically explored for its potential contribution to abiotic stress adaptation in wheat. A good example comes from Mexico where landraces were introduced by Spanish settlers, grown over a range of rain-fed environments throughout the country, and eventually collected as a genetic resource. Effectively, the populations were subjected to 500 generations of natural and human selection and the surviving genotypes were recently screened for their adaptation to abiotic stress adaptation in controlled field conditions.

Case study 4:
The screening of over 3,000 Mexican landraces for yield under drought suggested considerable phenotypic diversity, and DNA fingerprinting confirmed significant variation between landraces and checks as well as among landraces themselves (Reynolds *et al.,* 2007b). Specific drought adaptive characteristics of the selected landraces were determined and included ability to extract water from the deeper parts of the soil profile. As mentioned above, selected landraces have already been employed in CIMMYT's drought crossing program using biparental and single backcross approaches and have resulted in advanced lines with superior drought adaptation. This example illustrates how physiological interventions can be used with success to pinpoint individual genotypes that show favorable expression of specific PTs to augment the genetic base for stress adaptive characteristics in conventional gene pools.

Secondary gene pool

Inter-specific hybridization with closely-related genomes is a more challenging approach for introducing new allelic variation for stress-adaptive traits. Tetraploid durum wheat has been hybridized with *Aegilops tauschii*, the ancestral donor of the D-genome, to recreate hexaploid bread wheat (Trethowan and Mujeeb-Kazi, 2008). Although the so-called "synthetic" or "re-synthesized" wheat possess significant new variation for adaptation to moisture limited environments, the primary synthetics do not necessarily express better yield under drought compared with modern cultivars; however, they hybridize freely with conventional cultivars and the progeny show a high frequency of drought adaptation.

Case study 5:
Recent physiological studies compared synthetic derivative lines with their recurrent parents under moisture-stressed and irrigated conditions; larger yield and biomass were associated with an increased uptake of soil moisture at all depth profiles down to 1.2 m, resulting in an average 11% increase in water use (Reynolds *et al.,* 2007b).

Such results can be applied to screen primary synthetics for the same characteristic in spite of the fact that they may show no yield advantage in un-adapted backgrounds.

Tertiary gene pool

The tertiary gene pool is composed of related genera of annual and perennial grasses from which transfer of genes requires special techniques. Technical difficulties aside, the main problem with exploiting these materials is the uncertainty with which phenotypic expression can be extrapolated from one genome to another. However, genetically more simple PTs (e.g., osmotic adjustment, transpiration efficiency and heat tolerant metabolism) and perhaps characteristics such as root depth are less likely to show genome interaction than complex agronomic traits, so tertiary gene pool species can potentially be screened for some traits relating to abiotic stress adaptation.

Quantifying the potential of genetic resources

When considering a broad range of genetic backgrounds, many stress-adaptive traits, for example transpiration efficiency, soluble stem carbohydrates concentration and access to water deep in the soil, frequently show superior expression in respective genetic resources compared to checks (Reynolds *et al.,* 2007a).

Case study 6:

An attempt to quantify the theoretical value of these traits, were they to be expressed in check backgrounds, suggested modest yield improvements for traits individually (Figure 2.5) as well as the potential for synergy when combined. The full results of this experiment are reported in Reynolds *et al.* (2007a). However, in summary, traits associated with harvest index (including harvest index itself and potential for remobilization of stem carbohydrates during grain-filling) were those showing the most favorable expression in genetic resources compared with checks. Cooler canopies compared with check lines were also expressed in certain genetic resources under drought and heat stress and suggested potential for increasing water use under drought, and improving radiation use efficiency (via increasing stomatal conductance) under heat stress. In addition, the traits transpiration efficiency and the spectral index RARSc (Ratio Analysis for Reflected Spectra associated with photo-protective carotenoid pigments) were favorably expressed in certain genetic resources under drought and heat stress, respectively. Furthermore, principal component and multiple regression analyses confirmed the idea that traits in different groups show independent expression and could therefore be expected to combine favorably if combined in a single background. Although it cannot be predicted with any degree of certainty that specific combinations of traits will be cumulative or synergistic due to the complex nature of gene action, these data nonetheless show that a relatively large proportion of the phenotypic variation in performance under drought and heat can be explained by a small number of traits. Estimates of broad-sense heritability (e.g., 0.82 for vegetative CT under drought) for these traits and their genetic correlation (e.g., –0.90 for vegetative CT under drought with yield) indicated that in many cases they would be amenable to reliable quantification in parents and verification of expression in segregating progeny and, therefore, result in genetic gain in yield (Reynolds *et al.,* 2007a).

This kind of information has been used for several years in physiological breeding at CIMMYT to strategically design crosses (Reynolds *et al*., 2009) and represents a rapid means of evaluating genetic resources including material that has become isolated from mainstream genepools.

Strategic physiological research

Disciplinary research in physiology can be used strategically in a number of ways, including: (i) developing conceptual models of stress-adaptive response; (ii) dissection of G×E interaction of trait expression to further refine understanding; and (iii) underpinning genetic studies aimed at developing molecular markers and revealing the genetic basis of PTs, through precision phenotyping.

Developing conceptual genotype models

The word 'model' is defined as: "A schematic description of a system that accounts for its known or inferred properties and may be used for further study of its characteristics. A preliminary work or

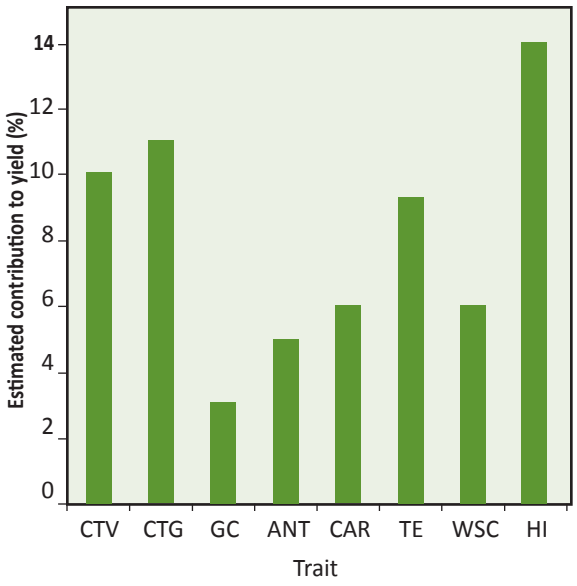

Figure 2.5. Estimated potential contribution to yield when the best trait expression among 25 genetic resources is extrapolated to check (Weebil 1): CTV/CTG = canopy temperature during vegetative/grain-filling stages; GC = ground cover at crop establishment; ANT = days to anthesis, CAR = carotenoid concentration of leaves estimated with spectral reflectance; TE = transpiration efficiency estimated with carbon isotope discrimination; WSC = water soluble carbohydrate concentration in stems shortly after anthesis; HI = harvest index (using data presented in Reynolds *et al*., 2007a).

construction that serves as a plan from which a final product is to be made" (dictionary.com).

When developing conceptual models for crop genotypes there are several possible approaches that will determine which traits are included, depending upon how the model is to be used, which is itself often a function of how much information is available about the crop environment. Examples include the following:

- *General model*; encompasses a comprehensive set of traits whose value will depend largely on the environment in which they are deployed (Figure 2.1).
- *Generic model*; includes a core set of traits that are theoretically useful across a range of environments – for example over a range of heat or drought-stressed environments or combined stresses for which common physiological/genetic strategies may be useful (Figure 2.6).
- *Environment-specific model*; includes all traits that are adaptive in a specific target environment (this should factor in crop responses to climate, soil, biotic and agronomic factors).
- *Simulation models*; in theory these can be applied to any of the above types of models with the following objectives:
    - *To interpolate value of traits across a range of environments/years.*
    - *To hypothetically extrapolate the value of extreme trait expression.*
    - *To put a theoretical value on new trait combinations.*

General model for drought adaptive traits

A general model for drought adaptation was developed at CIMMYT encompassing most of the traits for which evidence has been presented of a potential role in dry environments.

Case study 7:

Traits are grouped according to the main drivers of yield (Figure 2.1) and are, therefore, expected to be relatively discrete genetically. While based on an incomplete knowledge of drought adaptation and its genetic basis, the model helps to establish a broad conceptual framework. The following traits are included related to: (a) *water uptake*: access to water as a result of root depth or intensity that would be indicated by a relatively cool canopy (Reynolds *et al.,* 2007b) and rapid ground cover to shade the soil from evaporation (Richards *et al.,* 2002); (b) *water use efficiency*: constitutive transpiration efficiency (TE = biomass per mm water transpired) estimated by CID of well watered leaves (Condon *et al.,* 2002), and photo-protective anatomical traits such as leaf wax (Richards *et al.,* 2002); (c) *harvest index*: avoidance of reproductive failure that otherwise results in inability to partition assimilates to yield (Turner, 2004), accumulation of soluble carbohydrates in the stem from jointing onwards and their remobilization for grain-filling (Blum, 1998), and alternate *Rht* alleles (Ellis *et al.,* 2005; Rebetzke *et al.,* 1999).

In addition to its value in making crossing decisions, the conceptual platform can also be used as a decision support tool for activities such as: (i) defining suitably contrasting parents for the development of molecular mapping populations; (ii) quantifying the potential benefits of enhanced trait expression, thus indicating targets for exploration of genetic resources; and (iii) identifying common physiological bases between drought and other abiotic stresses (Figure 2.6).

Generic model for stress-adaptive traits

Figure 2.6 presents a generic conceptual model of a core-set of traits for adaptation to dry as well as hot irrigated environments in wheat. It is clear when considering the groups of traits that a number of physiological mechanisms are likely to be of benefit in more than one situation. For example, rapid ground cover is a useful trait for avoiding the wasteful evaporation of soil water under pre-anthesis drought stress (Loss and Siddique, 1994), while under hot, irrigated conditions rapid early growth increases light interception thereby avoiding losses in crop assimilation associated with reduced-tillering at high temperatures (Rawson, 1986). Accumulation of stem carbohydrates and their subsequent remobilization in the post-anthesis period provide an extra source of assimilates for grain growth when either heat or drought stresses are experienced during grain-filling (Blum, 1998). Similarly, root growth that permits better access to soil water has obvious benefit under drought, while enabling heat-stressed canopies to match the high evaporative demand associated with hot, low relative humidity environments, resulting in higher leaf gas exchange rates and heat escape through cooler canopies (Reynolds *et al.,* 2001).

Case study 8:

Using this approach, CIMMYT in collaboration with CSIRO Plant Industry, have designed experiments using the Seri/Babax mapping population (Olivares-Villegas *et al.,*

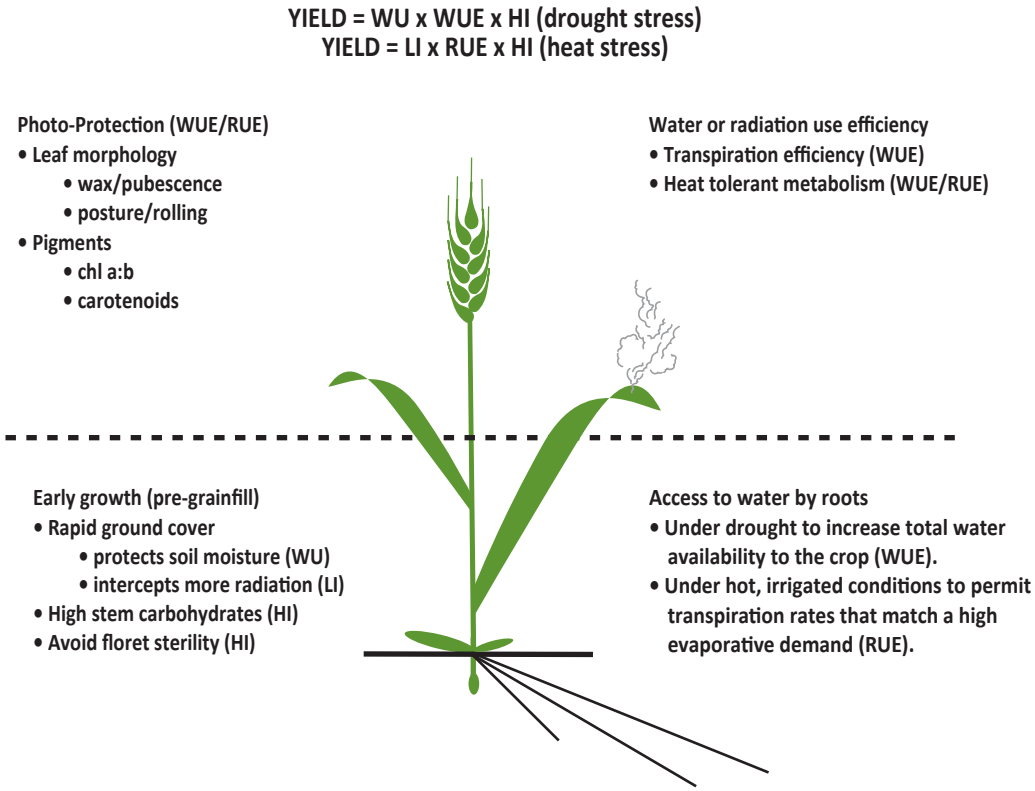

Figure 2.6. Conceptual model for generic traits associated with adaptation to moisture-stressed and/or hot-irrigated environments. Where: HI = harvest index; LI = light interception; RUE = radiation use efficiency; WU = water uptake; and, WUE = water use efficiency. Adapted from Reynolds *et al*., 2007a.

2007) where QTLs for CT have been evaluated under both hot irrigated and drought environments; QTLs on chromosomes 1B, 3B, and 4A indicate a common genetic basis for cooler canopies in both environments (Pinto *et al.,* 2010).

Understanding G×E interaction

Climate change accompanied by even greater variations in temperature and rainfall are making it more imperative to understand G×E interaction, even when developing cultivars targeted for a single locality, as year-to-year variability increases. With this objective, research is aimed at dissecting G×E interaction of yield into its most sensitive components i.e., a particular trait (T) expressed in response to a specific environmental (E) factor at a given phenological (P) stage – abbreviated here as TEP. With reliable phenotypic and environmental data, statistical techniques permit variance associated with G×E to be partitioned to discrete TEPs (Vargas *et al.,* 1998; Brancourt-Hulmel *et al.,* 2003; Crossa *et al.,* 2004; Reynolds *et al.,* 2004; Lillemo *et al.,* 2005). Identification of TEPs paves the way for more detailed dissection of the cellular and molecular mechanisms and genetic basis of yield variation. Such research could potentially lead to breakthroughs in genetic improvement in yield stability, or at least improve predictions of how genotypes may respond to a defined and varied range of environments.

Designing experiments to understand the genetic basis of abiotic stress-adaptive traits

The identification of consistent QTL in wheat mapping studies has been limited by two main factors. The first is the fact that studies involving genetically random populations encompass, by definition, many genotypes with substandard agronomic adaptation; therefore, complex stress-adaptive effects are likely to be masked by unfavorable alleles that would be routinely discarded in early generation plant selection. This first factor can be overcome by employing either the association genetics approach where populations are carefully selected to avoid confounding traits or by

designing crosses more carefully using elite parents that contrast in physiological strategies but not in alleles for height for example.

The second confounding factor is genetic variation in flowering date. This is not generally considered to be problematic if the population's overall maturity class fits the target environment, however, this is often a false assumption and the most likely reason why QTL studies frequently identify *Ppd* and *Eps* loci as those most strongly associated with adaptation to drought. While early flowering is a well-established drought avoidance strategy, many other physiological mechanisms have been documented in crops. In order to determine the genetic basis of these mechanisms, which are likely to be complex, the confounding effect of phenology must be controlled experimentally. It is well established in wheat that key developmental processes such as kernel set are determined within relatively narrow developmental windows and can be especially sensitive to environmental conditions including moisture stress (Fischer, 1980; Fischer, 1985; Abbate *et al.,* 1997). Therefore, genotypes growing side-by-side but which pass through key developmental stages on different dates are likely to trigger different signal transduction pathways and different stress-adaptive responses at the whole plant level due to variation in simple weather parameters. Therefore, the only way to eliminate this confounding factor is to minimize differences in flowering time in the experimental population (Reynolds *et al.,* 2009). This is relatively easy using the association genetics approach especially if pre-existing data on flowering time is available over a range of environments. If using RILs or doubled-haploids it is necessary to characterize potential parents for their phenotypic expression and G×E for flowering time in the appropriate range of target environments as well as genotype the lines for *Ppd*, *Vrn* and *Eps* alleles as much as possible. Yet despite efforts to minimize confounding variation in development with performance under drought, examples exist where even a relatively small range in flowering (e.g., less than 10 days in Rebetzke *et al.,* 2010) still show confounding effects in traits related to grain yield (e.g., CT or transpiration efficiency) and/or grain yield *per se*.

Once populations have been developed that are not confounded by agronomic traits, the same high-throughput approaches as used in early generation screening can be used for precision phenotyping in order to genetically dissect the traits of interest (Olivares-Villegas *et al.,* 2007; Pinto *et al.,* 2010).

Genetic control of performance under abiotic stress

Genetic complexity

The availability of suitable populations, and development of robust phenotyping tools has led to an improved understanding of the genetic basis underpinning performance in environments limited by abiotic stress. Genetic control can vary from single major genes of large effect that improve tolerance to a subsoil constraint (e.g., salt; Huang *et al.,* 2006) to polygenic control of many genes of small effect such as occurs with transpiration efficiency, CT, stem carbohydrates or coleoptile length (Table 2.1). Selection of a major gene to overcome root disease (e.g., Lagudah *et al.,* 1997), or improve root growth in a chemically-hostile soil (e.g., Delhaize *et al.,* 2004) provides a simple means of increasing root biomass to explore and acquire soil nutrients and water for biomass and reproductive growth. These will not be considered here but are nonetheless critical in pre-adapting a crop to a broad range of soils commonly encountered by a new variety.

Many yield and fitness-related traits have been demonstrated to be genetically complex whether investigated in crop breeding or natural populations (e.g., Juenger *et al.,* 2005). Fortunately, gene action is commonly such that selection for improvement in a trait can be readily achieved if the correlation of phenotype and genotype is strong; i.e., heritability is high to increase breeder confidence. Where this correlation is not strong and heritability is low, the phenotype may reflect influence of environment and/or sampling by the breeder. No genetic gain here is possible as selection is on an environmental covariance and not based on favorable alleles transmitted from parent to progeny. Thus, successful selection is contingent on populations containing adequate additive genetic variance and high narrow-sense heritability. The target trait/s should also be simple and inexpensive to measure particularly in mass selection where many families may be evaluated. Narrow-sense heritability (h^2) can be calculated on a line-mean basis as:

$$h^2_{Line\text{-}mean} = \sigma^2_A / (\sigma^2_A + \sigma^2_{AE}/n_e + \sigma^2_{Residual}/n_r n_e) \quad \text{Equation 2.1}$$

Where: σ^2_A , σ^2_{AE} , and $\sigma^2_{Residual}$ are estimates of the additive, additive × environment and residual variances, respectively, and n_e and n_r are the number of environments and replications per environment, respectively.

Table 2.1. Traits with potential for improving wheat performance in water-limited environments. Details are also provided on genetic control for each trait.

Trait	Ease of screening	Heritability	Chromosomal location of genes	Source
Drought stress				
Phenology	Simple	High	2A, 2B, 2D, 3A, 3B, 5A, 5B, 5D, 6A, 6B, 7A, 7B	Snape *et al*. (2001)
Early vigor	Simple	High	2D, 4B, 4D, 5A	Rebetzke *et al*. (2001)
Leaf rolling	Simple	High	Unknown	Sirault *et al*. (2008)
Restricted-tillering	Simple	High	1A	Spielmeyer and Richards (2004)
Canopy temperature	Simple	Moderate	1B, 2B, 3B, 4A	Pinto *et al*. (2010)
Coleoptile length	Simple	Moderate	2B, 2D, 4A, 4B, 4D, 5D, 6B	Rebetzke *et al*. (2007)
Glaucousness	Simple	Moderate	2B, 2D	Tsunewaki and Ebana (1999)
Photosynthetic capacity	Simple	Moderate	1B, 1D, 2D, 3B, 4A, 4B, 4D, 5B, 6B, 7A, 7B	Rebetzke unpub. data
Carbon isotope discrimination (leaf)	Difficult	High	1B, 1D, 2D, 3B, 4A, 4B, 4D, 5A, 7A, 7B	Rebetzke *et al*. (2008b)
Carbon isotope discrimination (grain)	Difficult	High	1D, 2A, 2D, 4B, 4D, 6D, 7B	Rebetzke unpub. data
Harvest index	Difficult	High	2B, 2D, 4B, 4D	Ellis *et al*. (2002)
Osmotic adjustment	Difficult	Moderate	7A	Morgan and Tan (1996)
Staygreen	Difficult	Moderate	2B, 2D	Verma *et al*. (2004)
Stem carbohydrates	Difficult	Moderate	1A, 2B, 2D, 3B, 4B, 5B, 6B, 7A, 7B	Rebetzke *et al.* (2008a)
Root biomass	Difficult	Low	1B	Waines and Ehdaie (2007)
Stomatal conductance	Difficult	Low	1B, 2A, 2B, 2D, 4A, 4B, 4D, 7A, 7B	Rebetzke *et al.* (2010)
Rate-of-grain-filling	Difficult	Uncertain	Unknown	Whan *et al*. (1996)

The additive genetic variance provides a measure of the effect of substituting one allele at a locus for another and can be estimated through measures of the level of inbreeding and the genetic relationship among sibs via the covariance among relatives (Falconer and Mackay, 1996). Where heritability is low, alternate populations containing greater additive genetic variance (and high mean) should be considered or sampling modified to reduce non-genetic variation.

Assessing value of physiological traits in breeding

We have demonstrated that other attributes confer adaptation to improve yield in water-limited environments. These may improve water-use and/or water-use efficiency to increase biomass, or partitioning to grain to increase harvest index (Passioura, 1977). These surrogate traits are often inexpensive to measure, have high heritability and/or contribute new genetic variance in a manner that can be targeted and without disrupting the framework or desired ideotype for the stress environment. If the additive genetic correlation (r_A) between two traits (X and Y), and their narrow-sense heritabilities h^2_x and h^2_y are known, the correlated response of trait Y to selection on trait X ($\Delta_{GY.X}$) can be predicted by:

$$\Delta_{GY.X} = k\, \sigma_{py}\, h_y\, h_x\, r_A \qquad \text{Equation 2.2}$$

Where: k is the standardized selection differential, and σ_{py} is the phenotypic standard deviation for trait Y (Falconer and Mackay, 1996). If $h_y < h_x r_A$ then selection for trait X will result in a greater change in trait Y than direct selection for Y. The genetic correlation for two variables can be readily estimated from analysis of covariance. Linkage disequilibrium (population type), chromosomal linkage and pleiotropy can all contribute toward two traits being genetically correlated. However, owing to recombination, only pleiotropic effects are likely to maintain a genetic association over cycles of crossing and selection in a breeding program. Most studies report only phenotypic correlations, which differ from genetic correlations as phenotypic correlations also contain environmental and sampling covariance components. Only the genetic covariance component of this correlation is responsive to selection.

A number of traits have been reported as under pleiotropic control with other traits at one or more loci. For example, CID (measured prior to anthesis) shows a strong additive genetic correlation with grain yield and biomass (Rebetzke *et al.,* 2002). Indirect selection for yield and biomass is more effective using CID when assessment is made on single plots. This is because large G×E interaction and residual variances reduce narrow-

sense heritability for yield and biomass in unreplicated plots. The relative benefit of correlated genetic gain is reduced with replicated testing over blocks and environments to increase heritability of yield but less so for biomass. Notwithstanding, CID offers potential for screening unreplicated families in the early stages of yield testing such as occurs when little seed is available or while early generations are still genetically heterogeneous. By selecting families with low CID, the breeder is restricting the costly stages of replicated yield-testing across environments to those families with a greater likelihood of high yield in droughted environments. Importantly, by fixing favorable alleles for CID early, greater emphasis can be placed on selection for other genes important for adaptation to water-limited environments.

Quantitative trait loci

Remarkably, little is known or understood of the genetic basis of wheat performance under drought. The development and availability of molecular markers, and their integration into genetic maps across a number of genotyped populations is confirming the genetic complexity understood from traditional quantitative genetic studies. However, good genetic maps can allow for trait dissection into component QTL, and insight into the underlying genetic basis for variation and covariation in a trait. For example, numbers of genes and their interactions, and gene action can be determined even for low heritability traits. Information around breeding complexity including linkage, linkage disequilibrium and pleiotropy may also be gleaned through mapping studies particularly if extended to multiple populations (e.g., Rebetzke *et al.,* 2007). Key processes involved in plant growth and development can then be inferred from what is already known of the physiology. For example, development of sequence-based perfect markers for the *Rht-B1b* and *Rht-D1b* dwarfing genes has confirmed their effects on tissue-insensitivity to endogenous gibberellins, and reduction in both coleoptile and early leaf area development (Rebetzke *et al.,* 2001).

Perhaps the greatest opportunity for use of linked markers in breeding for performance under drought lies in their use for selection of difficult, low heritability, recessive (gene expression) or expensive to measure traits such as transpiration efficiency, CT or stem carbohydrate concentration (Table 2.1). In the absence of recombination, markers linked to target QTL have a heritability of 100% thereby allowing rapid generation advance. From the indirect selection formula above, the benefit of using a 100% heritable molecular marker over direct selection for grain yield (i.e., $\Delta_{GY.X}$ /Δ_{GY}) equals r_A/h_{yield}. This can be extended to multiple QTL so that the sum of absolute genetic correlations with yield must exceed h_{yield}. Assuming the marker is diagnostic (robust marker and perfect gene association), and evaluation and generation costs are similar to that of yield, marker-aided selection in variable environments should produce greater genetic gain for yield. However, this is likely to be limited to traits controlled by fewer QTL and the likelihood of identifying genotypes containing all or most QTL in smaller populations commonly used by breeders (Bonnett *et al.,* 2005). Enrichment strategies may aid in overcoming large numbers of QTL in biparental –and backcross– based breeding programs.

Marker-assisted selection is likely to provide the greatest benefit to breeders targeting novel traits from distantly-related germplasm. For example, markers are extremely useful in identifying recombinants when targeting positive genes linked in repulsion to genes of negative effect. A number of QTL have been identified with potential for selection of improved performance in water-limited environments (Table 2.1). Critical to successful implementation of linked markers in breeding are well-defined QTL for improved productivity. Populations under evaluation should be representative of the target populations under selection; genotyping should be adequate in development toward a good molecular map with even marker coverage and quality mapping (good markers and map development); phenotyping is sound and repeatable, and commonly represents evaluation in environments representative of the target environment. Molecular marker types affect ease and cost of implementation in breeding. For example, it is easier to implement microsatellites than diversity arrays technology (DArTs) and amplified fragment length polymorphism (AFLPS). Single nucleotide polymorphisms (SNPs) are most amenable to very high-throughput but little SNP information is currently available for wheat. DArTs are showing potential as an inexpensive high-throughput marker tool and provide opportunity toward whole genome mapping for use by breeders. Finally, methods are being developed aimed at efficient marker implementation strategies in breeding programs. These strategies may vary depending on breeding program structure and goals, genetic complexity of traits, cost and type of markers etc. (Wang *et al.,* 2007).

Case study 9:
Access to populations developed for improved performance under drought has facilitated yield dissection into component traits. For example, the Cranbrook/Halberd population was developed from a cross between a CIMMYT-sourced, widely adapted variety, Cranbrook, and the widely-adapted Australian variety Halberd. Doubled-haploid lines from this population were assessed under irrigated conditions in three years for CT and stomatal conductance (after Rebetzke *et al.,* 2010). After genotyping of individual lines, a multi-environment QTL analysis was undertaken. Stomatal conductance was assessed using a viscous-flow porometer permitting all 160 lines to be assessed in less than 2 hours. Of the 24 sampling dates, stomatal conductance was assessed for pre- versus post-flowering events, and prior to and following irrigation. Many QTL were identified for CT and stomatal conductance alike. Further, QTL for stomatal conductance commonly co-located independently of stage of development and/or soil water status. Importantly, many stomatal and CT QTL coincided, confirming the importance of transpiration as a driver for genotypic differences in CT.

Summary of physiological applications

The main areas in which physiological research, focused at the whole plant and field level, can be applied in breeding have been addressed. In summary, (i) physiological characterization of potential parents provides background information that can be used in strategic crossing to accumulate stress-adaptive genes; the information can be added to the catalogue of information on generically important traits (such as quality and disease resistance) already used routinely in planning crosses; (ii) physiological traits can be selected for in early generation progeny to increase favorable gene frequencies and discard physiologically inferior lines before expensive yield testing; because of the large numbers of lines involved and the fact that information will generally have a one-time use the traits should be quick and inexpensive to measure; and (iii) characterization of genetic resources may permit the identification of valuable genotypes that have superior physiological traits despite being expressed in substandard or obsolete agronomic backgrounds.

In terms of strategic research, gene discovery can be facilitated by: (i) dissecting stress-adaptation conceptually into measurable physiological components, (ii) applying rigorous phenotyping procedures in well designed experimental populations thereby controlling factors such as phenology that otherwise may confound trait expression, and (iii) gaining a better understanding of adaptive processes through combining these two approaches with statistical procedures that permit variance associated with G×E to be partitioned into TEP combinations.

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Chapter 3: Nitrogen and phosphorus use efficiency[#]

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Abstract

Globally, the nitrogen use efficiency of wheat is only 35%. This low efficiency means that a large proportion of the N that is applied by farmers is lost, increasing the cost of production and having important negative consequences on the environment. The efficiency of P fertilizer ranges from around 10 to 30% in the year that it is applied. Breeding and agronomic management are the two main strategies that can help improve nutrient use efficiency. Characterizing wheat germplasm for uptake and utilization efficiency will be important to better understand the mechanisms associated with improved efficiency. If uptake is the dominant trait then root characteristics such as root length density, root depth, the production of root exudates or the ability of the roots to associate with vesicular-arbuscular mycorrhiza (VAM) should be analyzed. In contrast, if utilization efficiency is the most important trait, then harvest index and biomass production efficiency should be studied. The breakup of nutrient use efficiency into uptake and utilization should also facilitate the identification of molecular markers that can be later used in a breeding program.

Introduction

The improvement of nutrient use efficiency in wheat cropping systems can be achieved through two main strategies:

1) By adopting more efficient crop management practices (such as nutrient rate, timing, source, and placement); and
2) Breeding more nutrient use efficient cultivars.

Although both are important, this paper will focus on improving nutrient use efficiency (specifically nitrogen and phosphorus) through plant breeding. More detailed guidelines on how to improve nitrogen use efficiency in wheat through crop management have been described elsewhere (Ortiz-Monasterio, 2002).

It is important to clearly define nutrient use efficiency before describing methods for improving it. We have found the definition proposed by Moll *et al.* (1982) useful for looking at genetic differences in nitrogen use efficiency among wheat cultivars – though the concept was developed using nitrogen as an example, it can also be applied to phosphorus. These authors define nitrogen and phosphorus use efficiency in wheat as grain yield per unit of nutrient supplied (from the soil and/or fertilizer). They divide nutrient use efficiency into two components: (1) uptake, or the ability of the plant to extract the nutrient from the soil, and (2) utilization efficiency, or the ability of the plant to convert the absorbed nutrient into grain yield.

Hence:

Nutrient Use Efficiency = Uptake Efficiency × Utilization Efficiency

$$Gw/Ns = Nt/Ns \times Gw/Nt \qquad \text{Equation 3.1}$$

Where: Gw = grain dry weight, Nt = total above-ground plant nutrient at maturity, and Ns = nutrient supplied. All units are in g m^{-2}. Utilization efficiency can also be subdivided into two components, as suggested by Ortiz-Monasterio *et al.* (1997a), and expressed as:

Utilization Efficiency = Harvest Index × Nutrient Biomass Production Efficiency

$$Gw/Nt = Gw/Tw \times Tw/Nt \qquad \text{Equation 3.2}$$

Where: Tw = total above-ground plant dry weight at maturity. Utilization efficiency can also be expressed as:

Utilization Efficiency = Harvest Index × Inverse of Total Nutrient Concentration in the Plant

$$Gw/Nt = Gw/Tw \times 1/Nct \qquad \text{Equation 3.3}$$

Where: Nct = total nutrient concentration in the plant as a percentage.

[#] This chapter does not attempt to make an exhaustive review of the literature but rather presents practical information based on CIMMYT Wheat Program experience working on nitrogen and phosphorus use efficiency.

The definition for nitrogen use efficiency (NUE) proposed by Moll *et al.* (1982) can be used for both low and high input situations. However, there are other nutrient efficiency classification systems that take into account performance both in the presence and in the absence of nutrient stress as, for example, the system proposed by Gerloff (1977), which separates cultivars into four groups based on their response to P. The groups are: (1) efficient, responder; (2) inefficient, responder; (3) efficient, non-responder, and; (4) inefficient, non-responder. An efficient cultivar has higher yield than the other cultivars under low nutrient supply, while a responder cultivar has higher yield under high nutrient supply. This classification system groups cultivars based on performance under low (efficient vs. inefficient) and high (responder vs. non-responder) nutrient supply, and allows the identification of those cultivars with adaptation to a range of soil nutrient conditions.

CIMMYT and its predecessor have been generating wheat germplasm for the developing world since the 1940s. CIMMYT bread wheats were first and most rapidly adopted in irrigated areas of the developing world (e.g., the Yaqui Valley in Mexico, the Indian Punjab, and the Pakistani Punjab) (Byerlee, 1996). Fertilizer is widely applied (sometimes at sub-optimal levels) by farmers in those areas as a way to correct nutrient deficiencies. However, in other target environments farmers do not apply fertilizers because they cannot afford them or because inputs are simply not available. It is therefore essential that CIMMYT wheats be widely adapted and able to grow in different (low and high) soil nutrient situations.

In this chapter we will discuss how studying the individual components of nutrient use efficiency (uptake vs. utilization) under different nutrient levels can help us gain a better understanding of the opportunities and limitations of breeding for nitrogen and phosphorus use efficiency.

Nitrogen

Bread wheat

With the adoption of the input-responsive and lodging tolerant semidwarf wheat cultivars that launched the green revolution in the 1960s, the use of nitrogen fertilizer rapidly increased, as did yields. Thanks to the introduction of this new genetic material, the amount of grain produced per unit of N applied has increased significantly (Figure 3.1).

We have documented the changes in the nitrogen use efficiency of CIMMYT bread wheats developed between 1950 and 1985 under medium to high levels of N fertility. Results show that more recent CIMMYT cultivars out yield both earlier semidwarfs and old tall cultivars at all nitrogen levels (Ortiz-Monasterio *et al.,* 1997a). This indicates that the current strategy of selecting and evaluating under medium to high N levels has resulted in germplasm that produce higher yield when grown under low or high levels of N fertility. CIMMYT bread wheats from 1950 to 1985 gradually became not only more responsive to N inputs, but also more efficient in their use, according to Gerloff's classification (1977). As a result, CIMMYT bread wheats do not require more N than the old tall cultivars; in fact, they often need less N to produce the same yield. In addition, since CIMMYT bread wheats are more responsive to N application, the optimum economic rate is higher than that for the old tall cultivars (Ortiz-Monasterio *et al.,* 1997a).

Although our current breeding strategy has been successful in addressing the needs of both low input and high input wheat-producing environments, we are interested in identifying alternative selection methods that might be even more successful. To that end, we characterized relevant CIMMYT germplasm for two main components of nitrogen use efficiency: nitrogen uptake and utilization efficiency. We found that there is genetic diversity for both traits.

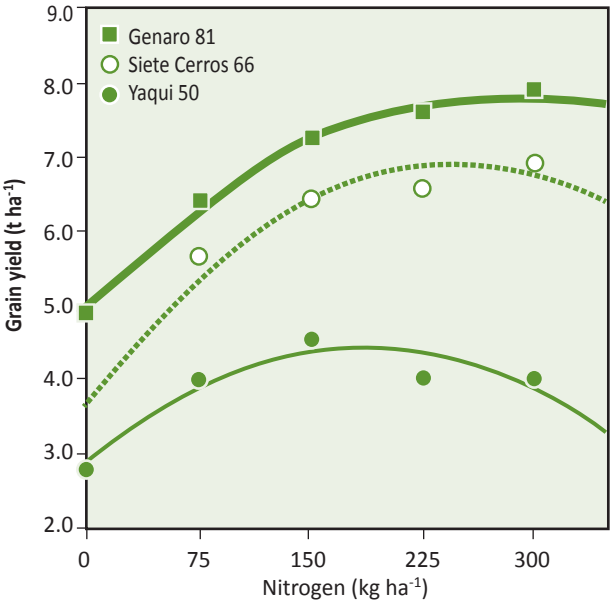

Figure 3.1. Response of tall (Yaqui 50) and semidwarf spring wheat cultivars to increasing levels of nitrogen fertilizer.

Our work and that of others has shown that the level of N in the soil plays a very important role in the expression of uptake and utilization efficiency (Dhugga and Waines, 1989; Ortiz-Monasterio *et al.* 1997a). However, the effect of different soil N levels on the expression of a given component of nitrogen use efficiency in spring wheat may be affected by genotype and/or location. Dhugga and Waines (1989) found better expression of uptake efficiency under high soil N and better expression of utilization efficiency under low N. In contrast, Ortiz-Monasterio *et al.* (1997a) found better expression of uptake efficiency under low N conditions and better expression of utilization efficiency under high N conditions. These findings notwithstanding, available information has shown that the level of soil N may be manipulated together with genetic variability to develop cultivars with improved performance under both low and high input conditions (Ortiz-Monasterio *et al.,* 1997a; van Ginkel *et al.,* 2001).

Nitrogen uptake vs. utilization efficiency

In view of the above, an important aspect of our current research is to identify the best selection strategies for developing genotypes that produce higher grain yields as a result of their improved uptake and/or utilization efficiency. The question is which component to emphasize.

Utilization efficiency has ecological appeal, since it means either higher yields with the same nutrient levels in the plant or the same yield with lower nutrient levels in the plant, which requires fewer resources. As indicated earlier, utilization efficiency can be broken down into harvest index (HI) and biomass production efficiency. If we analyze which component has been most associated with utilization efficiency gains in the past, we find that most progress has been associated with improvements in HI rather than in biomass production efficiency (Equation 3.2). However, Fischer (1981) and Calderini *et al.* (1995) suggest that the possibilities of further improving HI as a way to increase grain yield are limited.

There are two main routes for making further progress in grain yield through better utilization efficiency: (1) to increase grain yield while maintaining or reducing nutrient concentration in the plant, and (2) to reduce total nutrient concentration in the plant while increasing or maintaining grain yield (Equation 3.2). Most CIMMYT high yielding wheats grown under a wide range of N levels tend to have, on average, a nitrogen HI of around 75%. In other words, 75% of the plant's total N is found in the grain at maturity. This means that cultivars with higher utilization efficiency, which is not associated with HI (assuming a constant HI), will have lower protein concentration in the grain. This can negatively affect the grain's bread making quality and nutritional value, unless the percent protein reduction is compensated by a proportional improvement in protein quality.

We should point out that bread making quality, which is a key issue for breeding programs in the developed world, is now gaining significance for breeding programs in developing countries. The original focus of many wheat breeding programs in developing countries—i.e., generating sufficient yield increases to feed their rising populations—has expanded to include fulfilling farmers' need to produce high quality grain that competes well on the market.

The nutritional value of wheat grain is another issue that is gaining in significance due to its perceived potential to better the nutrition of developing country's populations. The nutrient content of wheat grain is negatively affected by lower protein concentration in the grain. Studies in Mexico and Argentina have shown that protein concentration in the grain has decreased as grain yield has increased throughout the history of breeding (Calderini *et al.,* 1995; Ortiz-Monasterio *et al.,* 1997b). This reduction in protein N has been associated with higher utilization efficiency. Thus an important challenge for breeding programs in both developed and developing countries will be to continue to improve nitrogen use efficiency and, at the same time, maintain or improve the bread making quality and/or nutrient content of wheat grain.

A similar dilemma arises when uptake efficiency, the other component of nutrient use efficiency, is implemented as a strategy to improve grain yield. For resource poor farmers who cannot afford fertilizers and grow wheat under low input conditions, the development of cultivars with high N uptake efficiency may not be desirable because it may accelerate soil nutrient mining. In contrast, in high input environments, high uptake efficiency is a very desirable trait because residual soil N (soil N not absorbed by the crop) may either leach through the soil to pollute waterways with soil nitrates or escape into the atmosphere as N_2, N_2O, NO_X, or NH_3.

Nitrate leaching has been well documented for many years in many developed countries (CAST, 1985; Keeney, 1982). The problem tends to be associated with the application, especially in sandy soils, of more

nitrogen than is required for producing maximum yield. However, nitrogen losses to the environment are also being reported in developing countries because of the increased use of nitrogen in these countries. These types of reports are more likely to increase in the future since in 2006, 90.86 million tons of N fertilizer was applied onto crops, and of that approximately 70% was applied in the developing world (Heffer, 2009).

There are wheat production systems in the developing world where very high rates of N fertilizer are already being applied —for example, in certain wheat growing areas of Mexico, Egypt and particularly in China. In the high input wheat systems of northwestern Mexico, where farmers apply an average of 250 kg N ha^{-1}, researchers have recorded large N leaching losses (Riley *et al.,* 2001), high emissions of greenhouse gases into the atmosphere (Matson *et al.,* 1998), and it has been shown that fertilization and irrigation events by farmers in the Yaqui Valley coincide with algae blooms in the Golf of California (Beman *et al.,* 2005). If cultivars and crop management systems remain as they are now, as N rates increase, the problems of N leaching and greenhouse gas emissions (N_2O), common in many industrialized countries, will also become widespread in the high input areas of developing countries.

Strategies for improving nitrogen use efficiency

Grain yields of CIMMYT bread wheats developed between 1950 and 1985 have gradually increased. We evaluated these wheats at N levels commonly applied by farmers in irrigated areas of the developing world (75–150 kg N ha^{-1}) and found that 50% of the yield gains were associated with higher nitrogen uptake efficiency and the other 50% with better utilization efficiency (Ortiz-Monasterio *et al.,* 1997a). This clearly shows that improvements in both uptake and utilization efficiency have been important in the past and most likely will continue to be in the future.

Hence, it is important to select and evaluate for nitrogen use efficiency under both low and high nutrient conditions; this allows the researcher to identify genotypes that perform well under nutrient stress (low input, efficient) and genotypes that respond well to high input conditions (responder) (Figure 3.2).

In a study that evaluated the selection of segregating populations using CIMMYT's shuttle breeding under five N selection treatments (low, medium, high, alternating high-low, and alternating low-high N levels), we found that the highest yielding germplasm tested in medium or high N input environments was obtained by alternately selecting (from F_2 to F_7) under high in Yaqui and low N conditions in Toluca. No differences between N selection treatments were observed when the resulting lines were evaluated in low N environments (van Ginkel *et al.,* 2001).

We conclude that the relative importance of both uptake and utilization efficiency will vary according to the needs of different production systems. Given that wide adaptation is a primary objective in breeding CIMMYT germplasm, we will continue to improve both components.

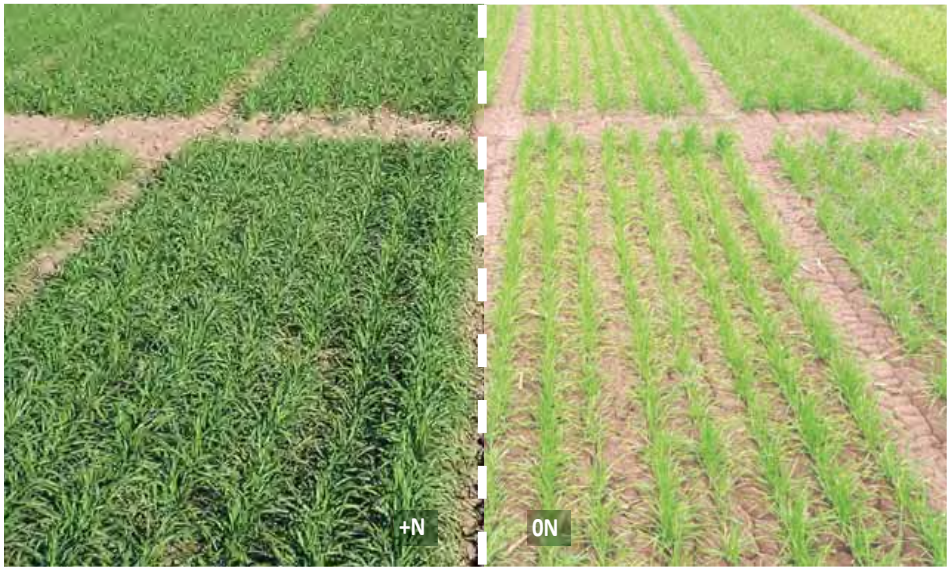

Figure 3.2. Varieties with (+N; at 250 kg N ha^{-1}) and without (0N) nitrogen application, at CENEB, Yaqui Valley, Sonora, Mexico. (Photograph: JI. Ortiz-Monasterio).

Durum wheat

A three year field study looking at genetic gains for nitrogen use efficiency in durum wheat from 1940 to 1989 at four levels of N (0, 75, 150 and 300 kg N ha^{-1}) showed very limited progress for nitrogen use efficiency under the 0 N treatment which represented severe nitrogen stress. Grain yield at the 0 kg N ha^{-1} level was only 39% of that achieved under 300 kg N ha^{-1}. Genetic gain at the other three levels of N (75, 150 and 300 kg N ha^{-1}) were statistically significant and increased as the N rate increased. This gradual increase in genetic gains as the N rate increased can partially be explained by lodging of the tall variety (*Barrigion Yaqui*), particularly at the 150 and 300 kg N ha^{-1} (Ortiz Monasterio *et al.,* unpublished data). In contrast, bread wheat breeders have made the same level of relative progress for nitrogen use efficiency under low, medium or high levels of nitrogen (Ortiz Monasterio *et al.,* 1997a). This is interesting since durum and bread wheat breeders at CIMMYT have been selecting in the same locations (e.g., Yaqui and Toluca) in Mexico when applying the shuttle breeding program and they have used similar selection methodologies in the field. However, durum wheat seems to be lagging behind bread wheat in terms of genetic gains under severe nitrogen stress. This suggests that perhaps there is less genetic variability for this trait in durum, which could be limiting progress.

Phosphorus

Many soils have large reserves of total phosphorus, but low levels of "available" phosphorus. Al-Abbas and Barber (1964) reported that total soil P is often 100 times higher than the fraction of soil P available to crop plants. Our objective in breeding for P efficient and responsive cultivars has been to identify wheat cultivars that can access P not usually available to the average cultivar under low P conditions (P efficiency), but also respond to P applications (P responsiveness).

As in the case of N, CIMMYT has been breeding under medium to high levels of P in the soil. Preliminary results suggest that phosphorus use efficiency in CIMMYT bread wheat cultivars between 1950 and 1992 has improved under low as well as high levels of P fertility (Ortiz-Monasterio *et al.,* unpublished data). Again, using Gerloff's (1977) definition, CIMMYT bread wheat germplasm has become more efficient as well as more responsive to P applications during that time period.

There is little information on the contribution of uptake and utilization to total P use efficiency in wheat. In a CIMMYT study, the relative importance of uptake and utilization in spring wheat was evaluated in two different environments: a rainfed area with Andisols in the central highlands of Mexico and an irrigated, low-altitude area with Vertisols in northwestern Mexico. Uptake and utilization were characterized in a set of CIMMYT lines. Results showed that in an acid Andisol with no Al toxicity, uptake was more important than utilization in explaining P use efficiency. In contrast, in the same group of genotypes utilization efficiency was more important when evaluated in an alkaline Vertisol (Manske *et al.,* 2001). In these two different environments it was shown that there was genetic diversity for both uptake and utilization efficiency in the CIMMYT material tested.

This study shows that, as in the case of N, the environment where a given set of genotypes is evaluated plays a very important role in the expression of P uptake and utilization efficiency. However, in the case of P, what influenced the expression of uptake vs. utilization was not low P vs. high P, but rather the effect of location. At this point it is not clear how much of the location affect is due to soil effects and how much is due to above-ground effects (radiation, temperature, etc.) (Manske, 1997). Also to be determined is why the same genetic material expresses genetic diversity for uptake efficiency in some environments but not in others.

Evaluating germplasm under both low and high nutrient conditions allows the identification of genotypes that perform well under nutrient stress (low input) and genotypes that are responsive to high input conditions (Figure 3.3 and 3.4). Preliminary data suggest that evaluating advanced genetic materials under low P conditions is useful for identifying exceptional germplasm for P stress conditions. When advanced genetic materials are evaluated only under high input conditions, sometimes we fail to identify genotypes that are outstanding under low P conditions. This germplasm might be discarded if it is tested only under high input conditions (Trethowan *et al.,* unpublished data). Hence the importance of selecting and evaluating under both low and high nutrient conditions.

In acid soils, P deficiency is often accompanied by Al and Mn toxicity, especially when soil pH is below 5.4. Evidence available so far indicates that genes controlling adaptation to Al and Mn toxicity and tolerance to P deficiency appear to be independently inherited and recombinable (Polle and Konzak, 1990). Therefore the recommendation is

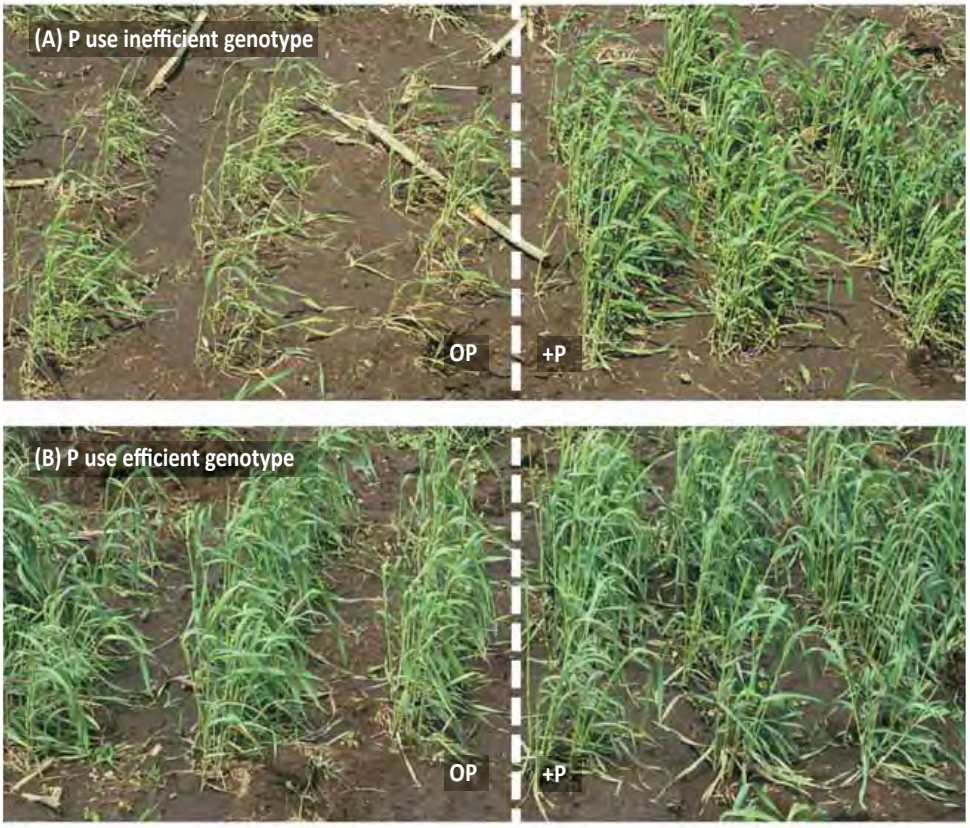

Figure 3.3. Screening plots for phosphorus use efficiency, Patzcuaro, Michoacan, Mexico. In both pictures, plants on the right received 80 kg P_2O_5 ha^{-1} (+P), while those on the left received none (0P): (A) P use efficient genotype, (B) P use inefficient genotype. (Photographs: JI. Ortiz-Monasterio).

Figure 3.4. Screening plots for phosphorus use efficiency at CENEB, Yaqui Valley, Sonora, Mexico. The plants at the bottom of the picture received 80 kg P_2O_5 ha^{-1} (+P), while those at the top received none (0P). (Photograph: JI. Ortiz-Monasterio).

that screening for P use efficiency be done first in soils without Al or Mn toxicity, if possible. Once elite materials have been selected for P use efficiency in the field, they can be screened for Al and/or Mn toxicity either in the field or in hydroponics.

We suggest that screening for P uptake efficiency under nutrient culture conditions be avoided until a satisfactory correlation between performance in the field and in nutrient cultures has been shown. This is particularly important for P, given that very little of the crop's P requirement is provided by mass flow (transpiration flow). Diffusion is more important, but difficult to simulate in solution culture. It is generally recognized that nutrient culture should be limited as a screening environment primarily because of the low correlation of the results with those of field tests. Nutrient cultures cannot simulate the soil–plant interface properly.

Phosphorus uptake vs. utilization efficiency

Phosphorus utilization efficiency (grain yield per unit P in the plant) is dependent on the plant's internal P requirement. Increased harvest index, P harvest index, and low P concentration in grain may improve P utilization efficiency (Jones *et al.,* 1989; Batten, 1992).

Most CIMMYT high yielding wheats have a P harvest index of about 80% under irrigated conditions. As in the case of N, breeding for higher P utilization efficiency, given the small margin to breed for higher HI, will result in lower P concentration in the grain. Selection for wheat genotypes that remove small amounts of P from the soil due to their low P grain concentration can contribute to sustainable land use (Schulthess *et al.,* 1997). Genotypic differences in grain P concentration are fairly consistent across environments (Schulthess *et al.,* 1997). If breeders in Australia, which is a major exporter of wheat grain but has soils that are poor in P availability, can reduce the P concentration in the grain of wheat cultivars, farmers will be able to purchase substantially less P to replace the P exported with the grain.

However, the strategy of reducing P concentration in the grain has a limit. There is evidence that excessively low P concentration in the grain affects seed vigor, particularly in P deficient soils. A study on a set of historically important CIMMYT semidwarf bread wheats showed that P concentration in the grain decreased significantly over the years as a result of breeding (Manske, 1997). Similar information is available from a wheat breeding program in Argentina (Calderini *et al.,* 1995). As in the case of N, this reduction in P concentration in the grain is associated with gains in utilization efficiency.

Most nitrogen absorbed by plants comes from mass flow (i.e., soil water moves towards the roots as the plant loses water through transpiration), but phosphorus is absorbed mainly by diffusion through gradients created by root absorption. Phosphate concentrations in soil solution are small (<0.05 μ g^{-1}) compared to nitrate-N concentrations (100 μ g^{-1}), and very little phosphate is moved to the roots by capillary water movement. The amount of P extracted is limited by P concentration at the root–soil interface, which means that wheat roots have to grow to come into contact with new soil from which they can extract phosphate. Root length is thus a major determinant of the absorbing surface area.

Wheat genotypes with greater root length density are able to take up more phosphorus (Manske *et al.,* 2000). When P supply is low, the correlation between root length density and P uptake or grain yield is usually 0.50–0.60, but with adequate P supply this correlation is lower. In some environments, P uptake can be more important than utilization efficiency. In areas where uptake is the main component associated with P use efficiency, P uptake efficiency holds great promise for improving P use efficiency, since soils with relatively high levels of total P in the soil often have low levels of available P.

Strategies for improving phosphorus uptake efficiency

Different approaches can be used to enhance P uptake (Polle and Konzak, 1990; Johansen *et al.,* 1995) and these are detailed below:

Increasing the root surface/soil contact area
This can be achieved by modifying root morphology. For a constant level of root biomass, roots with higher specific root length (i.e., roots with smaller diameter) can cover a larger surface area. A second approach for achieving the same objective is through increased hair root development. Root fineness or branching is an important determinant of P uptake efficiency in wheat (Jones *et al.,* 1989). This route seems promising given that there is evidence of large genetic variability for this trait in wheat. However, the time consuming and labor intensive methodologies currently in use limit its application in breeding programs where large numbers of genotypes need to be screened.

Increasing the effective root area

Root symbiosis with arbuscular mycorrhizal fungi (AMF) has been shown to enhance P absorption by increasing the effective root area (Hayman and Mosse, 1971). AMF infection improves P influx (P uptake per unit root length). On the other hand, the information available discussing the genetic diversity present among wheat cultivars to associate with vesicular-arbuscular mycorrhiza is not consistent (Vlek *et al.,* 1996). There are reports that show differences in mycorrhizal association among wheat cultivars (Vlek *et al.,* 1996). In contrast, extensive screening of CIMMYT's spring wheat cultivars for mycorrhizal association found very small differences among genotypes; the differences were not strongly associated with higher P absorption (Manske *et al.,* 2000).

Increasing nutrient availability through rhizosphere modification

Root exudates, ranging from protons to complex organic molecules, can influence nutrient availability and uptake. Phosphatases have been reported to transform poorly available organic phosphorus, which usually accounts for 40–50% of a plant's total P supply, into inorganic forms available to the plant (Randall, 1995). There are genotypic differences in root phosphatase excreted or bound at the root surface (McLachlan, 1980). Our work in an Andisol showed an association between acid phosphatases and P uptake in different wheat and triticale cultivars (Portilla-Cruz *et al.,* 1998).

As in the case of N, most opportunities for breeding for higher utilization efficiency probably lie in improving biomass production efficiency (BPE) rather than HI. In this case biomass production will have to either increase with the current levels of P in the plant or be maintained with a lower concentration of P in the plant. Utilization efficiency is associated with the efficiency with which plants use absorbed P; this, in turn, is a function of: (1) how efficiently P is distributed to the functional sites, and (2) the P requirement of the cells at those sites (Loneragan, 1978).

Calculating nutrient uptake efficiency

As defined earlier, uptake efficiency refers to the ability of the crop to extract or absorb nutrients from the soil. Hence:

$$\text{Uptake Efficiency} = Nt/Ns \qquad \text{Equation 3.4}$$

Where: Nt = total above-ground nutrient in the plant at maturity; Ns = nutrient supplied.

Uptake efficiency can be measured at any stage of development, but particularly useful information can be collected at anthesis and physiological maturity. The steps below describe the means in which you can measure uptake efficiency.

First, a biomass sample is collected by either harvesting all the above-ground biomass in a given area (a minimum of 0.5 m^2 is suggested) or harvesting a predetermined, representative number of plants (a minimum of 50 stems is suggested) at random. Detailed methods for doing this type of sampling at different stages of development are described by Bell and Fischer (1994) and in the accompanying volume.

If the sample is collected right before or shortly after anthesis, there is no need to separate the grain from the rest of the plant for N or P analysis. However, if the sample is collected at or around physiological maturity, it is important to separate the grain from the rest of the biomass for N analysis. This is because there is a large difference in % nutrient concentration between the grain and non-grain biomass (leaves, stems, chaff). In well fertilized spring wheat crops under irrigated conditions, we have observed values of approximately 2% N in the grain and 0.8% N in non-grain biomass. Therefore it is best to take a weighted average to calculate total nutrient in the plant, using the following formula:

$$Nt = (Gw \times Gn) + (Bm \times Bn) \qquad \text{Equation 3.5}$$

Where: Nt = total above-ground nutrient in the plant at maturity; Gw = grain weight at 0% moisture (g m^{-2}); Gn = nutrient concentration in the grain (%); Bm = non-grain biomass at 0% moisture (g m^{-2}); Bn = nutrient concentration in non-grain biomass (%).

Total nutrient in the plant is then divided by the amount of nutrient supplied (g m^{-2}) as fertilizer. If soil samples are collected and the amount of soil available nutrient is known, this can be added to the amount supplied as fertilizer.

Nutrient absorption is dependent on root characteristics, especially for immobile plant nutrients in the soil, such as phosphorus.

Calculating nutrient utilization efficiency

Nutrient utilization efficiency is defined as a crop's ability to convert the absorbed nutrients into grain yield. Hence:

Utilization Efficiency = Tw/Nt Equation 3.6

Where: Tw = total above-ground plant dry weight at maturity and Nt = total aboveground plant nutrient at maturity. To measure uptake efficiency, certain information needs to be collected. First, calculate the harvest index (HI), as follows:

HI = Gw/Tw Equation 3.7

Where: Gw = grain weight at 0% moisture, and Tw = total plant biomass at 0% moisture.

This can be done either on an area or a plant basis, as suggested by Bell and Fischer (1994) and detailed in the accompanying volume. Finally, BPE is calculated as:

BPE = Gw/Nt Equation 3.8

Conclusions

Bread wheat breeding work at CIMMYT has shown that selection and evaluation of genetic material under medium to high nitrogen levels results in genetic gains expressed when this material is tested under low, medium, or high nitrogen levels. In other words, selecting for high yield potential under optimum conditions has resulted in germplasm with higher nitrogen use efficiency under low, medium, or high nitrogen fertility conditions. Now there is evidence that breeding under alternating low and high nitrogen levels may produce germplasm that is even more efficient and responsive to nitrogen.

It is clear that nutrient use efficiency and nutrient responsiveness are under genetic control. Some researchers consider these traits as two different breeding objectives, but it has been shown that they are not incompatible. One of the best pieces of evidence for this is the results achieved by bread wheat breeders at CIMMYT. During the last several decades, CIMMYT has been breeding wheat under medium to high levels of nitrogen and phosphorus and has developed cultivars that are not only more responsive to nitrogen and phosphorus, but also more efficient in their use.

To characterize and better understand the mechanisms associated with higher N and P use efficiency:

- Use the definition of N and P use efficiency suggested by Moll *et al.* (1982);
- Distinguish between efficiency and responsiveness. This will require that all germplasm be evaluated under low as well as high N and P conditions;
- Establish the importance of uptake vs. utilization efficiency in the target environment;
- Understand the mechanisms associated with higher uptake (more roots, phosphatases, etc.) or utilization efficiency (BPE vs. HI). If these mechanisms are well understood, they can be used as selection criteria; and
- Once genetic markers for genes controlling these traits are identified, selection for these traits could be done in the laboratory.

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Chapter 4: Opportunities to improve genetic wheat yield potential

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Abstract

Wheat yield potential could be increased by 50% or more –theoretically– through the genetic improvement of radiation use efficiency (RUE). However, to achieve agronomic impacts, structural and reproductive aspects of the crop must also be improved. Attempts to increase RUE will focus on increasing the efficiency of Rubisco, introduction of C4-like traits such as CO_2 concentrating mechanisms, and improvement of light interception and photosynthesis at the spike and whole canopy levels. For extra photo-assimilates to translate into increased grain yield, reproductive aspects of growth must be fitted to a range of agro-ecosystems to ensure that stable expression of a high harvest index (HI) is achieved. Adequate partitioning among plant organs will be necessary to achieve favorable expression of HI, and to ensure that plants with heavier grain have strong enough stems and roots to avoid lodging. Trait-based crossing strategies will aim to achieve their simultaneous expression in elite agronomic backgrounds and wide crossing will be employed to augment genetic diversity where needed. Genomic selection approaches will be employed, especially for difficult-to-phenotype traits. Products will be delivered to national wheat programs worldwide via CIMMYT's international nursery systems and are expected to make a significant contribution to global food security.

Introduction

Increases in wheat productivity have been achieved worldwide as a result of adoption of Green Revolution technologies (Evenson and Gollin, 2003). Nonetheless, the challenges of increasing production to feed a world population of 9 billion by mid century are considerable. Less developed countries are particularly vulnerable in terms of food security for three main reasons: firstly, most are net importers of cereals (Dixon *et al.,* 2009). Secondly many of their national wheat programs lack sufficient capacity to meet demand (Kosina *et al.,* 2007). Finally, the majority are located in climate vulnerable regions (Lobell *et al.,* 2008). While international public wheat breeding has focused in recent decades on increasing resistance to disease and abiotic stress (Reynolds and Borlaug, 2006; Braun *et al.,* 2010), efforts to raise genetic yield potential *per se* have received little attention. In fact the fundamental bottleneck to raising productivity, namely radiation use efficiency (RUE), has barely changed.

Research in photosynthesis suggests that improvements in yield are theoretically possible (Long *et al.,* 2006; Parry *et al.,* 2007; Zhu *et al.,* 2010). These consider the inefficiency of carbon fixation in C3 crop and compare it with that of C4 crops which show up to 50% greater RUE. In wheat, genetic modification of Rubisco and its regulation are major targets to improve photosynthetic efficiency (Parry *et al.,* 2007); a more ambitious approach in rice is to introduce the characteristics of C4 photosynthesis (Furbank *et al.,* 2009). While increasing photosynthetic potential will require research to focus at cellular and sub-cellular processes, this must go in parallel with genetic modification of structural and reproductive aspects of growth, since these determine the net agronomic benefit of increased RUE. Specifically, adaptation of the reproductive processes to variation in seasonal and other environmental factors, while relatively poorly understood, determines the efficiency with which photo-assimilates are converted to yield (Reynolds *et al.,* 2009a). Furthermore, even at current levels of yield potential, a significant portion of wheat yield worldwide is already lost due to lodging (Berry *et al.,* 2004). In summary, to achieve impacts under agronomic conditions, the following broad objectives must be tackled simultaneously: (i) increase crop biomass through modification of RUE, (ii) improve targeted adaptation of reproductive processes to major wheat agro-ecosystems thereby permitting increases in RUE to be consistently translated to grain weight, and (iii) enhance plant structural characteristics to ensure that grain yield potential and quality are not sacrificed due to lodging.

To achieve these objectives, the International Maize and Wheat Improvement Center (CIMMYT) began consulting with crop experts worldwide culminating in the formation of a Wheat Yield Consortium (WYC) (Reynolds *et al.,* 2011). The remit of the WYC is, through linking ongoing research worldwide, to develop a cohesive portfolio of research activities to maximize the probability of impact in farmers' fields (Figure 4.1).

Overview of research approaches to raise the yield potential of wheat

WYC includes expertise within three linked themes:

1) Increasing photosynthetic capacity and efficiency.
2) Optimizing partitioning to grain yield while maintaining lodging resistance.
3) Breeding to accumulate yield potential traits and delivery of new germplasm.

Within each of these, a set of sub-projects (SPs) has been developed (Table 4.1) in a way that capitalizes on pre-existing knowledge and ongoing research. In the following three sections, the broad objectives of the three themes are presented in context of how research products will translate into new traits for use in breeding and eventually the delivery of new wheat cultivars. Further details for all three Themes have been published separately (Parry *et al.,* 2011; Foulkes *et al.,* 2011; Reynolds *et al.,* 2011).

Theme 1: Increasing photosynthetic capacity and efficiency

To achieve a quantum increase in crop yield potential a major improvement in photosynthetic capacity and/or efficiency will be required. In rice, potential grain number has increased markedly in the new rice types but only around 40% of these florets are fertilized and filled (Sheehy *et al.,* 2007), indicating "source" limitation by insufficient provision of photosynthate at key developmental stages. In wheat, while "sink" strength of grain and photosynthetic

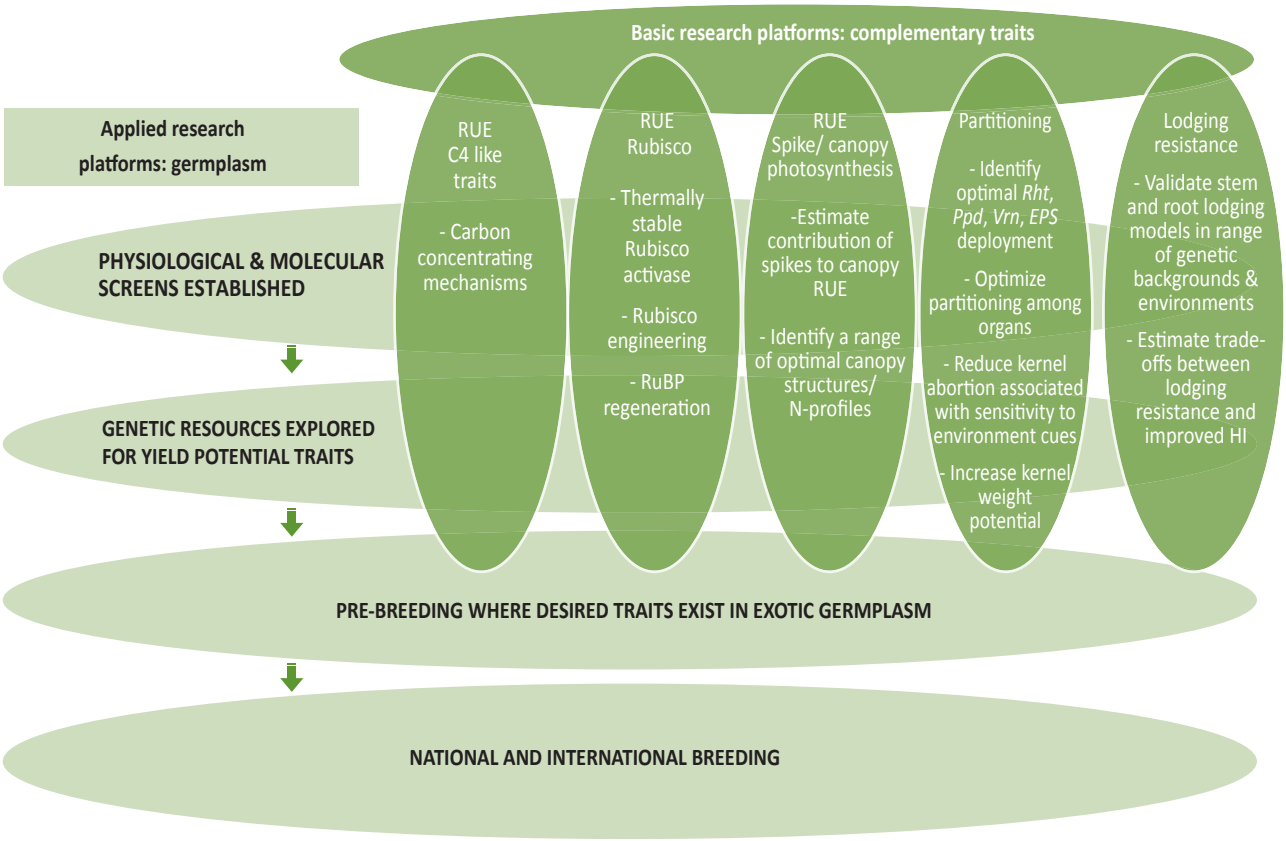

Figure 4.1. A research strategy to improve yield potential of wheat. Where: HI = harvest index; RUE = radiation use efficiency.

capacity may be more in balance, there is also evidence that historic gains in wheat yield potential have been associated with increased photosynthesis (Fischer *et al.,* 1998). Furthermore, basic research in photosynthesis has confirmed that substantial improvements are theoretically possible (Parry *et al.,* 2007; Zhu *et al.,* 2010).

Two approaches will be investigated for increasing total crop biomass. They both aim to increase photosynthetic efficiency and capacity by targeting the first step of CO_2 fixation in C3 photosynthesis, catalysed by Rubisco, and the subsequent regeneration of the co-substrate for this enzyme, Ribulose-1,5-bisphosphate (RuBP). Rubisco operates at low catalytic efficiency and also catalyses an oxygenation reaction (initiating photorespiration) which wastes carbon and energy (See Zhu *et al.,* 2010). The first approach has multiple components (Table 4.1). A component intended to achieve application in the 5-year timeframe is to target the properties of the Rubisco protein and associated photosynthetic machinery (Parry *et al.,* 2007) by phenotypic screening of diverse sources of germplasm for photosynthetic performance at the whole leaf or canopy level using direct measurement coupled with mathematical modeling. This phenomics approach will define sets of germplasm with variation in Rubisco properties and associated regulatory proteins such as Rubisco activase (Table 4.1, SPs 1.1, 1.2 and 1.3). This will be coupled with a project to screen for improved photosynthetic capacity in spikes which can contribute a large proportion of grain carbon (see Gebbing and Schneider, 1999; Table 4.1, SP 1.2).

Table 4.1. Sub-projects (SPs) of the Wheat Yield Consortium.

Theme 1: Increasing photosynthetic capacity and efficiency	
SP1.1	Phenotypic selection for photosynthetic capacity and efficiency
SP1.2	Capturing the photosynthetic potential of spikes
SP1.3	Optimizing canopy photosynthesis and photosynthetic duration
SP1.4	Chloroplast CO_2 pumps
SP1.5	Optimizing RuBP Regeneration
SP1.6	Improving the thermal stability of Rubisco Activase
SP1.7	Replacement of LS Rubisco
Theme 2: Optimizing partitioning to grain while maintaining lodging resistance	
SP2.1	Optimizing harvest index through increasing partitioning to spike growth and maximizing grain number
SP2.2	Optimizing developmental pattern to maximize spike fertility
SP2.3	Improving spike fertility through modifying its sensitivity to environmental cues
SP2.4	Improving grain-filling and potential grain size
SP2.5	Identifying traits and developing genetic sources for lodging resistance
SP2.6	Modeling optimal combinations of, and tradeoffs between, traits
Theme 3: Breeding to accumulate yield potential traits	
SP3.1	Trait and marker based breeding
SP3.2	Wide crossing to enhance photosynthetic capacity
SP3.3	Genomic selection to increase breeding efficiency
SP3.4	Germplasm evaluation and delivery

Genetic engineering will be used to improve RuBP regeneration, Rubisco activase (Table 4.1, SP 1.4) or to introduce Rubisco subunits with enhanced catalytic properties (Table 4.1, SP 1.6). Under conditions of low light and elevated CO_2 the regeneration of RuBP limits photosynthetic carbon assimilation. There is clear experimental evidence that manipulation of RuBP regeneration by over expressing sedoheptulose-1,7-bisphosphatase (SBPase) can increase plant productivity in controlled environments. Modeling approaches also suggest increased benefit from the over-expression of fructose-1,6-bisphosphate aldolase (FBPA). In SP 1.5 (Table 4.1), RuBP regeneration capacity in wheat will be increased by over-expressing both SBPase and FBPA which is predicted to increase yields by around 10% (Reynolds *et al.,* 2009a). For Rubisco activase, genetic variation in heat stability has been established in a range of plant species and the residues responsible mapped in *Arabidopsis* (Salvucci and Crafts-Brander 2004; Kurek *et al.,* 2007). In SP 1.6 (Table 4.1) Rubisco activase will be reengineered to increase its thermotolerance, with the aim of broadening the temperature range for photosynthesis.

Much progress has been made in identifying natural variation in the catalytic properties of Rubisco from different species and in developing the tools for introducing Rubisco genes into plants. Modeling suggests that very large increases in photosynthetic performance should be possible (Parry *et al.,* 2007) by introducing existing Rubisco variants from other plant species. In a longer term approach, SP 1.7 (Table 4.1) recognizes this potential and will develop plastid transformation for wheat.

Another approach is to mimic systems that already exist in nature which concentrate CO_2 in the compartment where Rubisco is located, eliminating photorespiration and ensuring Rubisco operates close to its catalytic optimum. These systems are present in C4 plants, where a biochemical CO_2 concentrating mechanism has evolved many times, capable of elevating CO_2 at the site of Rubisco up to 10-fold over atmospheric levels (von Caemmerer and Furbank, 2003). There is currently an International Consortium attempting to install a C4

pathway in rice (Furbank *et al.,* 2009), however, the complexity of the anatomical and biochemical traits necessary for this mechanism to operate is daunting and the minimal set of genes necessary unknown. In many algae and cyanobacteria, however, CO_2, in the form of bicarbonate, is pumped across membranes to elevate CO_2 to even higher levels than those seen in C4 plants (Price *et al.,* 2008). Only one or two genes are required for this transformation and these are now cloned and functionally validated (Price *et al.,* 2008). If these transporter proteins could be placed in the chloroplast membrane of wheat and the system functions as it does in algae and cyanobacteria, large increases in photosynthetic efficiency would result (Table 4.1, SP 1.4).

In summary a range of options –both transgenic and non-transgenic– exist to raise RUE in wheat, some of which may be physiologically complementary or genetically additive. Further exploration of genetic diversity within and outside the Triticeae tribe will eventually determine which approaches are most likely to be implemented in breeding.

Theme 2: Optimizing partitioning to grain yield while maintaining lodging resistance

Adaptation of reproductive processes to environment is still considered among the most challenging aspects of cereal improvement (Barnabas *et al.,* 2008). While increases in harvest index (HI) have been achieved since the Green Revolution period (Sayre *et al.,* 1997; Shearman *et al.,* 2005) their physiological and genetic basis is not well established. For wheat, this is in part because it is grown across widely divergent temperature regimes and latitudes, and in extreme cases, poor adaptation can result in negligible yield despite the expression of a significant crop biomass. Key physiological components include developmental response to vernalization, photoperiod, and other environmental factors that influence intra-plant competition for growth resources (Fischer, 1985; Slafer and Rawson, 1994; Ugarte *et al.,* 2007; Ghiglione *et al.,* 2008). It has been shown that spike fertility can be improved by increasing the availability of assimilates to the developing spike (Fischer, 1985), thereby reducing the early abortion of grains (Miralles and Slafer, 2007) or by increasing grain weight potential (Calderini and Reynolds, 2000; Duggan and Fowler, 2006). Both processes are affected by photosynthetic capacity, intra-plant competition between organs for assimilates, and their interaction with environmental signals that respond to photoperiod, temperature, water and nutritional status. The photosynthetic capacity of contemporary germplasm may not even be utilized efficiently if spike fertility is not optimized (Reynolds *et al.,* 2009a).

One candidate gene that has been identified for spike fertility *per se* –Gn1a in rice– codes for cytokinin oxidase which through its regulation of cytokinin levels influence numbers of reproductive organs in the panicle (Ashikari *et al.,* 2005). The apparent involvement of growth regulators in determining grain number suggests that a better understanding of plant signaling (Davies *et al.,* 2005) may be the route to explaining the interaction of spike fertility with environment and its genetic basis.

SPs 2.1, 2.2, 2.3, and 2.4 (Table 4.1) specifically aim to better understand these interactions and identify reliable physiological and marker-based selection criteria so that improvements in RUE can be translated into greater agronomic yield potential. In this context, the use of perfect markers associated with height reduction and photoperiod and vernalization responses are expected to provide a valuable genetic underpinning to the research. A principal research target will be to maximize the partitioning of assimilates to the developing spike to increase spike fertility –i.e., potential grain number and grain weight potential. However, plants with increased photosynthetic rate and a larger biomass are likely to require more efficient if not larger root systems. Therefore, the potential tradeoffs associated with different partitioning strategies must be carefully evaluated in the context of which resource is most likely to limit yield.

Adequate partitioning among plant organs is also key to ensuring that plants with heavier grain weight have strong enough stems and roots to avoid structural failure (Berry *et al.,* 2007). Lodging is already a common phenomenon in wheat which can reduce yield by as much as 80% as well as reducing grain quality (Easson *et al.,* 1993; Berry *et al.,* 2004). Any comprehensive strategy to improve wheat yield potential must include lodging resistance since heavier yielding crops will require stronger plants (Table 4.1, SP2.5). Lodging resistance traits are prime candidates for development of molecular markers since at least some of the traits involved (e.g., crown root spread, material strength of stem) are expected to be relatively heritable, yet are not easy to phenotype in the field.

In summary, many traits are involved in optimizing agronomic performance whose genetic basis is independent of increasing biomass or RUE *per se*. Their physiological mechanisms, complex interactions, and

genetic basis will be dissected in this theme. Simulation modeling of these interactions (Table 4.1, SP2.6) will be used to refine the conceptual models used to make breeding decisions in Theme 3. The main output of Theme 2 will be a toolkit –consisting of phenotyping approaches and molecular markers– to facilitate hybridization strategies and progeny selection, such that expression of HI and lodging resistance is optimized in germplasm targeted to major wheat agro-ecosystems systems.

Theme 3: Breeding to accumulate yield potential traits

Trait selection is the foundation of plant breeding and has made continual progress through incorporating the following types of traits: simply inherited agronomic characteristics such as height and flowering time, resistance to a spectrum of common diseases, quality parameters determined by end use, and yield based on multi-location trials (Braun *et al.,* 2010). To accelerate genetic gains in yield in the future, complex physiological traits (PTs) must now be incorporated as additional criteria. The main objective of Theme 3 is to combine PTs deterministically whereby progeny will encompass both strategic traits that improve RUE with those alleles necessary to maximize agronomic impact at the system level –including PTs associated with HI and lodging resistance– into elite agronomic backgrounds (i.e., disease resistant, appropriate quality parameters, etc). Physiological trait-based breeding approaches have been implemented successfully by CIMMYT, leading to international distribution of a new generation of elite drought adapted lines (Reynolds *et al.,* 2009b). These principles will be adapted to a conceptual platform for designing crosses that combine PTs for yield potential (Figure 4.2) whose progeny will be selected using a combination of visual criteria, precision phenotyping, and molecular marker-assisted approaches (SP3.1). Whole genome selection will also be evaluated in this context –given its utility in maize breeding (Bernardo and Yu, 2007)– since it provides a potentially powerful mechanism for accumulating alleles associated with complementary PTs (SP3.3).

The primary wheat gene pool (i.e., *Triticum aestivum*) may need to be complemented with traits from more exotic sources in cases where conventional ones lack adequate diversity. In fact, inter-specifc and inter-generic crosses within the Triticeae are already routine procedures in wheat breeding (Skovmand *et al.,* 2001;

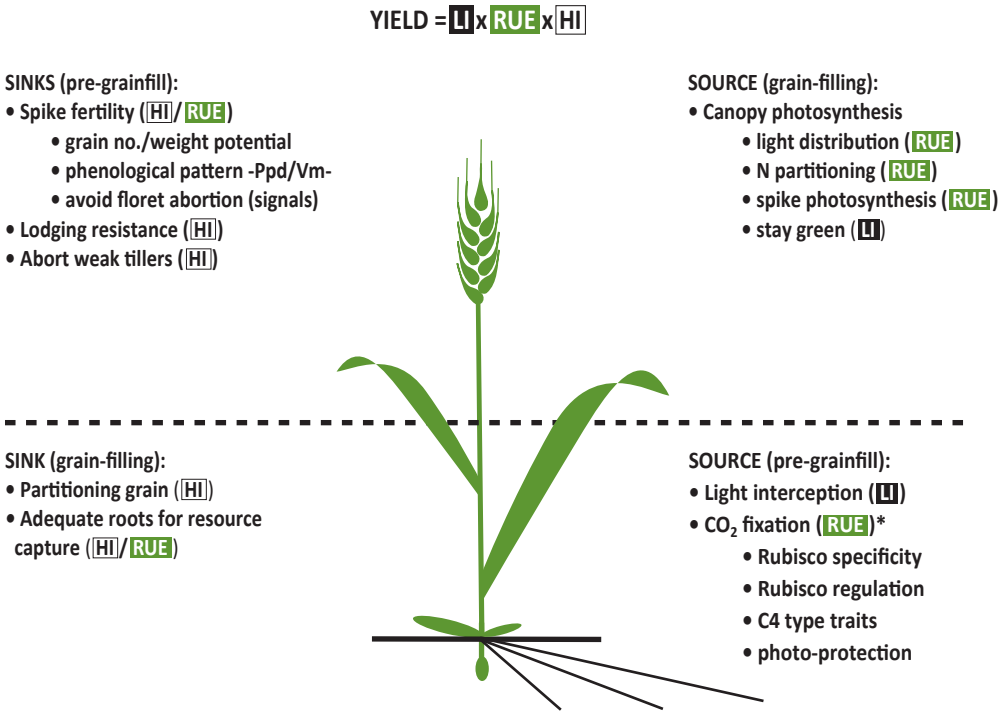

Figure 4.2. A conceptual platform for designing crosses that combine complementary yield potential traits in wheat (based on traits reviewed in Reynolds *et al*., 2009a). Traits are categorized as either net sources or sinks of photo-assimilates and their predominant expression is considered either before or during grain-filling, and in some cases both*. Where: HI = harvest index; RUE = radiation use efficiency.

Trethowan and Mujeeb-Kazi, 2008) (Table 4.1, SP3.2). In addition to the many photosynthetic traits, these might include sources of spike fertility and lodging resistance, traits for which sources are already known. Sources of other 'yield improving' traits may yet have to be identified as the limitations of current levels of expression in conventional gene pools are defined by research in Themes 1 and 2.

Both wheat/alien introgression for introducing exotic chromatin and whole genome fusion (to create synthetic polyploids) from wide crosses have had major agronomic impacts throughout the world (Ortiz *et al.,* 2008; Trethowan and Mujeeb-Kazi, 2008). While the introduction of genes from outside of the Triticeae tribe is not a routine procedure in wheat breeding, chromatin from C4 species (maize, *Zea mays* L.; and *Tripsacum dactyloides*) has been introduced into wheat but so far not proven to be stably integrated and transmitted (Laurie and Bennett, 1989; Comeau *et al.,* 1992; Li *et al.,* 1996; Brazauskis *et al.,* 2004). Greater success has been achieved in oat (*Avena sativa* L.) with the production of a complete set of disomic additions of each of the maize chromosomes (Kynast *et al.,* 2001). Expression of C4 photosynthetic enzymes in some of these oat–maize chromosome addition lines has been reported (Knowles *et al.,* 2008). These precedents and the availability of advanced molecular techniques allowing earlier, higher throughput screening and identification of putative introgressions, suggest that with appropriate investment, wide crossing may be able to introduce all of the chromatin into wheat required for full expression of C4 photosynthesis, although this would clearly require considerable effort.

The impact of the above work will depend on effective delivery of products. The International Maize and Wheat Improvement Center –the coordinating institute of the WYC– has for over 45 years coordinated an international wheat breeding effort and through its international nursery system delivers approximately 1,000 new genotypes per year, targeted to the varying needs of national wheat programs in less developed countries (Reynolds and Borlaug, 2006; Braun *et al.,* 2010). Impacts at the farm level are well documented (Lipton and Longhurst, 1989; Evenson and Gollin, 2003). These approaches will be applied and modified as necessary to ensure that new high yielding cultivars are delivered to farmers via their national programs in as short a timeframe as possible (Table 4.1, SP3.4).

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Chapter 5: Searching genetic resources for useful variation in physiological traits

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Abstract

Plant genetic resources for food and agriculture are crucial for feeding the world's population. They are the raw material that farmers and plant breeders use to improve the quality and productivity of our crops. The future of agriculture depends on international cooperation and on the open exchange of crops, and their genes, that farmers all over the world have developed and exchanged for over 10,000 years. No country is sufficient in itself. All depend on crops and the genetic diversity within these crops from other countries and regions. This chapter will discuss the nature and availability of genetic resources in wheat and some of the potential applications in breeding. Over 80 germplasm collections, holding in excess of an estimated 800,000 wheat accessions, have been established globally. The genetic resources available for wheat improvement and research are found in several Triticeae gene pools. Gene transfer within species of the primary gene pool is not difficult. Unfortunately, many populations of the annual wild relatives of wheat, particularly those at the extremes of their distribution that are of special interest for breeding purposes, are under threat because of changing patterns of land use. At the same time, new technologies have made the use of the annual wild relatives as a germplasm source easier which has generated an interest and need for representative collections of annual wild relatives to be maintained in accessible collections. For these reasons the annual wild relatives should clearly be afforded a greater priority, accessing new sources of genetic variability. The International Treaty for Plant Genetic Resources for Food and Agriculture covers wheat and its related species. The Treaty is vital in ensuring the continued availability of the plant genetic resources that countries will need to feed their people. We must conserve for future generations the genetic diversity that is essential for food and agriculture.

Introduction

Three main approaches can be employed to widen gene pools, namely: (i) introgression from germplasm with compatible genomes, (ii) wide crosses involving inter-specific or inter-generic hybridization, and (iii) genetic transformation. Both landraces and products of interspecific hybridization have been used in wheat breeding, mainly to introduce traits associated with resistance to a range of biotic stresses (Dwivedi *et al*., 2008; Ortiz *et al*., 2008; Trethowan and Mujeeb-Kazi, 2008). Relatively few wild relatives of crops have been used to improve yield potential or stress adaptation (Hajjar and Hodgkin, 2007; Trethowan and Mujeeb-Kazi, 2008).The transgenic approach is theoretically unlimited in its potential to exploit genetic diversity across taxonomic groups, and much data has been collected for candidate genes that improve survival of both model and crop species under drought in controlled environments (Umezawa *et al.,* 2006). This chapter will discuss the nature and availability of genetic resources in wheat and some of the potential applications in breeding.

Due to the strategic importance of wheat in food security and trade in many countries, and the critical importance of breeding in ensuring national industries remain competitive, over 80 autonomous germplasm collections holding in excess of an estimated 800,000 accessions have been established globally. These collections vary in size and coverage; the largest have over 100,000 accessions and the smallest only a few hundred. They also vary greatly in coverage. Most collections evolved from breeders' working collections and carry predominantly local or regional cultivars – advanced, obsolete or landrace– as well as introduced cultivars of interest to national or regional breeders (Table 5.1).

The genetic resources available to plant physiologists and breeders are found in several Triticeae genepools recognized by Von Botmer *et al.* (1992) and described as concentric circles (Figure 5.1). The concept of the genepool was first proposed in 1971 by Harlan and de Wet (Harlan, 1992), who suggested a circular way of demonstrating the relationships among genepools. The primary genepool consists of a given biological species

† Deceased

Table 5.1. Major collections of a Global Network of Wheat Genetic Resources.

Country	Institute	No. of accessions
Global	International Maize and Wheat Improvement Center (CIMMYT), El Batan, Mexico	111,681
USA	United States Department of Agriculture (USDA)-Agricultural Research Service (ARS), National Small Grains Facility, Aberdeen, Idaho	56,218
Russia	N.I. Vavilov Research Institute of Plant Industry (VIR), St. Petersburg	39,880
Global	International Center for Agricultural Reserach in the Dry Areas (ICARDA), Aleppo, Syria	37,830
India	National Bureau of Plant Genetic Resources (NBPGR), New Delhi	32,880
Australia	Australian Winter Cereals Collection, Tamworth	23,917
France	INRA Station d'Amelioration des Plantes, Clermont-Ferrand	15,850
Iran	National Genebank of Iran, Genetic Resources Division, Karaj	12,169
Czech Republic	Research Institute of Crop Production, Prague	11,018
Ethiopia	Plant Genetic Resources Centre, Institute of Biodiversity Conservation and Research, Addis Ababa	10,745
Bulgaria	Institute for Plant Genetic Resources "K. Malkov", Sadovo	9,747
Germany	Genebank, Institute for Plant Genetics and Crop Plant Research (IPK), Gatersleben	9,633
United Kingdom	Department of Applied Genetics, John Innes Centre, Norwich	9,584
Cyprus	National Genebank (CYPARI), Agricultural Research Institute, Nicosia	7,696
Japan	Genetic Resources Management Section, NIAR (MAFF), Tsukuba	7,148
Switzerland	Station Federale de Recherches en Production Vegetale de Changins, Nyon	6,996
Turkey	Plant Genetic Resources Department, Aegean Agricultural Research Institute, Izmir	6,381
Netherlands	Centre for Genetic Resources, Wageningen	5,529
Canada	Plant Gene Resources of Canada, Winnipeg	5,052
USA	Wheat Genetics Resource Center, Kansas State University, Manhattan	5,000
Japan	Plant Germplasm Institute, Graduate School of Agriculture, Kyoto University	4,378
Spain	Centro de Recursos Fitogeneticos, INIA, Madrid	3,183
Sweden	Nordic Gene Bank, Alnarp	1,843
Total	**23 Institutes**	**434,358**

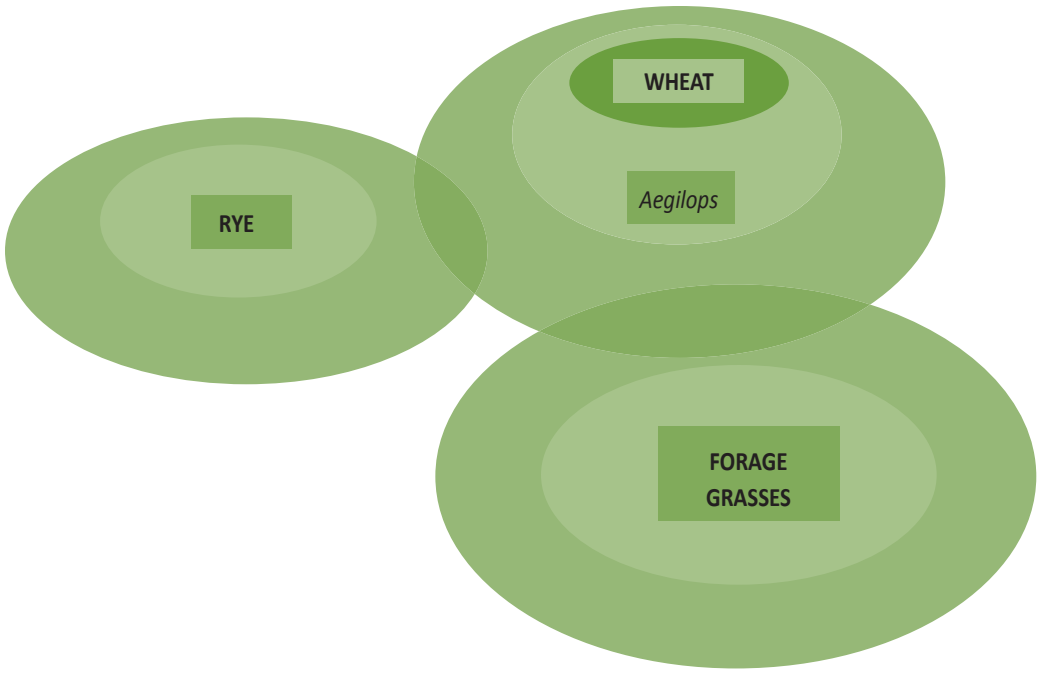

Figure 5.1. Schematic diagram of the concentric circles illustrating genepools of the Triticeae. Adapted from von Botmer *et al*. (1992).

including, in the case of a crop species, its cultivated, wild, and weedy forms. Gene transfer within species of the primary genepool is not difficult.

An important issue in the use of wheat genetic resources is the diversity of available accessions (see Merezhko, 1998). One extreme view would be to use only the primary gene pool –the cultivated species and the closely related species with which they can be readily hybridized. The other extreme is that in the modern world of transgenics all biological species are potential genetic resources for wheat breeding and the concepts of primary, secondary and tertiary genepools are quaint and outmoded. It is suggested here following Merezhko (1998) we should restrict our focus to *Triticum* species and related genera of the Triticeae. This coverage aligns with the intention of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA, 2011).

Modern and obsolete improved cultivars are generally well conserved in global wheat germplasm collections because many such collections either were derived from breeders' working collections or were primarily established to service local or regional breeding programs, and these were the accessions most sought by breeders. In fact many important cultivars are conserved in the majority of national and international collections.

Landrace varieties have received priority for collection, conservation and documentation in recent years supported by the efforts of the Food and Agriculture Organization (FAO), the Consultative Group on International Agricultural Research (CGIAR) and others because of the increasing threat to their continued existence by the spread of improved modern cultivars. Nevertheless, such cultivars are poorly represented in world collections compared to modern and obsolete cultivars and will likely remain important sources of genetic variability.

The wild relatives of wheat are also generally poorly represented in global wheat germplasm collections. There are several reasons for this. Firstly, wild relatives are more difficult to use in conventional breeding programs than cultivars of the same species and usually require an extensive period of germplasm enhancement. They tended therefore to be collected and used by the small number of specialist institutes concerned with interspecific hybridization. Secondly, they are more difficult to seed increase and maintain because of their tendency to shatter their seed than crop cultivars. For this reason also the distribution and use of some wild species is limited because of their potential as weeds. Finally, wild species, because of their capacity to self-reproduce in nature, were seen as under less threat of extinction than the cultivated landraces.

Unfortunately many populations of the annual wild relatives of wheat, particularly those at the extremes of their distribution that are of special interest for breeding purposes, are under threat because of changing patterns of land use. At the same time, new technologies have made the use of the annual wild relatives as a germplasm source easier which has generated an interest and need for representative collections of annual wild relatives to be maintained in accessible collections. For these reasons the annual wild relatives should clearly be afforded a greater priority accessing new sources of genetic variability.

The growing size and sophistication of genetic and molecular stock collections is testimony to their increasing contributions to enable the effective utilization of the variation conserved in "traditional" germplasm collections. The role of genetic stock collections as sources for useful genetic variability should be re-evaluated and they should be afforded a higher priority in a rationalized system than they have been accorded in the past.

The cultivated species of *Triticum* and their genomic constitution are given in Table 5.2. It will be noted that there are two valid biological species at each ploidy level. The diploid *T. monococcum* has both cultivated and wild forms, while *T. urartu* only exists in the wild. Both tetraploid forms exist in both cultivation and the wild, while both hexaploid species only exist in cultivation. The distribution of these species is described by Gill and Friebe (2002).

Aegilops is the most closely related genus to *Triticum* and has been widely used in wheat improvement. All *Aegilops* are annuals (Table 5.3). Their taxonomy and distribution is discussed by van Slageren (1994).

Dasypyrum [Haynaldia] villosum is among the Triticeae species as genetic resources for wheat breeding. It is an annual with V genome and is easily hybridized to *Triticum aestivum* or *T. turgidum.* Each of the chromosomes has been added to common wheat by E. Sears.

In addition to *Aegilops*, a host of more distantly related annual and perennial members of related genera in the Triticeae have potential as a source of germplasm in wheat breeding including cultivated rye and barley and their near relatives as well as a host of perennial grasses.

Access to wheat genetic resources

In a collaborative effort between CGIAR genebanks, Bioversity International, the Global Crop Diversity Trust, and the Secretariat of the ITPGRFA, the GeneSys gateway to genetic resources global web portal (www.genesys-pgr.org) has been developed to improve global information exchange. The portal enables users to search on passport, environmental, morphological, chemical, disease, insect, stress, growth, and phonological types of data. Currently, GeneSys provides access to over two million accessions records, made available through partnership with EURISCO, CGIAR/System-wide Information Network for Genetic Resources (SINGER) and the United States Department of Agriculture (USDA)/Germplasm Resources Information Network (GRIN).

Use of wheat genetic resources as sources for new genetic variability

A schematic diagram of the effort needed to transfer traits from genetic resources to farmers' fields is given in Figure 5.2. Within the primary genepool, the utilization cost increases as the genetic distance increases. Within a species there are also levels of genetic resources (from current high yielding cultivars to landraces) that may determine the cost of using those resources.

As one moves away from the primary genepool, the effort required to utilize genetic resources in the secondary and tertiary genepools increases geometrically. It is difficult to release a commercially acceptable cultivar that does not have previously released cultivars in its pedigree (Sanjaya Rajaram, personal communication) because crosses with species in the secondary and tertiary genepools tend to disunite favorable gene complexes, which affects performance. Technology extends the genepools and reduces costs, as for example, embryo rescue has done in the recent past and genetic engineering promises to do in the future. Also, species in the secondary genepool, such as *Aegilops tauschii*, can now be used as readily as species in the primary genepool through the production of hexaploid synthetic wheats using embryo rescue followed by chromosome doubling using colchicine (Mujeeb-Kazi, 1995).

Table 5.2. Species of genus *Triticum* and their genomic constitution.

Species	Genomic constitution	
	Nuclear	Organellar
Triticum aestivum L.	ABD	B (rel. to S)
Triticum aestivum subsp. *aestivum* (common or bread wheat)		
Triticum aestivum subsp. *compactum* (Host) Mackey (club wheat)		
Triticum aestivum subsp. *macha* (Dekapr. & A. M. Menabde) Mackey		
Triticum aestivum subsp. *spelta* (L.) Thell. (large spelt or dinkel wheat)		
Triticum aestivum subsp. *sphaerococcum* (Percival) Mackey (Indian dwarf wheat)		
Triticum turgidum L.	AB	B (rel. to S)
Triticum turgidum subsp *carthlicum* (Nevski) A. Love & D. Love (Persian wheat)		
Triticum turgidum subsp *dicoccoides* (Korn. ex Asch. & Graebn.) Thell. (wild emmer)		
Triticum turgidum subsp *dicoccum* (Schrank ex Schubl.) Thell. (emmer wheat)		
Triticum turgidum subsp *Durum* (Desf.) Husn. (macaroni or durum wheat)		
Triticum turgidum subsp *paleocolchicum* A. Love & D. Love		
Triticum turgidum subsp *polonicum* (L.) Thell. (Polish wheat)		
Triticum turgidum subsp *turanicum* (Jakubz.) A. Love & D. Love (Khorassan wheat)		
Triticum turgidum subsp *turgidum* (pollard wheat)		
Triticum zhukovskyi Menabde & Ericz.	A^tA^mG	A (rel. to S)
Triticum timopheevii (Zhuk.) Zhuk.	A^tG	G (rel. to S)
Triticum timopheevii subsp *armeniacum* (Jakubz.) Slageren (wild form)		
Triticum timopheevii subsp *timopheevii* (cultivated form)		
Triticum monococcum L.	A^m	A^m
Triticum monococcum subsp *aegilopoides* (Link) Thell. (wild form)		
Triticum monococcum subsp *monococcum* (einkorn or small spelt wheat)		
Triticum urartu Tumanian ex Gandilyan (wild form)	A	A

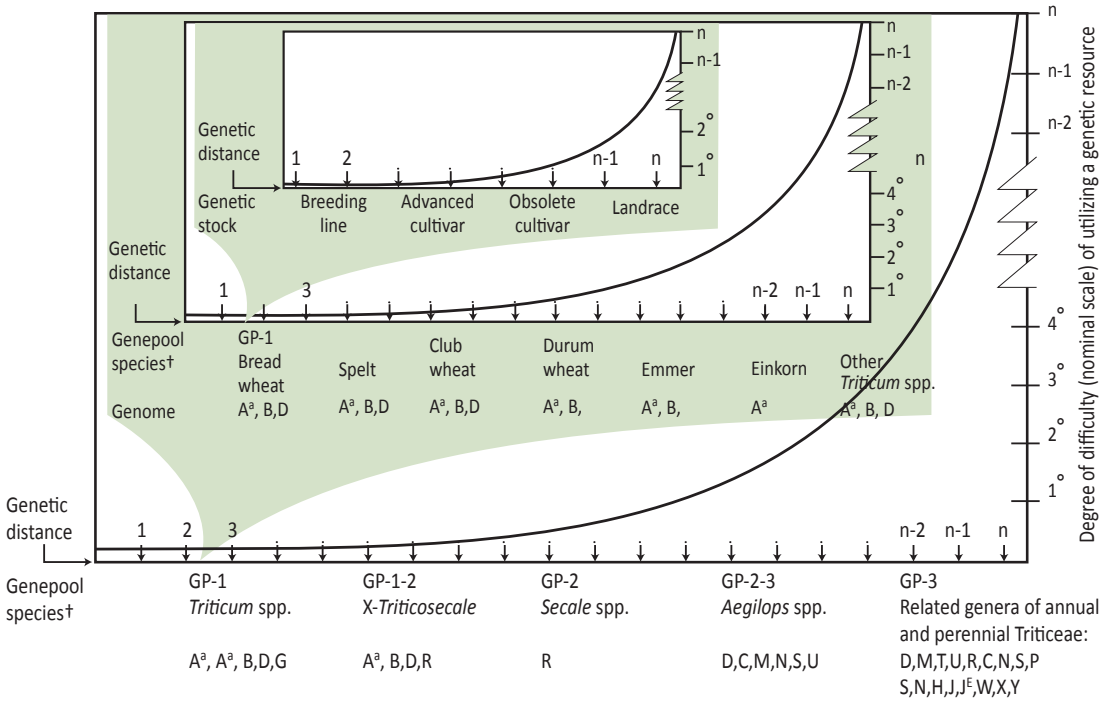

Figure 5.2. Schematic diagram of the effort required to transfer adaptive traits from genepools of wheat to farmers' fields.
† Not in strict phylogenetic order.

Table 5.3. Species of genus Aegilops and their genomic constitution.

Species	Genomic constitution	
	Nuclear	Organellar
Aegilops bicornis (Forssk.) Jaub. & Spach	S^b	S^b
Aegilops biuncialis Vis.	UM (UM^o)	U
Aegilops caudata L.	C	C
Aegilops columnaris Zhuk.	UM (UX^{co})	U
Aegilops comosa Sm. in Sibth. & Sm. var. heldreichii	M	M
Aegilops crassa Boiss.	$D^{c1}M^c(D^{c1}X^c)$	D^2
var. glumiaristata	$D^{c1}D^{c2}M^c$ ($D^{c1}D^{c2}X^c$)	-
Aegilops cylindrica Host	D^cC^c	D
Aegilops geniculata Roth (syn. Ae. ovata)	UM (UM^o)	M^o
Aegilops juvenalis (Thell.) Eig	DMU ($D^cX^cU^j$)	D2
Aegilops kotschyi Boiss.	US (US^1)	S^v
Aegilops longissima Schweinf. & Muschl.	S^1	S^{12}
Aegilops mutica Boiss.	T	T,T^2
Aegilops neglecta Req. ex Bertol. (syn. Ae. triaristata)	UM (UX^n)	U
var. recta (Zhuk.) Hammer	UMN (UX^tN)	U
Aegilops peregrina (Hack. in J. Fraser) Maire & Weiller (syn. *Ae. variabilis*)	U<u>S</u> (US^1)	S^v
Aegilops searsii Feldman & Kislev ex Hammer	S^s	Sv
Aegilops sharonensis Eig	S^{sh}	S^1
Aegilops speltoides Tausch	S	S,G,G^2
Aegilops tauschii Coss. var. tauschii, var. strangulata	D	D
Aegilops triuncialis L.	UC^t	U,C^2
Aegilops umbellulata Zhuk.	U	U
Aegilops uniaristata Vis.	N	N
Aegilops vavilovii (Zhuk.) Chennav.	DMS ($D^cX^cS^v$)	D^2
Aegilops ventricosa Tausch	D^vN^v	D

Note: Underlined genomes are modified at the polyploid level; those in brackets were deduced from DNA analysis. Gill and Friebe (2002) modified from Dvorak (1998) based on chromosome pairing and DNA analysis.

Genetic resources with desirable traits usually need to be tested and improved to be of use in wheat improvement (Figure 5.2). Most often these resources have many undesirable characteristics, such as extreme disease susceptibility, low yield, and highly specific environmental adaptation, in addition to the required trait. These resources therefore need to undergo pre-breeding before they can be used in improvement work. Figure 5.3 demonstrates two pre-breeding schemes, each with a different purpose: the open-parent, cyclical crossing program and a backcrossing program aimed at producing isogenic lines. These two programs have different purposes and different end results; moreover, the first is progressive, while the second is unprogressive in terms of yield potential.

The open-parent, cyclical crossing program described by Rasmusson (2001) is utilized when introgressing a trait known to be of value. Rasmusson was striving to introgress characters from two-row barley into six-row barley and found that the initial cross yielded germplasm with no putative candidates for cultivar release, with the best lines yielding about 20% less than the improved parent. The second cycle of the program, where the improved parent was the best current cultivar, produced progenies that yielded about 98% of the best parent's yield. The third cycle, again using the best current cultivar as a parent, yielded 112–119% of the checks. Using this scheme, germplasm with the desired trait is produced that could be competitive in a cultivar-release program.

A backcrossing program to generate isogenic lines is applied when the identified trait has as yet no proven value. The recurrent parent is crossed repeatedly to the genetic resource with the desired trait. In each backcross generation, selection is done for the tails of the populations, i.e., lines with the trait and lines without the trait. Lines that differ genetically only for the trait in question are the end result of this program. Additional trials can be conducted to assess the value of the trait, but bearing in mind that the germplasm produced will not outperform the recurrent parent.

Successful use of genetic resources in breeding

Landraces have been used extensively to introduce adaptive genes into wheat mainly associated with biotic stresses (Smale *et al.,* 2002) while recent work has shown their potential to contribute drought and heat adaptive traits like deep roots (Reynolds *et al.,* 2007). On the other hand, despite extensive use of inter-specific and inter-generic hybridization to introgress genes for biotic stress (Dwivedi *et al.,* 2008), in general only few wild relatives of crops have been used to improve adaptation to abiotic stress (Hajjar and Hodgkin, 2007). In this context, however, wheat has been a relatively good model for alien introgressions. The evolution of hexaploid wheat resulting from hybridization between tetraploid wheat and diploid *Aegilops tauschii* created a genetic bottleneck that can be overcome by re-synthesizing the hybridization using a spectrum of diploid and tetraploid accessions (Trethowan and Mujeeb-Kazi, 2008). Drought-adaptive traits associated with *A. tauschii* have been used for semi-arid environments in Australia and by the International Maize and Wheat Improvement Center (CIMMYT) (Trethowan and Mujeeb-Kazi, 2008).

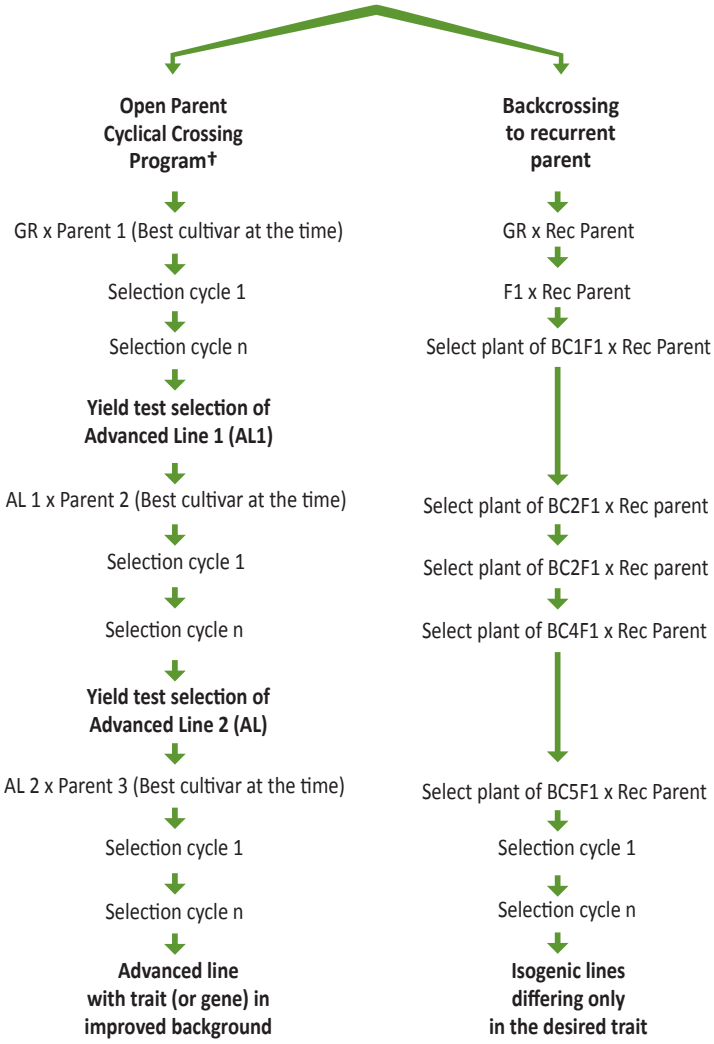

Figure 5.3. Utilization of genetic resources: prebreeding schemes (Rasmusson, 2001). †Not in strict phylogenetic order.

Comparison of synthetic derivative lines with recurrent parents showed increased water uptake associated with a root system that was more responsive to moisture stress than conventional varieties, changing its relative depth profile according to moisture availability (Reynolds *et al.,* 2007).

Alien translocation from *Thinopyrum elongatum* that carries leaf rust resistance gene *Lr19* has been shown to increase wheat yield potential and biomass by up to 15% under favorable conditions and is associated with increased spike fertility and photosynthetic rate (Reynolds *et al.,* 2001). For further details of successful use of wide crossing in wheat see Trethowan and Mujeeb-Kazi, (2008) and Ortiz *et al.* (2008).

Molecular approaches to screen genetic resources

A new initiative to explore genetic resources has recently been established at CIMMYT called *Seeds of Discovery* (SeeD). This will apply new genomics tools to identify and enable the use of undiscovered alleles in seed banks. Phenotypic and molecular descriptions of wheat and maize biodiversity –held in CIMMYT and other germplasm banks– will be developed using high-throughput phenotyping technologies and next-generation DNA sequencing platforms, respectively. The information generated by SeeD is anticipated to have application in all aspects of wheat and maize breeding though a major focus will be to identify genetic variation that will assist with adaptation to climate change (http://masagro.cimmyt.org/index.php/areas-prioritarias/descubriendo-la-diversidad-genetica-de-las-semillas).

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Phenotyping

Chapter 6: Canopy temperature and plant water relations traits

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Abstract

Water drives most of the processes involved in plant growth and significant relationships exist between crop performance and many water-relations related traits such as leaf water potential, water uptake by roots, stomatal conductance, transpiration efficiency, osmotic adjustment, etc. Understanding of these relationships has permitted the identification of efficient tools that are used in plant selection for adaptation to water limited environments; including canopy temperature (CT) which is related to root depth and hydration status, and carbon isotope discrimination which –when measured on non-water stressed tissue– is related to intrinsic transpiration efficiency. These tools have also been applied in identifying QTLs for drought adaptation. Knowledge of water relations traits has also been used to identify complementary parents in breeding for improved adaptation of wheat to water limited environments. Both CT and leaf conductance can be used as surrogates for measuring photosynthetic rate, and have application in breeding for irrigated environments, especially where yield is source limited, by heat stress for example. Water relations traits such as leaf water potential, relative leaf water content, root characteristics, and osmotic adjustment are generally too time consuming to be applied in routine breeding but are useful experimentally as accurate indicators of stress levels in field trials.

Introduction

Since water drives most of the physiological processes involved in plant growth and development, measurement of plant water status can be a powerful means of assessing the adaptive potential of crop cultivars. Screening methodologies for water status involve measurement of physiological traits that integrate plant–water relations such as: stomatal conductance (G_s), canopy temperature (CT), carbon isotope discrimination (CID), and relative water content (RWC), as well as direct measurement of leaf and soil water potential ($\Psi_{leaf;}$ Ψ_{soil}), osmotic adjustment (OA), and roots. The theoretical basis for expression of these traits and their application in breeding will be discussed below. Details of how to measure them can be found in the accompanying volume.

Canopy temperature

Many plant traits and environmental variables play roles in the energy balance of the plant canopy affecting its temperature (Figure 6.1). The main environmental variables are: (i) incident radiation which warms the plants directly –and which is obviously mitigated by cloud cover–, (ii) soil moisture in the active root zone which determines the potential for transpiration rate and therefore evaporative cooling, (iii) wind which can increase transpiration rate through reducing boundary layers around plant structures –that would otherwise insulate the plant from atmospheric effects–, and (iv) relative humidity of the air which influences the vapor pressure deficit (VPD) between plant organs and the air –warm, dry air being most conducive to evaporative cooling– (Idso *et al.*, 1977). In terms of plant characteristics that determine genotypic differences in CT –in a given environment– the most important traits are: (i) the vascular system –of leaves, shoots and roots– which determines the capacity for transpiration, (ii) stomatal aperture which regulates transpiration rate and may be influenced by hormonal signals i.e., from roots (Davies *et al.*, 2005), (iii) root depth which determines access to water –especially under drought– (Lopes and Reynolds, 2010), (iv) metabolism which if constrained for any reason (e.g., by heat stress) will cause feedback inhibition of CO_2 fixation and therefore influence stomatal aperture (e.g. Reynolds *et al.*, 2000), and (v) source–sink balance since a strong demand for assimilates (e.g., from a large number of fast growing grains) will result in increased CO_2 uptake associated with larger stomatal conductance (Reynolds *et al.*, 2005). Many studies have confirmed that CT is associated with crop yield (Figure 6.2) (e.g. Blum *et al.*, 1982; Reynolds *et al.*, 1994; Olivares-Villegas *et al.*, 2007) as well as a range of physiological traits including stomatal conductance (Amani *et al.*, 1996), plant water status (Blum *et al.*, 1982), and deep roots (Figure 6.3). As such, CT is a

versatile measurement that can complement breeding because it is highly integrative of the many physiological functions necessary to ensure adaptation to a given environment.

In terms of practical application, infrared (IR) thermometry was first used for scheduling crop irrigation in the 1970s (Jackson *et al.,* 1977) while Blum demonstrated the use of CT in drought screening in the early 1980s (Blum, 1982). The use of CT in CIMMYT's breeding research began in the early 1990s for hot, irrigated environments (Reynolds *et al.,* 1994) and has also been used as a parental selection tool for strategic crossing and in early generation selection under drought (i.e., from F_3 generation onwards) culminating in the release of physiological trait (PT) based cultivars in the 17th SAWYT (Semi-Arid Wheat Yield Nursery) (Reynolds *et al.,* 2009).

The main advantages of CT as a selection trait are the speed of measurements (≈10 seconds per plot), simplicity (aim and hold the trigger) and low cost (an IR thermometer costs less than US$200). It is also integrative of the whole canopy due to scoring many plants at once, thus reducing error associated with plant-to-plant variation. In addition, measurements of

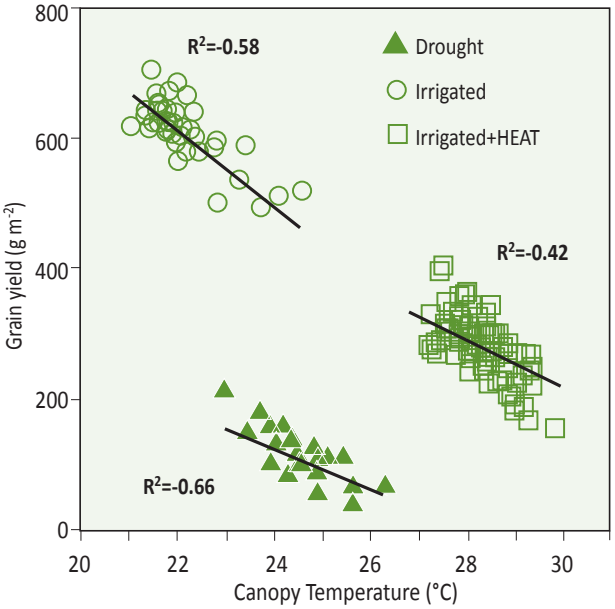

Figure 6.2. Relationship between grain yield and canopy temperature for selected data sets of wheat genotypes growing under different environmental conditions during 2011 in the Yaqui Valley, NW Mexico.

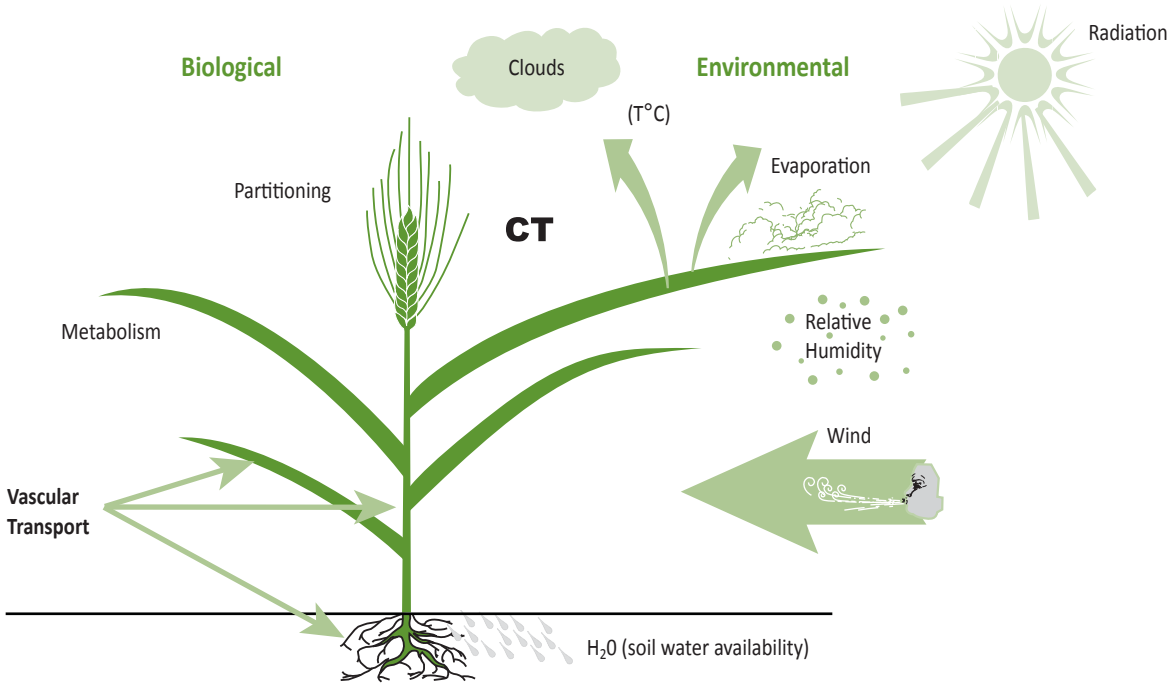

Figure 6.1. Biological (physiological) and environmental factors affecting canopy temperature (Adapted from Reynolds *et al*., 2001).

CT on plants do not interfere with the sensitive stomata; in comparison with other methods that estimate leaf conductance such as porometry and other gas exchange approaches. In recent years a price decline for IR imaging systems has extended the scope of canopy temperature measurement, allowing the development of new phenotyping platforms. These may include accurate estimation of the temperatures of different organs of a single plant or the simultaneous capture of CT of all plots in a large trial (Jones *et al.*, 2009; Munns *et al.*, 2010).

Caution is obviously needed when interpreting CT data in crop breeding. For example, there may be no association of CT with yield under drought conditions where water is not available at soil depth as in many Mediterranean conditions (Royo *et al.,* 2002). On the other hand, while a cool CT may be related directly to the genetic potential of root's capacity to explore soil moisture (Lopes and Reynolds, 2010), factors such as micro-element deficiency or soil-borne disease that affect root growth may confound the relationship. This is supported by the fact that CT is associated with expression of *Septoria tritici* and spot blotch diseases which affect the vascular system (Eyal and Blum, 1989; Rosyara *et al.*, 2008).

Tolerance to environmental stresses (mainly drought and heat) and its genetic control were explored in mapping populations at CIMMYT during recent years (Olivares-Villegas *et al.*, 2007; Pinto *et al.*, 2010). The latter

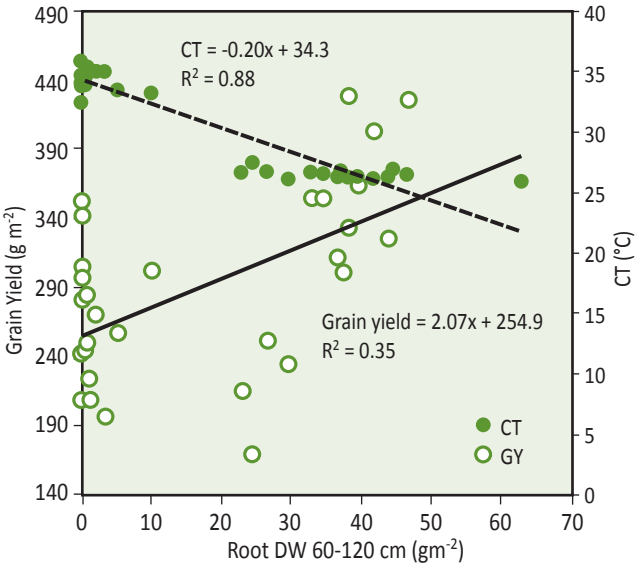

Figure 6.3. Relationship between rooting depth and: (i) yield, and (ii) canopy temperature in wheat (adapted from Lopes and Reynolds, 2010).

reported that a QTL located on chromosome 4A explained around 30% of the variation in CT of wheat under hot and irrigated environments, while 14% of variability in CT was explained by another QTL located on 3B. Pinto *et al.* (2010) found several QTL in common for adaptation to both dry and hot, irrigated conditions, suggesting that in both environments a good root system is a key adaptive trait. QTLs associated with CT under two contrasting water conditions were found in rice (Liu *et al.*, 2005) showing significant epistatic effects on spikelet fertility and grain number determination.

Stomatal conductance

There is a strong relationship between stomatal conductance (G_s) and CT since stomatal conductance has a direct effect on transpirational cooling (Amani *et al.*, 1996). Therefore, both traits are affected by many of the same environmental and physiological factors (Figure 6.1). Under well-watered conditions, stomatal regulation maintains optimal levels of internal CO_2 concentration to feed the demand for CO_2 fixation from the Calvin cycle. However, under soil water deficit there will be a trade-off with the need to maintain a functional water status of leaves (Cornic, 2000; Lawlor and Cornic, 2002). Therefore, under such conditions differences in the VPD between canopy and atmosphere as well as chemical signals synthesized in dehydrating roots, mediate stomatal aperture and therefore water flux to the atmosphere. The closure of stomata may increase leaf temperature depending mainly on the radiation load on the canopy but will result in a better water economy or increased transpiration efficiency (TE) (Condon *et al.*, 2004).

Stomatal conductance can be monitored instantaneously or over a growth period using different methodologies depending on the research objectives. Instantaneous assessment can be performed using leaf porometers, IR thermometers or infrared gas analyzers (IRGAs), while the use of isotopic discrimination gives an integration of the G_s over a growth period. Hand-held porometers assess the quantification of variability in G_s although that assessment is not as quick and integrative as CT due to a restriction in the number of leaves that can be measured. Therefore, under uniform canopy conditions, a single CT measurement provides a faster and more accurate estimation of the rate of transpiration and G_s of the whole plot. As mentioned, a close relationship exists between CT and G_s at moderate to high VPD conditions (Amani *et al.*, 1996). Oxygen isotopic composition in leaf and grain materials has also been recommended as an integrative way to estimate G_s under irrigated conditions,

being a less tedious technique than porometry, but a more expensive approach than both porometry and CT (Barbour *et al.*, 2000).

Positive relationships are observed between grain yield and G_s under irrigated environments for wheat and other crops (Reynolds *et al.*, 1994; Amani *et al.*, 1996; Fischer *et al.*, 1998) (Figure 6.4). Condon *et al.* (2008) measured leaf porosity in experimental breeding populations under irrigated conditions and found that approximately 50% and 65% of the grain yield variability was explained by G_s before and after anthesis, respectively. In irrigated environments, high G_s indicates high transpiration rates and thus the potential for biomass accumulation. Under drought environments, low G_s during vegetative stages may be beneficial due to higher TE and to a postponement of water use to late growing stages (Condon *et al.*, 1990; Morgan and LeCain, 1991) when water use is associated almost entirely with grain-filling (Kirkegaard *et al.*, 2007). However, the use of leaf porometers even under water stress is not feasible since instruments are not able to accurately measure low flux rates. It is worth noting that the slope of the relationship between CT and G_s depends on the VPD (Figure 6.5) and may explain why the relationship is not equally strong across environments (Blum *et al.*, 1989; Araghi and Assad, 1998).

Leaf water potential and relative water content

Water status in plants can be defined by two main components: the content of water *per se*, and the energy status. One of the fundamental methodologies to assess energy status of plant water is water potential (ψ). The ψ is the water energy status at a determined time resulting from the combination of osmotic potential (ψ_s), matric potential (ψ_m) and pressure potential (ψ_p). In most cases, ψ_m is minimized and leaf water potential (LWP) can be expressed as:

$$\psi = \psi_s + \psi_p \qquad \text{Equation 6.1}$$

In general, the screening of ψ in wheat is conducted on leaves. Due to time restrictions it is only carried out in targeted or check genotypes to assess the stress level of a trial at a given growth stage. In wheat, significant cultivar differences can be found in LWP under environmental conditions that limit water extraction or vascular capacity (e.g., drought, heat, and salinity). LWP values under irrigated conditions are typically between -0.5 and -1.1 MPa (Saini and Aspinall, 1981; Gusta and Chen, 1987) for wheat. Mechanisms like leaf elongation or apical growth and spikelet formation begin to be affected by values of LWP around -1.0 MPa (Barlow *et al.*, 1977; Munns and Weir, 1981). Values of -1.2 MPa or lower were

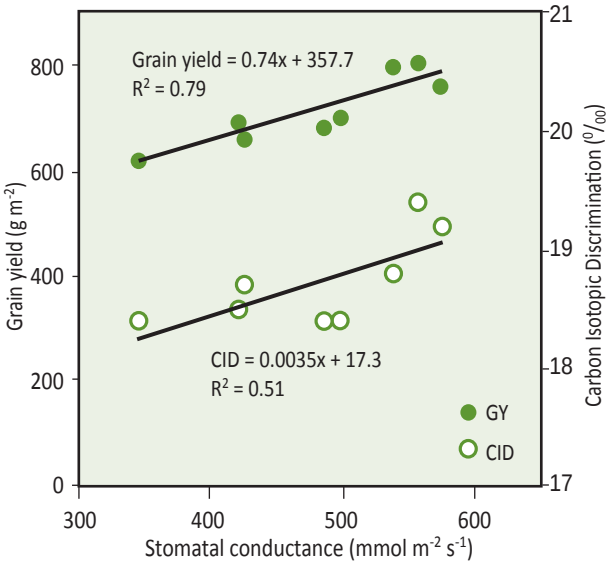

Figure 6.4. Relationship between stomatal conductance of different genotypes and grain yield (left Y axis) or carbon isotopic discrimination (CID) (right Y axis) in NW Mexico (adapted from Sayre *et al.*, 1997; Fischer *et al.*, 1998).

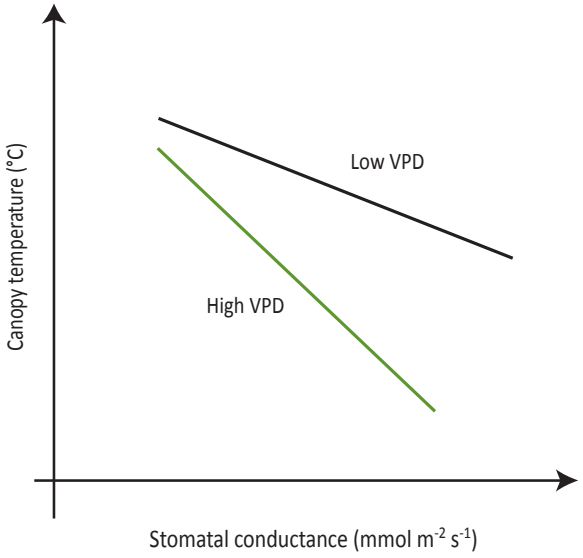

Figure 6.5. Schematic relationship between canopy temperature and stomatal conductance in two different vapor pressure deficit (VPD) environments.

associated with a reduced maximum leaf area, number of spikelets per spike and grains per unit land area (Saini and Aspinall, 1981; Gusta and Chen, 1987). A decrease in transpiration rate of leaves and spikes was found in wheat and barley as a consequence of low LWP (Johnson *et al.*, 1974). A decrease to minimum values of transpiration was observed in flag leaves and spikes when plants were at -2.8 and -3.1 MPa, respectively. Stress conditions have been found to induce embryo abortion, as well as affecting structures associated with both pollen sterility and embryo survival (Ji *et al.*, 2010), when low LWP occurs during the critical period for grain yield determination. In addition, complete stomatal closure during grain-filling was observed in wheat plants when exposed to conditions of -3.1 MPa (Frank *et al.*, 1973).

RWC indicates the hydration status of plant tissue. It is expressed in relative terms as a percentage of maximum water content at full turgor. RWC is related to ψ although both are dependent on the growth stages and water history of the plant (Hsiao, 1973). RWC in wheat shows genetic variability and intermediate heritability, and also can be measured at low cost, however, it is time consuming to measure (Khan *et al.*, 2010). In the case of bread wheat, additive gene effects were identified under different drought tolerant lines (Dhanda and Sethi, 1998).

Root system

Root systems determine the potential volume of soil that can be mined for water and nutrients. Since roots are less studied than above-ground organs, some authors suggest root physiology research as the new frontier for advancing cereal productivity in the future (Lynch, 2007; Foulkes *et al*., 2009). The interaction between environmental and genetic factors strongly determines the development of effective root systems (Passioura, 1983). Kirkegaard *et al*., (2007) indicated that access to subsoil water (deeper than 1.2 m) after anthesis may increase grain yield by improving marginal water use efficiency. In wheat crops, root growth occurs during the period between germination and anthesis. In wheat, root growth rate varies from 0.5 to 3.0 cm day^{-1} in primary and adventitious roots (Barley, 1970; Evans *et al*., 1975). Results for soybean indicate that the effect of root size and its architecture on grain yield will depend on both soil moisture and the level of competition for the resources within the canopy (King *et al*., 2009).

Vigorous and deeper root systems are recommended as one of the target traits for breeding cereals for improved water productivity in resource scarce conditions (Richards *et al.*, 2010). However, extensive root systems also have higher respiration costs for plants. While genotypic variability exists in root length under favorable conditions, the benefits of a more extensive root system are more important when plants are exposed to drying soils (Blum, 2005). Furthermore, genotypes with efficient root systems (capturing more resources with lower investments in root system) should be prioritized over abundant but inefficient systems (Lynch, 2007). Screening of root architecture, distribution and anatomy, enhanced adventitious rooting and greater dispersion of lateral roots may help to improve genetic gains for cereals under stress conditions. An alternative way to increase rooting depth and root distribution is to increase the period of root growth achievable by earlier sowing or the use of genotypes with delayed flowering. Disadvantages of these approaches are the possible negative effect of temperature (e.g., frost in early sowing, or heat stress in delayed flowering) on grain yield.

Methodologies for root trait evaluation under field conditions involve the use of coring methods (Nissen *et al.*, 2008) or uprooting plants (e.g., "shovelomics" in maize; Trachsel *et al.*, 2010) to estimate root mass and architecture (Sanguineti *et al.*, 1998). Soil coring methods are a tedious and impractical job for large populations. Misinterpretation of root trait data in field studies may be related to: (i) heterogeneity of growth in the soil, (ii) physical and chemical impedance to root growth, (iii) the plasticity of roots in response to soil physical conditions, and (iv) the occurrence of biotic infections (e.g., nematodes and fungal diseases). On the other hand, in order to reduce the variability observed in field studies, root screening can be made under controlled environments using rhizotrons (Johnson *et al.*, 2001), pots, hydroponics, or gel-filled containers. Associations between results obtained in field and controlled conditions have been reported (Landi *et al.*, 2001) for maize. See the chapters by Herrera *et al.* (this volume, Chapter 9) and Lopes *et al.* (the accompanying volume, Chapter 17) for more details on root function and sampling methods, respectively. Since CT has been shown to be associated with the ability of roots to extract water under drought (Lopes and Reynolds, 2010), then selecting for CT may increase gene frequencies for root-related traits in environments where water is available at depth.

Due to the constraints of root sampling, mapping populations are poorly explored for root traits compared with above-ground traits. Genetic control of root traits and its variability has been reported on wheat (Sanguineti *et al.*, 2007; Sayar *et al.*, 2007). Sanguineti

et al. (2007) reported a number of chromosome regions significantly associated with variability in root architecture and major effects were found on 2AL, 7AL and 7BL regions using length and weight of seminal roots.

Osmotic adjustment

Osmotic adjustment (OA) is the process by which plants accumulate solutes in their cells to minimize water loss and maintain cell function under drought conditions. The value of OA for stress tolerance has been demonstrated in C3 as well as C4 species (Ali *et al.*, 1999; Blum *et al.*, 1999; Chimenti *et al.*, 2006). The capacity of plants to accumulate solutes is one of the more important adaptive responses to drought at the cellular level. OA has been identified as a mechanism to maintain grain yield under stressed conditions (e.g., drought, salinity) by allowing root growth and maintaining water and nutrient capture (Turner, 1986).

Osmotic adjustment is an inappropriate trait for field-based evaluation due to confounding effects generated by genotypic differences in soil water exploration by roots. However, measurement of OA in leaves under controlled conditions in a glasshouse can identify genetic variation (Morgan, 1988). Ali *et al.* (1999) found that wheat shows a large expression of OA in comparison to other species in response to drought treatments. Genotypic variability in OA is expressed in wheat genotypes exposed to periods of water stress, and can be used as a selection criterion for drought conditions (Morgan, 1977; Morgan, 1983; Rekika *et al.*, 1998). Genes involved in osmoregulation were found to be associated with increased yield under conditions of high VPD for wheat (Morgan, 1991; Morgan, 2000). Regarding the genetic control of OA, Peleg *et al.* (2009) found 11 different quantitative trait loci (QTLs) for ψ_s and four different loci (2A, 2A, 3B, 5B) on the G18-16 allele associated with low ψ_s. Previous work of Morgan and co-workers (Morgan, 1983; Morgan, 1991; Morgan and Tan, 1996) suggests that this trait could be controlled by a single recessive gene related to K^+ accumulation in leaves.

Carbon isotope discrimination

CID ($\Delta^{13}C$) provides an integrative assessment of leaf transpiration efficiency (TE) in C3 species (Farquhar *et al.*, 1982). It involves biochemical and physiological processes such as stomatal aperture, internal conductance of gases in plant tissue, and external environmental conditions that modify gas exchange and CO_2 fixation (Farquhar *et al.*, 1989). CID ($\Delta^{13}C$) is a measure of the $^{13}C/^{12}C$ ratio in plant material relative to the value of the same ratio in the air on which plants feed, and its positive values reflect the C3 discrimination (Δ) against the heavier stable carbon isotope (^{13}C) during photosynthesis. This favored discrimination of ^{13}C is positively associated with carbon dioxide levels in the intercellular air spaces of the photosynthetic tissues and, given a constant tissue-to-air VPD, is also positively associated to water uptake but negatively associated with TE. Greater overall stomatal aperture allows increased rates of leaf gas exchange, allowing the plant to favor ^{12}C but with higher water losses (Condon *et al.*, 1990).

CID has been identified as a useful trait to measure the changes in TE and CO_2 concentration at the intercellular level of the leaf (Farquhar *et al.*, 1982; Condon *et al.*, 1987; Condon *et al.*, 1990; Rebetzke *et al.*, 2008) and also has been successfully used in breeding programs for improving productivity under water scarce conditions (Richards *et al.*, 2010). In fact, CID has been used as a selection tool and commercial varieties have already been released based on selection for high TE (Richards, 2006).

Under favorable environments, high CID (measured in grains or biomass) is positively associated with grain yields. Genotypes with high CID usually transpire more water to produce grain yield under well-watered conditions at the risk of using water with low efficiency (Condon *et al.*, 1987; Araus *et al.*, 2002). Therefore, selecting genotypes with low CID (conservative water use) may help to improve productivity by increasing water use efficiency under rain-fed conditions. Variability in the response of grain yield to CID can be observed under stressed conditions and it therefore depends on the phenological stage of the crops and the level of stress explored. In general, positive relationships were reported for CID and grain yield under drought conditions (Condon *et al.*, 1987; Ehdaie *et al.*, 1991; Sayre *et al.*, 1995; Araus *et al.*, 1998; Araus *et al.*, 2003; Condon *et al.*, 2004). Under drought conditions, the high CID can be interpreted as a higher capacity to sustain G_s (Condon *et al.*, 2004), probably related to greater access to water at depth. Therefore, when crop growth is determined by access to water, high CID can be associated with high yield. Alternatively, under environments where yield depends on the stored water, low CID may be used to detect better genotypes (Richards *et al.*, 2001) due to conservative water use behavior.

Genotypic variability has been reported in small grain cereals in leaves as well as in grains (Condon *et al.*, 1990; Merah *et al.*, 2001; Rebetzke *et al.*, 2002). The genetic control for CID has been studied in different populations for wheat in terms of QTL analysis (Rebetzke *et al.*, 2008; Peleg *et al.*, 2009). Rebetzke *et al.* (2008) mapped three different populations in more than three different

environments without water limitations indicating that QTL effects account for less than 10% of the total additive variance and suggest a number of chromosomal regions are related to this trait (1BL, 2BS, 3BS, 4AS, 4BS, 5AS, 7AS and 7BS). The confirmed polygenic control of CID in their results, in addition to the small size of the QTL, could be interpreted as a limitation in the use of CID for breeding wheat through marker-assisted selection (MAS). Peleg *et al.* (2009) used recombinant inbred lines of durum wheat under four different water availability conditions and reported twelve different QTLs for CID, supporting previous findings of the polygenic control (Rebetzke *et al.,* 2008).

Conclusion

As briefly reviewed above, there are different approaches for estimating plant water status; the choice of which will be a function of breeding targets, scale of operation, and resources available (Table 6.1). Within well-watered environments the main breeding targets are likely to be yield potential and/or heat tolerance. For these situations photosynthetic capacity and efficiency are important traits to improve (Parry *et al.*, 2010). Since photosynthesis *per se* is a time consuming trait to screen for using IR gas analysis, both CT and G_s offer high-throughput surrogates (Fischer *et al.*, 1998). Both traits show good association with photosynthesis and performance under hot, irrigated environments (Gutiérrez-Rodríguez *et al.*, 2000; Reynolds *et al.*, 2000).

Selection for drought targets should include traits that indicate a better use or capture of water, including root traits. However, current methodologies for screening root traits are still tedious and time consuming. On the other hand, CT estimates the relative water-uptake capacity of roots much more efficiently than root sampling (Reynolds *et al.*, 2007; Lopes and Reynolds, 2010). The selection of genotypes with more conservative water use (high TE) can be achieved by analyzing CID (of non-stressed tissue). In addition –though estimates are only feasible under controlled conditions– the evaluation of OA for determining differences in dehydration tolerance potential is also a useful trait for adaptation to limited 'stored soil water' conditions. Other traits like RWC or LWP have restricted application in breeding due to their non-straightforward protocols. However, they are recommended to quantify the evolution of stress levels over a cycle, or during a day, using a fixed number of genotypes identified as checks.

Table 6.1: Environments and recommended water relations traits for use in parental selection and screening.

Environment	Traits
Irrigated	CT***, G_s ***
Drought (water at depth)	CT***, Roots*, RWC*
Drought (restricted stored water)	CT*, CID***, OA**, Roots*, RWC*
Drought (unpredictable rainfall)	CT**, CID**, OA**, Roots*, RWC*
Irrigated + hot temperatures	CT***, G_s ***, Roots*

Note: ***, **, and * indicate high, intermediate, and low priority, respectively. CT = canopy temperature; G_s = stomatal conductance; CID = carbon isotope discrimination; OA = osmotic adjustment; RWC = relative water content.

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Chapter 7: Spectral radiometry

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Abstract

Through a detailed understanding of the electromagnetic spectrum and the optical properties of plant canopies, researchers have been able to take a large leap forward in the efficiency of screening physiological traits. These technologies are now able to identify the very best genotypes from early generation material, before expensive yield trials are conducted, and enhance selection from already superior advanced lines. This chapter outlines the theoretical framework behind spectroradiometry and the important considerations that should be understood when performing remote sensing measurements and interpreting spectral results. It also details spectral reflectance indices, which relate to various aspects of plant physiology, such as vegetative growth, water status and photochemistry. Spectroradiometry is a powerful selection tool that lends itself to the high-throughput screening of traits. It has the potential to enhance future genetic gains, increase wheat yield potential and improve the response of crops to stress.

Introduction

The application of spectral reflectance indices is a practical means of adopting physiological trait selection within crop improvement and research programs. Breeding approaches for improving abiotic stress tolerance in crop species have evolved at a rapid rate, with the development of molecular technologies and transgenic approaches becoming a prominent part of many research initiatives. However, in order to surpass the current limits for yield potential and stress adaptation in wheat, new variation for physiological traits needs to be identified and physiological screening tools for the integration of the traits into field research and breeding programs need to be developed. During recent years spectroradiometrical technologies have offered a quantum leap forward in the efficiency of physiologically screening traits that have typically been laborious and time-consuming and have consequently had limited application within breeding programs. These technologies are now able to identify the very best genotypes from early generation material, before expensive yield trials are conducted (Reynolds *et al.*, 1999), and enhance selection from already superior advanced lines. The prospect of future genetic improvements, through utilizing spectroradiometry to identify and track physiological traits, provides plant breeding programs with new opportunities and genetic diversity from which to increase yield potential and to improve the response of crops to stress (Knipling, 1970; Shorter *et al.,* 1991).

The electromagnetic spectrum

Spectral-radiometry is the study of electromagnetic radiation and its interaction with matter, as a function of wavelength. The electromagnetic spectrum is comprised of an infinite range of possible frequencies of electromagnetic radiation. Hence, the electromagnetic spectrum of an object, such as a leaf or crop canopy, is the characteristic distribution of electromagnetic radiation emitted, or absorbed, by that particular object. Furthermore, the electromagnetic spectrum is continuous; with waves of radiation able to be characterized and grouped by their wavelength and frequency. The commonly referenced spectral regions are indicated in Figure 7.1. When measuring crop canopies, primary focus is given to electromagnetic radiation within the visible (VIS) and near-infrared (NIR) regions of the electromagnetic spectrum (350 nm–750 nm and 750 nm–2500 nm, respectively).

Spectral reflectance: theory and application

Spectral radiometry of a crop canopy is obtained from measurements of reflected radiation. The ability to detect reflected radiation is derived from the fact that when a single light wave collides with a material it is restricted to three physical processes. It can be reflected from the surface, absorbed by the object, or transmitted through the object (Figure 7.2). However, with crop canopies in the field, many or all wavelengths of radiation are incident towards the surface of an object. In this case, objects will selectively reflect,

absorb and transmit the radiation depending on its frequency and the nature of the atoms of the object. The reflection of light occurs because the frequencies of the light waves do not match the natural frequencies of the vibration of the object. If the object is opaque, then the vibrations of the electrons are not passed from atom to atom through the bulk of the material, but instead vibrate for short periods of time and then re-emit the energy as a reflected light wave. Through measuring this unique reflectance signature, researchers are able to characterize aspects of crop structure and function.

Spectral reflectance from leaves at different wavelengths gives a unique spectral signature as it is influenced by the optical properties of the plant (e.g., proteins, lignin, cellulose, sugar, starch, etc.). Pigments in leaves absorb light strongly in the photosynthetically active radiation (PAR) region of the electromagnetic spectrum, but not in the NIR (750 nm–2500 nm) region (Knipling, 1970). This results in a lower reflection of radiation in the PAR compared to the NIR region of the spectrum. For example, chlorophylls *a* and *b* (Chl*a* and Chl*b*, respectively), which are present in green leaves, strongly absorb light in the red (RED) region of the spectrum (≈690 nm), while cell walls strongly scatter (reflect and transmit) this light in the NIR (≈850 nm) region of the spectrum (Figure 7.3). This generates an absorption contrast between these two spectral regions, which can be represented by various indices. Spectra that are reflected by plant canopies provide information that may be used to determine a range of parameters. These may include an estimation of green biomass, photosynthetic size of the canopy, the amount of PAR absorbed by the canopy and its photosynthetic potential (Wiegand and Richardson, 1990; Baret and Guyot, 1991; Price and Bausch, 1995; Araus *et al.,* 2001; Reynolds *et al.*, 2001). The physiological status of the canopy may also be measured via spectral reflectance. Reflectance measurements can be used to assess the effects of nutrient deficiencies and environmental stresses through estimations of chlorophyll and carotenoid concentrations, photosynthetic radiation use efficiency (RUE) and water content (Peñuelas *et al.,* 1993; Peñuelas *et al.,* 1995a; Peñuelas *et al.,* 1995c; Araus *et al.,* 2001; Reynolds *et al.,* 2001; Babar *et al.,* 2006a; Prasad *et al.,* 2007). Grain yield has also been estimated using spectral reflectance indices during different growth stages of the crop (Aparicio *et al.,* 2002; Osborne *et al.,* 2002; Babar *et al.,* 2006a).

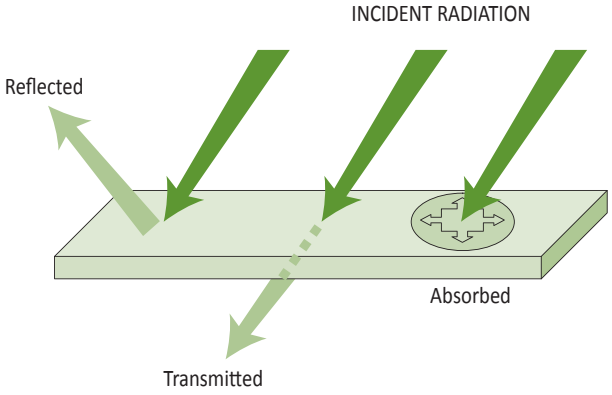

Figure 7.2. Incident radiation is equal to reflected radiation + absorbed radiation + transmitted radiation.

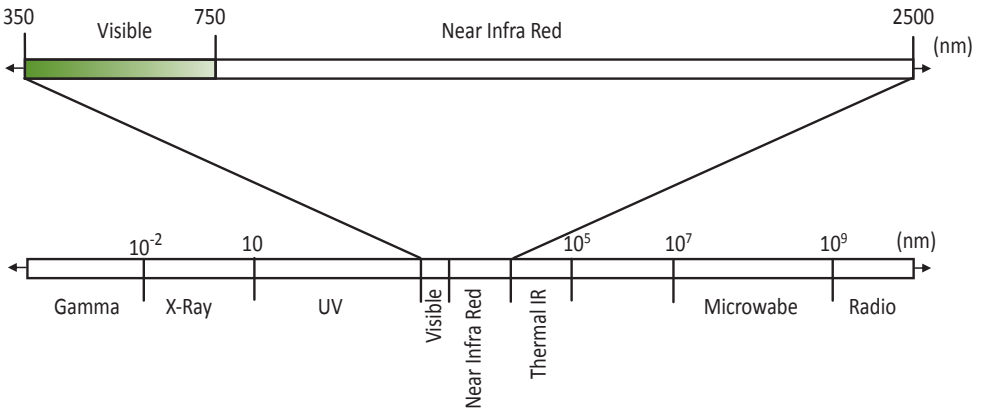

Figure 7.1. Range of the electromagnetic spectrum, with the region measured using canopy spectral reflectance indices enlarged above.

Methods and techniques

When taking spectral reflectance measurements, two key pieces of equipment are required: (1) a field spectro-radiometer, equipped with an appropriate foreoptic lens, and (2) a white spectral reference panel, with the necessary support for maintaining a fixed and horizontal position in the field (Figure 7.4). Field spectrometers are commonly manufactured with a spectral range of 350 nm–1100 nm, or with a more extended range of 350 nm–2500 nm. This continuous range encompasses the visual and near-infrared regions of the electromagnetic spectrum and is sufficient for measuring the wavelengths used for most canopy related indices. The advantage of measuring a continuous range is that numerous indices may be calculated from the one spectral reflectance measurement. However, the quantity of data that can be generated from large trials demands an efficient approach to data management.

Reflected radiation from the canopy is received by a foreoptic lens, and relayed to the spectroradiometer via a fiber-optic cable. The foreoptic lens provides a defined field of view (FOV), which is usually between 10° and 25°. The optimal FOV will be determined by the crop species, experimental design and distance between the foreoptic and canopy; however, with manual measurements within field trials the 25° FOV is most commonly used. The foreoptic is generally held 1–2 m above the crop canopy, either by hand or with the assistance of a boom, and a constant vertical orientation is maintained during measurements. The same orientation is also used when measuring reflectance from the white reference panel, but with the foreoptic held closer to the panel to ensure the measurement is made using all of the FOV.

White reference panels should be Lambertian surfaces; these reflect incident radiation of all wavelengths equally in all directions, and are required to provide a

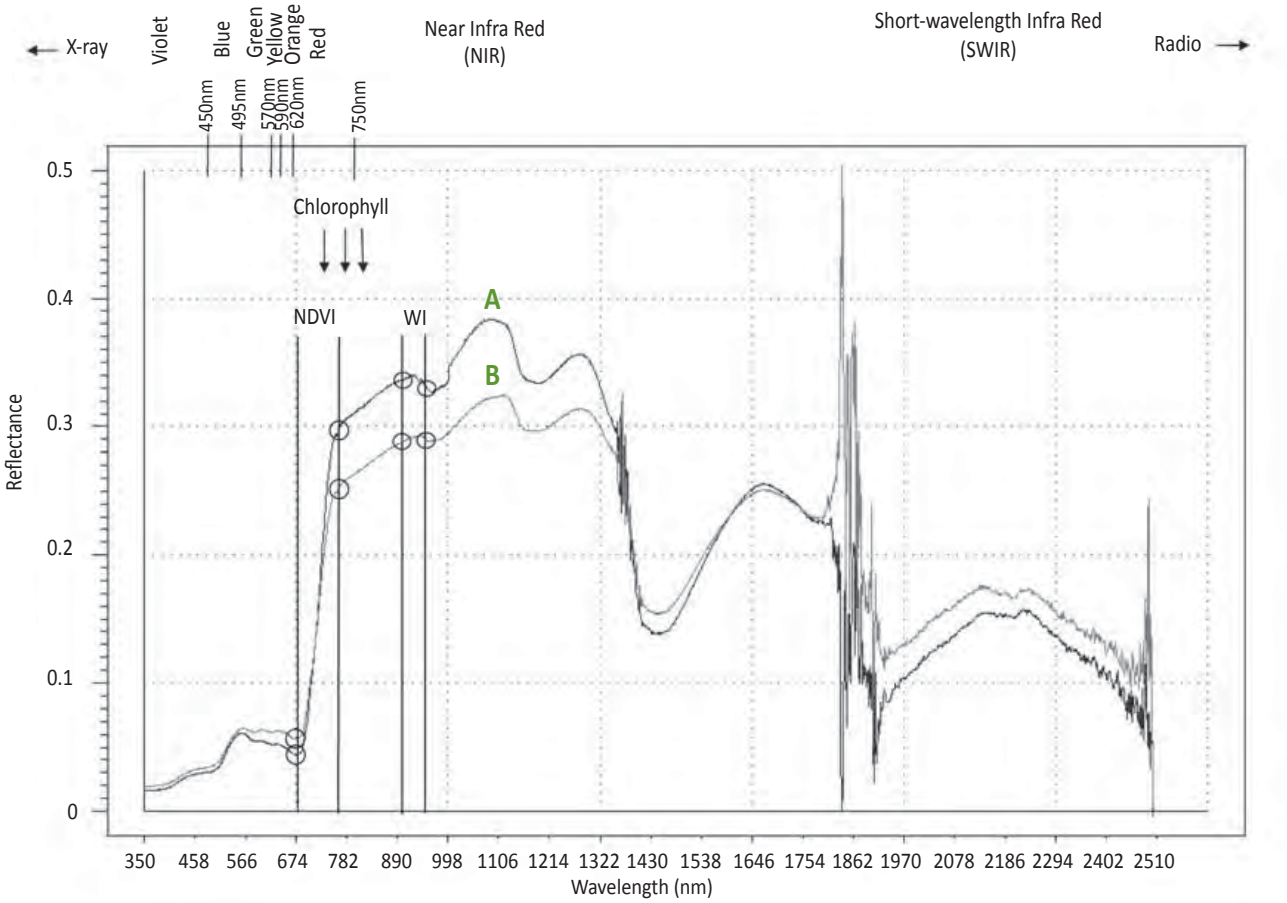

Figure 7.3. Example reflectance spectra from a spectroradiometer, with key spectral regions and indices marked. The spectra have been obtained from two wheat canopies with (A) high biomass, and (B) low biomass.

reference for the calculation of reflectance units (Araus *et al.,* 2001). As reference panels are not perfect, it is important to maintain a constant panel orientation, keeping the same angle with the foreoptics and the sun. Measurement of the reference panel provides a value for the spectra incident on the canopy, and is used to obtain a ratio with the spectrum reflected by the canopy. As the intensity of incident radiation is continuously changing with zenith angle and other environmental variables, it is important to perform regular measurements of the white reference panel.

There are several important considerations to be made when performing remote sensing measurements and interpreting spectral results. The reflectance of electromagnetic radiation from a canopy may be influenced by numerous factors, including the following:

- **Canopy structure and morphology** – Phenological differences between genotypes may affect spectral indices, and is a common factor to consider when sampling segregating populations at key phenotypic stages, such as during heading in wheat (Shibayama *et al.,* 1986). Likewise, the erectness of leaves in the canopy influence the direction of reflected incident radiation. Jackson and Pinter (1986) established that the vertical elements of an erectophile canopy trapped reflected radiation within the canopy. In contrast, in a more planophile canopy, a greater proportion of radiation is reflected vertically. Furthermore, differences in the surface of plant leaves may also affect spectral reflectance, as differences in glaucousness (waxiness) between genotypes have been shown to affect reflectance spectra (Febrero *et al.,* 1998).
- **Water status of the crop** – Plant leaves in the crop canopy may be composed of between 40 and 80% water by weight. As there are prominent liquid water absorption bands located at approximately 760 nm, 970 nm, 1190 nm, 1450 nm and 1950 nm, and water is highly absorbing in these regions, the spectral signatures used in indices may be masked by the water. Studies have shown the effects of differences in leaf water on NIR reflectance (Gao and Goetz, 1994) and as a result, indices that rely on spectral reflectance in regions in the NIR near water bands will be sensitive to variations in leaf reflectance that may be solely caused by leaf water content (Kokaly and Clark, 1999).
- **Degree of canopy cover** – When the fraction of canopy cover is not 100%, components such as soil, water or man-made objects have the ability to influence the reflected spectra. When using a spectrometer in the field the range of the instrument will usually include the visual spectrum (350 nm–750 nm), in which case a calculation of chlorophyll absorption can be used to estimate the fraction of coverage, and provide a means for data adjustment or quality control. Kokaly and Clark (1999) demonstrate in a study of a diverse collection of plant species that nitrogen estimates are relatively insensitive to soil background until a soil cover of 40% or greater is reached. Errors in lignin estimates are also relatively low until half the signal is comprised of the soil spectrum. However, errors in cellulose estimates were reported to increase substantially when soil cover exceeded 20% (Kokaly and Clark, 1999).
- **Geometry of incident radiation** – Changes in the position of the sun will affect the spectrum reflected from the crop canopy as it interacts with canopy structure and sowing orientation. This effect is most prominent in canopies with low leaf area index (LAI),

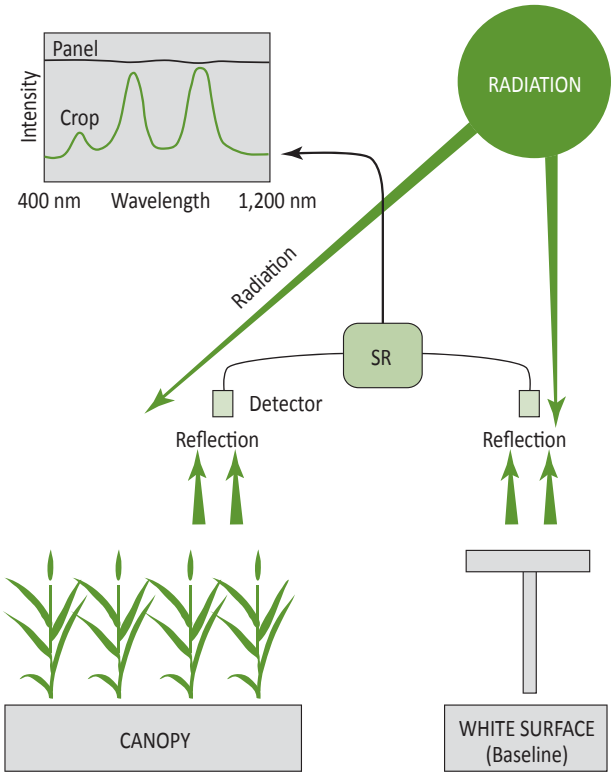

Figure 7.4. Spectral reflectance from crop canopies (Araus *et al*. 2001).

however, may be minimized by using a nadir viewing angle and sampling at noon. This approach is likely to minimize interactions between row orientation and sun position, but may be more affected by reflectance from soil background. When soil background effects are significant, the viewing angle may be altered by maintaining the angle between the sensor azimuth and the sun azimuth as 0–90° (Wardley, 1984).

- **Degree of shading** – The extent of shading within a crop canopy is influenced by canopy structure, sowing orientation, sun position and viewing angle of the spectrometer. Angular changes between plant row orientation and sun position alone may generate variation in the measured reflectance as high as 100% in RED and lower in NIR wavelengths (Kollenkark *et al.,* 1982). Leaves exposed to full incident radiation (i.e., in the upper portion of the canopy) reduce the light incident on shaded leaves in the wavelengths that have already been absorbed by those upper leaves, reducing their reflected spectra. Therefore, the greater the shading within the canopy for a particular field of view, the larger the difference in the canopy's reflectance spectra between regions where radiation is absorbed by photosynthetic pigments and regions where it is not (Araus *et al.,* 2001).
- **Clouds** – The presence of cloud cover will increase the amount of diffuse (indirect) radiation incident on the canopy. Increased diffuse radiation will increase canopy penetration and the amount of radiation absorbed by photosynthetic pigments. The estimation of vegetation indices will be overestimated under these conditions, and measurements will be more subject to error from changes in canopy structure (e.g., from wind) (Lord *et al.,* 1985). Incident radiation intensity is also likely to be more variable under cloudy conditions, requiring an increased frequency of white reference panel reflectance measurements to minimize error.
- **Presence of nearby objects** – The presence of objects close to the measured spectra can alter the reflectance of radiation from the canopy. Commonly, the instruments being used and the operator are the primary source of this interference, but effects may be minimized through keeping a maximum distance from the FOV, and coloring nearby objects as dark as possible to reduce the reflectance of radiation onto the canopy (Kimes *et al.,* 1983).

Spectral reflectance indices

Spectral reflectance indices are numerical indicators that use either specific wavelengths, or bands of the electromagnetic spectrum, to quantitatively relate changes in reflectance spectra to changes in physiological variables. Indices have the advantage of summarizing a large amount of information into a few numerical values, which may be evaluated simultaneously in each sample. These qualities make the use of spectral reflectance indices ideal for screening large germplasm sets for physiological responses to stress.

To improve interpretation, the electromagnetic spectrum sampled by a field spectrometer may be separated into three regions:

(i) Visible light region (VIS) – this incorporates the 350 nm to 750 nm region of the spectrum that contains Chl*a* and Chl*b*, carotene and xanthophyll pigments.

(ii) Near infrared (NIR) – this incorporates the wavelength spectrum between 750 nm and 1350 nm, and is affected by internal leaf structure.

(iii) Short-wave infrared – this incorporates the 1350 nm to 2500 nm region of the spectrum that is influenced to a small degree by leaf structure, but is highly affected by water concentration in leaf tissue, with strong absorption bands between 1450 nm and 1950 nm.

Vegetation indices

Vegetation indices are used to estimate the photosynthetic size of a canopy, and are based on spectral reflectance in the RED and NIR regions of the electromagnetic spectrum. Healthy vegetation absorbs most of the visible light that is incident upon it, while reflecting most of the NIR light. Conversely, unhealthy, or sparse vegetation, absorbs only a small portion of the visible and a larger proportion of NIR light (Araus *et al.,* 2001). Therefore, the larger the difference between NIR and RED reflectance, the more vegetation that is present in the sample. These characteristic spectral responses may be used to determine the behavior of plants through developing indices that are sensitive to specific wavelengths. Vegetation indices may be used to estimate LAI, green biomass, green leaf area index (GLAI) and more (Wiegand and Richardson, 1990; Baret and Guyot, 1991; Price and Bausch, 1995; Araus *et al.,* 2001). Periodic measurements during the crop growth cycle also allow the estimation of leaf area development as an indicator of stress and the total PAR absorbed by the canopy for yield predictions (Wiegand and Richardson, 1990).

Among the most commonly used vegetation indices are the simple ratio (SR) (Jordan, 1969) and the normalized difference vegetation index (NDVI) (Rouse *et al.,* 1973; Araus *et al.,* 2002) (Table 7.1). The SR is a simple index that divides the NIR reflectance by RED reflectance, while NDVI is a normalized index that accounts for changes in incident radiation. A normalized index facilitates comparisons between measurements taken under different light conditions, where measurements under high incident radiation (e.g., full sunlight) would have larger values (larger absolute difference between RED and NIR reflectance) than those taken under low incident radiation (e.g., cloudy sky).

The exact wavelengths used for RED and NIR reflectance and index calculation may vary due to experimental objectives and/or be dependent on the specifications of the radiometer. For example, Hall *et al.* (1990) used RED = 660 nm and NIR = 770 nm to estimate the fraction of absorbed photosynthetically active radiation, while Peñuelas *et al.* (1997a) used RED = 680 nm and NIR = 900 nm to assess the effects of salinity on barley. Furthermore, Carter (1998) modified the NDVI to improve correlations with leaf photosynthetic capacity, using RED = 520 nm and NIR = 701 nm. Essentially, the wavelengths sampled in the calculation of spectral indices may be adjusted to enhance their relevance to a specific research objective.

Adjustments in vegetation indices have incorporated compensation for the effect of soil background on measurements. The soil adjusted vegetation index (SAVI) (Huete, 1988) and the transformed soil adjusted vegetation index (TSAVI) (Baret and Guyot, 1991) were formulated to achieve improved repeatability between experimental locations that have large soil differences (Araus *et al.,* 2001) (Table 7.1). Additionally, indices have also been formulated to estimate LAI beyond the range at which NDVI will become saturated. Saturation of NDVI may begin at LAI = 1, and become completely insensitive to changes when LAI ≥2. The perpendicular vegetation index (PVI) is able to partly overcome this insensitivity to LAI, although becomes more sensitive to changes in spectroradiometer viewing geometry (Shibayama *et al.,* 1986) (Table 7.1).

The measurement of vegetation indices has been successfully applied to assessing LAI, biomass and vigor of wheat genotypes (Wiegand and Richardson, 1990;

Table 7.1. Vegetation indices.

Name	Abbreviation	Index calculation	Comments	Reference
Simple ratio (Ratio vegetation index)	SR (RVI)	R_{NIR} / R_{RED}	The index has a range of 0–inf where R_{NIR} is the reflectance at NIR and R_{RED} is the reflectance at red	Jordon, 1969
Normalised difference vegetation index	NDVI	$(R_{NIR} – R_{RED}) / (R_{NIR} + R_{RED})$	The index has a range of -1–1 where RNIR is the reflectance at NIR and R_{RED} is the reflectance at RED	Rouse *et al*., 1973
Soil adjusted vegetation index	SAVI	$[(R_{NIR} – R_{RED}) / (R_{NIR} + R_{RED} + L)] (1 + L)$	Where L is adjusted to minimize noise caused by soil for a large range of soil covers. For most crop conditions L=0.5, while for very low soil covers L=1 would be more appropriate and L=0.25 for very high covers	Huete, 1988
Transformed soil adjusted vegetation index	TSAVI	$a(R_{NIR} – aR_{RED} – b) / [R_{RED} + a(R_{NIR} – b) + 0.08 (1 + a^2)]$, where a is the slope and b is the intercept of the linear equation $R_{NIR\ soil}=a* R_{RED\ soil} + b$	An index that includes parameters obtained from the soil's reflectance spectrum	Baret and Guyot, 1991
Perpendicular vegetation index	PVI	$[(R_{RED\ soil} – R_{RED\ vegetation})^2 + (R_{NIR\ vegetation} – R_{NIR\ soil})^2]^{1/2}$	PVI partially overcomes the saturation problem associated with NDVI	Richardson and Wiegand, 1977

Where: R_{NIR} = Reflectance in the near infrared reflectance region of the spectrum; R_{RED} = Reflectance in the red reflectance region of the spectrum.

Baret and Guyot, 1991; Price and Bausch, 1995). A more practical application of these indices is in the prediction of yield from successive measurements taken during the growing season. NDVI has been used to predict grain yield in soybean, durum and winter wheat (Aparicio *et al.,* 2000; Ma *et al.,* 2001; Raun *et al.,* 2001), and in experiments across multiple locations, NDVI and SR were able to explain 50–65% of variability for wheat yield (Tucker *et al.,* 1980; Aparicio *et al.,* 2000; Serrano *et al.,* 2000; Raun *et al.,* 2001). Similarly, correlations (R^2) between vegetation indices and wheat grain yield of 0.5 and 0.66 have also been reported (Rudorff and Batista, 1990; Wiegand and Richardson, 1990).

Water indices

The water index is used to measure the water status of the canopy, including parameters such as stomatal conductance, leaf water potential, relative water content and canopy temperature (Peñuelas *et al.,* 1993; Babar *et al.,* 2006c;). Water absorbs several bands of wavelengths across the 950–2500 nm range that may be measured by field spectrometers. In the 1300–1500 nm region of the spectrum, water has a very high absorption of radiation. This limits the application of these wavelengths, as reflectance becomes saturated, even in canopies with low water content. However, water has a weaker absorption of radiation in the 950–970 nm region of the spectrum that may be used in the development of indices. A water index (WI) was first derived from spectral reflectance at 970 nm (Bull, 1991), with 900 nm used as a reference wavelength as it is similarly affected by canopy and leaf structures, but was not absorbed by water (Peñuelas *et al.,* 1993; Peñuelas *et al.,* 1997b) (Table 7.2). Good correlations between WI and relative water content (RWC) (R^2 = 0.55) have been reported for a range of species measured at different times of the year in their natural Mediterranean environment (Peñuelas *et al.,* 1997b). Furthermore, Peñuelas *et al.* (1997a) established a correlation between WI and canopy temperature depression in salt stressed barley, and Babar *et al.* (2006c) identified strong positive correlations between canopy temperature (CT) and WI in wheat. Gutiérrez-Rodríguez *et al.* (2004) and Babar *et al.* (2006b) also identify a strong association between WI and wheat grain yield under irrigated conditions.

New and improved indices have been developed to distinguish between high yielding genotypes with more accuracy. Strong phenotypic and genetic correlations were identified between two newly derived NIR-based indices (NWI-1 and NWI-2) (Table 7.2) and grain yield of wheat cultivars and random sister lines (F_5, F_6 and F_7 generations) under reduced (Babar *et al.,* 2006a) and full irrigation (Babar *et al.,* 2006b) conditions in northwest Mexico. In the reduced irrigation environment, a comparison of indices demonstrated that those based on NIR (WI, NWI-1, NWI-2) had consistently higher associations with grain yield, explained a higher proportion of the variability for grain yield, and identified a higher percentage of the top-yielding lines, compared with other spectral indices (NDVI and SR) (Babar *et al.,* 2006a). Under fully irrigated conditions, the NIR indices were also more highly associated with, and explained a greater proportion of the variation for, grain yield compared with the red normalized difference vegetation index (RNDVI), green normalized difference vegetation index (GNDVI), SR and photochemical reflectance index (PRI) indices (Babar *et al.,* 2006b). Furthermore, NWI-2 was identified as the most efficient index in selecting superior genotypes in different experiments (Babar *et al.,* 2006b). Two more normalized water indices (NWI-3 and NWI-4) (Table 7.2) were developed and were shown to detect a significant proportion of the highest yielding genotypes and an equal, or higher, correlated response than direct response for grain yield in winter wheat rain-fed environments (Prasad *et al.,* 2007).

Chlorophyll indices

Spectral reflectance indices have been developed to measure the concentration of leaf pigments, such as chlorophyll and carotenoids. There are several reflectance wavelengths that are sensitive to chlorophyll content. Spectral reflectance at 675 nm is very sensitive to changes in chlorophyll content, but this limits its use

Table 7.2. Water indices.

Name	Abbreviation	Index calculation	Reference
Water index	WI	R_{970} / $R6_{80}$	Peñuelas *et al*., 1993
Normalised water index - 1	NWI-1	$(R_{970} - R_{900}) / (R_{970} + R_{900})$	Babar *et al*., 2006b
Normalised water index - 2	NWI-2	$(R_{970} - R_{850}) / (R_{970} + R_{850})$	Babar *et al*., 2006b
Normalised water index - 3	NWI-3	$(R_{970} - R_{920}) / (R_{970} + R_{920})$	Prasad *et al*., 2007
Normalised water index - 4	NWI-4	$(R_{970} - R_{880}) / (R_{970} + R_{880})$	Prasad *et al*., 2007

to only samples with very low chlorophyll concentrations (<10 µg cm^{-2}) (Araus *et al.,* 2001). However, chlorophyll absorption at 550 nm is lower, meaning that it is less sensitive to chlorophyll changes, is not easily saturated and can be used to estimate chlorophyll contents in canopies or leaves across a greater range. To improve the estimation of chlorophyll content Chappelle *et al.* (1992) analyzed wavelengths that were more sensitive to changes in Chl*a*, Chl*b* and carotenoids in soybeans grown at different N levels. This research developed the ratio analysis of reflectance spectra (RARS) indices, RARSa, RARSb, and RARSc, which estimated Chl*a*, Chl*b* and carotenoids, respectively (Table 7.3). The indices have since been used to estimate chlorophyll and carotenoid contents in other species. For example, Babar *et al.* (2006c) showed a strong relationship between SPAD chlorophyll meter estimates of chlorophyll and PSSRa, RARSb and RARSc measurements. The SPAD-502 chlorophyll meter is a hand held single-leaf meter that measures chlorophyll using light transmittance at 650 nm and 940 nm (see the accompanying volume, Chapter 9). SPAD readings have been shown to be strongly correlated with extracted chlorophyll from plants (Yadava, 1986; Dwyer *et al.,* 1991). The PSSRa index was developed by Blackburn (1998) who reported that using 680 nm and 800 nm significantly improved the RARSa index in a range of species (Table 7.1). The estimation of chlorophyll content from canopy reflectance provides an integrated measure across all leaves in the canopy, with the added benefit of measuring additional parameters such as the content of other pigments, water status and canopy size.

Red edge indices

The position of the 'red edge' may be used to estimate leaf and canopy chlorophyll content in a wider and higher range of concentration than 675 nm or 550 nm. The red edge position (REP) refers to the specific wavelength where the change in reflectance is at its maxima when increasing the wavelength from RED to NIR and is in the 680–780 nm region of the spectrum. With increasing chlorophyll content, the REP changes to longer wavelengths and can be used to derive indices such as the wavelength of the red edge (λ_{re}), the maximum amplitude in the first derivative of the reflectance spectra (dR_{re}), and the sum of the amplitudes between 680 nm and 780 nm in the first derivative spectra ($\sum dR_{680-780}$) (Araus *et al.,* 2001) (Table 7.3). These REP-related parameters have the advantage that they are less affected by factors such as soil background, incident intensity and viewing geometry (Filella and Peñuelas, 1994).

Two more indices have been developed to measure chlorophyll content and its degradation in the canopy. The normalized phaeophytinization index (NPQI) can be used to detect chlorophyll degradation and was introduced as an indicator of pest attacks on apple trees (Peñuelas *et al.,* 1995b) and may indicate phenological states in wheat (Araus *et al.,* 2001) (Table 7.3). Additionally, the canopy chlorophyll content index (CCCI) (Barnes *et al.,* 2000) was developed to improve the estimation of the nitrogen status of crop canopies through the integration of a vegetation index (Table 7.3). The index combines estimates of NDVI, to estimate canopy cover, with the Normalized Difference Red Edge (NDRE), to measure leaf chlorophyll concentration. The approach attempts to isolate leaf chlorophyll contents from the soil background due to changes in cover (Barnes *et al.,* 2000; Tilling *et al.,* 2007).

Carotenoids

The carotenoid to chlorophyll ratio (Car:Chl) in leaves often increases when plants are stressed. Chlorophyll and carotenoids both absorb light in the blue region of the electromagnetic spectrum; however, only chlorophyll absorbs light in the red region of the spectrum. Indices that are derived from the combination of reflectance from these two regions may be used to estimate the Car:Chl and assess the extent of some plant stresses. Indices based on this ratio include the pigment simple ratio (PSR) and the normalized difference pigment index (NDPI). These indices were further improved through the development of the structural independent pigment index (SIPI), which was formulated in order to avoid errors associated with differences in leaf surface and structure (Table 7.3). The index uses reflectance at 800 nm as a reference, as neither carotenoids nor chlorophyll absorb at this wavelength and it is only affected by leaf structure (Peñuelas *et al.,* 1995c). The Car:Chl indices generally increase from vegetative growth through to the beginning of senescence, and may be used to assess the nutritional status of the crop (indicated by high ratio values when N is low) and the onset of pest attacks (Peñuelas *et al.,* 1995b).

Radiation use efficiency (RUE)

Vegetative and pigment indices may be used to estimate canopy photosynthesis, but this does not always correspond to actual photosynthesis. Actual photosynthesis becomes even more difficult to estimate from these indices when plants are grown in stressful environments. The PRI is correlated with

Table 7.3. Chlorophyll indices.

Name	Abbreviation	Index calculation	Parameter	Reference
Simple chlorophyll index (high sensitivity)	R675	R_{675}	Chlorophyll	Jacquemoud and Baret, 1990
Simple chlorophyll index (low sensitivity)	R550	R_{550}	Chlorophyll	Jacquemoud and Baret, 1990
Ratio of reflectance	R750/550	R_{750}/R_{550}	Chlorophyll	Lichtenthaler *et al*., 1996; Gitelson and Merzlyak, 1997
	R750/700	R_{750}/R_{700}	Chlorophyll	Lichtenthaler *et al*., 1996; Gitelson and Merzlyak, 1997
Green normalised difference vegetation index	$NDVI_{green}$	$(R_{NIR} - R_{540–570}) / (R_{NIR} + R_{540–570})$	Chlorophyll	Gitelson and Merzlyak,1997
Ratio analysis of reflectance Spectra (Chl*a*)	RARSa	R_{675} / R_{700}	Chlorophyll *a*	Chapelle *et al*., 1992
Ratio analysis of reflectance Spectra (Chl*b*)	RARSb	$R_{675} / (R_{650} * R_{700})$	Chlorophyll *b*	Chapelle *et al*., 1992
Ratio analysis of reflectance Spectra (Carotenoids)	RARSc	R_{760} / R_{500}	Carotenoids	Chapelle *et al*.,1992
Pigment specific simple ratio	PSSRa	R_{800} / R_{675}	Chlorophyll *a*	Blackburn, 1998
Wavelength of the red edge	λ_{re}	The maximum slope in the reflectance spectra between the RED and NIR regions.	Chlorophyll and N status	Filella *et al*., 1995
Amplitude in the 1st derivative of the reflectance spectra	dR_{re}	The maximum amplitude in the 1^{st} derivative of the reflectance spectra.	Chlorophyll and N status	Filella *et al*., 1995
Sum of the amplitudes (680–780 nm) in the 1st derivative of the reflectance spectra	$\sum dR_{680–780}$	Sum of the amplitudes between 680 and 780nm in the 1^{st} derivative of the reflectance spectra.	Chlorophyll and N status	Filella *et al*., 1995
Normalized difference red edge	NDRE	$(R_{790} - R_{720}) / (R_{790} + R_{720})$	Chlorophyll and N status	Barnes *et al*., 2000; Rodriguez *et al*., 2006
Normalized phaeophytinization index	NPQI	$(R_{415} - R_{435}) / (R_{415} + R_{435})$	Chlorophyll degredation	Peñuelas *et al*., 1995b
Canopy chlorophyll content index	CCCI	Calibrated index using NDRE as function of NDVI.	Chlorophyll and N status	Barnes *et al*., 2000; Fitzgerald *et al*., 2006; Rodriguez *et al*., 2006
Modified spectral ratio	MSR	$(R_{750} - R_{445}) / (R_{705} - R_{445})$	Chlorophyll concentration	Sims and Gamon, 2003
Pigment simple ratio	PSR	R_{430} / R_{680}	Carotenoid to chlorophyll ratio	Peñuelas *et al*., 1993
Normalized difference pigment index	NDPI	$(R_{680} - R_{430}) / (R_{680} + R_{430})$	Carotenoid to chlorophyll ratio	Peñuelas *et al*., 1993
Structural independent pigment index	SIPI	$(R_{800} - R_{435}) / (R_{415} + R_{435})$	Carotenoid to chlorophyll ratio	Peñuelas *et al*., 1995a
Photochemical reflectance index	PRI	$(R_{531} - R_{570}) / (R_{531} + R_{570})$	Radiation use efficiency	Peñuelas *et al*., 1995a
Modified spectral ratio planar index	MSRpi	Calibrated index using MSR as function of NDVI.	Nitrogen status	Rodriguez *et al*., 2006

Where: Chl*a* = chlorophyll *a*; Chl*b* = chlorophyll *b*; R_{NIR} = Reflectance in the near infrared reflectance region of the spectrum; R_{RED} = Reflectance in the red reflectance region of the spectrum.

photosynthetic radiation use efficiency (PRUE) of absorbed PAR, and gives a more responsive measure of canopy photosynthesis than vegetative indices (Peñuelas, 1995c) (Table 7.3). The index is derived from the fact that a portion of PAR absorbed by chlorophyll cannot be used for photosynthesis and so is lost to the system through heat dissipation. The xanthophyll cycle is involved in this process of radiation dissipation, and has been associated with leaf reflectance near 531 nm. Therefore, the PRI may be used to measure changes in the status of xanthophyll pigments without canopy-scale PAR manipulations (Araus, 1996). The PRI has been used to measure PRUE in different species as a result of different environmental factors such as nutritional state and midday reduction (Gamon *et al.,* 1997). However, limitations of the index include inaccurate estimations with canopy structural changes, such as leaf wilting (Gamon *et al.*, 1992), and the index is only valid for fully illuminated canopies as estimations are inaccurate across wide ranges of illumination (i.e., from sun to shade) (Gamon *et al.*, 1997).

Conclusion

Through a detailed understanding of the electromagnetic spectrum and the optical properties of plant canopies, researchers have been able to develop an extremely useful series of instruments, and measurements, for physiological trait selection. Spectroradiometry is an extremely powerful physiological selection tool due to its high-throughput capabilities and ability to sample a limitless number of traits simultaneously. As advances in this field of research continue, it is expected that the practical applications of the technology will become even more widely adopted within research and plant breeding communities. This has the potential to increase future genetic gains, increase wheat yield potential and improve the response of crops to stress.

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Chapter 8: Gas exchange and chlorophyll fluorescence – principles and applications

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Abstract

Photosynthesis is becoming one of the main targets for improving wheat yields, so a better understanding of techniques such as gas exchange and chlorophyll fluorescence is important. However, evaluating photosynthesis in different genotypes is not a trivial task. While theoretically we might expect a strong positive relationship between leaf photosynthesis and biomass and/or yield, this has not always been the case in field experiments. This lack of correlation is due to the fact that at any given period of time across several genotypes, photosynthetic rates change in parallel with leaf canopy size, architecture, leaf specific area and plant phenology. Further, translocation of assimilates from the leaf to stems, roots and reproductive structures (and maintenance of the plant as a living unit at minimum cost) are also important downstream contributors to yield. In other words, instantaneous measurements of leaf carbon dioxide (CO_2) fixation represent only a snapshot in time and space of total canopy photosynthesis over a crop cycle. The difficulties related to the lack of plant uniformity when evaluating photosynthesis are aggravated when assessing the responses to drought, since stomatal conductance is low and highly variable. However, under well irrigated and warm conditions there are examples where photosynthesis capacity has shown important positive contributions to yield. Therefore, evaluating photosynthesis under well irrigated and warm environments may provide an appropriate measurement of tolerance to those environments. Photosynthesis can be measured using gas exchange and estimated using chlorophyll fluorescence. A range of portable systems are now available allowing users to make real-time measurements of leaf photosynthetic CO_2 uptake, transpiration, leaf conductance, intercellular CO_2 mole fraction, efficiency of photosystem II photochemistry, photochemical and non-photochemical quenching and other chlorophyll fluorescence parameters that can be simultaneously determined. However, for appropriate use of these techniques, it is essential to have a basic knowledge of the principles, applications, measurements and limitations of both gas exchange and chlorophyll fluorescence systems. The principles and applications of these techniques are described in this chapter.

Abbreviations list

A- net CO_2 assimilation rate; ATP- adenosine triphosphate; ATPsyn- ATP synthase; C_a- atmospheric CO_2; C_i- intercellular CO_2; cyt b6/f- cytochrome b6/f complex; Φ_{PSII}- quantum yield of photosystem II photochemistry; F_o- minimal fluorescence from a dark adapted leaf; F_o'- minimal fluorescence from a light adapted leaf; F_m- maximal fluorescence from a dark adapted leaf; F_m'- maximal fluorescence from a light adapted leaf; F_s or F_t- steady state yield; F_v- variable fluorescence from a dark adapted leaf; F_v'-variable fluorescence from a light adapted leaf; F_v/F_m- maximum quantum efficiency of photosystem II photochemistry; F_v'/F_m'- photosystem II maximum efficiency; g_m- mesophyll conductance; *J*- photosynthetic electron transport rate; NADPH – reduced nicotinamide adenine dinucleotide phosphate; J_{max} – maximum capacity for electron transport rate; NPQ- non-photochemical quenching; PC- copper protein plastocyanin; PPFD- photosynthetically photon flux density; PQ- plastoquinone molecule; PSI- photosystem I; PSII- photosystem II; Q_A and Q_B- primary quinone electron acceptors; *qP*- photochemical quenching; R_d- day respiration; Rubisco- ribulose 1,5 –bisphosphate carboxylase/oxygenase; RuBP- ribulose 1,5 –bisphosphate; TPU- triose phosphate use; $V_{c,max}$- maximum carboxylation capacity of Rubisco.

Introduction

Photosynthesis drives plant productivity; however, plant physiologists realize the difficulty in obtaining a realistic evaluation of a large number of genotypes within an experiment. In addition, it is even more difficult to correlate the photosynthetic response with final yield. Reasons for this lack of correlation are related to the dynamic nature of photosynthesis and the importance/contribution of other interacting factors such as leaf area, respiration and assimilate partitioning to yield (Gaskel and Pearce, 1981; Mahon and Hobbs, 1981; Sinha *et al*., 1981; Amthor, 1989; Loomis and Amthor,

1999). In summary, plant productivity is driven by photosynthesis and the key elements in the system are:

(i) The interception of photosynthetically photon flux density (PPFD, 400–700 nm spectral band),

(ii) Use of this energy in the reduction of CO_2 and other substrates (in the process of photosynthesis),

(iii) Translocation of the assimilates from leaf to stem, roots and reproductive structures, and

(iv) Maintenance of the plant as a living unit at minimum cost (Loomis and Amthor, 1999).

Moreover, such instantaneous leaf photosynthetic rate measurements make it difficult to extrapolate from a single reading to whole canopy throughout the crop cycle (Reynolds *et al*., 2000). Failure to positively correlate photosynthesis with productivity has been shown in the literature and only a few examples are referred to here. For instance, evolution, higher level of ploidy and selection from the wild ancestor to the modern wheat have resulted in increased grain and leaf size, longer grain-filling duration (related to delayed senescence of upper leaves), and decreased net photosynthetic rate under saturating irradiance (Welbank *et al.,* 1966, 1968; Evans and Dunstone, 1970; Khan and Tsunoda, 1970; Austin *et al*., 1982). In another study, domestication and breeding of wheat has been reported to be associated with lower photosynthetic rates (Evans and Dunstone, 1970; Rawson *et al*., 1983). Consequently, the lack of correlation between leaf photosynthesis and yield, coupled with evidence that yield is sink limited rather than source limited have led to the idea that crop yields cannot be improved by increasing leaf photosynthetic rates (Long *et al*., 2006). However, the focus on atmospheric CO_2 enrichment has provided a good example of the contrary, i.e., that increased leaf photosynthesis may in fact increase yield (Long *et al*., 2006). There are, however, examples in the literature of positive correlations between leaf photosynthetic related traits and yield. For instance, stomatal conductance, maximum photosynthetic rate and canopy temperature depression, averaged over three seasons, were closely and positively correlated with progress in grain yield of eight representative semi dwarf spring wheat cultivars released in northwest Mexico between 1932 and 1988 under favorable management and irrigation conditions (Fischer *et al*., 1998). There are also reports of positive associations between photosynthesis and productivity (both biomass and yield) in wheat cultivars grown in a warm, irrigated environment indicating that higher leaf photosynthetic rates may have reflected a higher overall rate of net canopy photosynthesis under high temperatures (Reynolds *et al*., 2000). More recently, careful measurements of photosynthetic rates at different development stages, of 18 cultivars of winter wheat released in the period between 1945 and 1995 in China, (under well irrigated conditions in a continental warm environment) revealed a genetic increase in the rate of photosynthetic rates per unit leaf area (Jiang *et al*., 2003). Moreover, a positive association between photosynthesis/productivity under warm, irrigated environments may be related to the fact that photosynthesis is largely affected by high temperatures (Law and Crafts-Brandner, 1999) and, therefore, implies an important theoretic limit to productivity under those conditions. As a consequence, photosynthesis can be considered a promising tool to discriminate the response of different genotypes to high temperature.

This chapter is divided into three main sections: firstly, a brief review on the processes involved in photosynthesis is presented; then, the two main tools available for assessing photosynthesis –gas exchange and chlorophyll fluorescence based tools– are described; and finally, application of the most appropriate photosynthetic parameters, according to the target environment, are detailed. For practical aspects related to photosynthesis measurements, either by gas exchange or chlorophyll fluorescence, see the accompanying volume, Chapter 13.

Photosynthesis: the basics

In a very simplistic way, photosynthesis converts carbon dioxide into sugars. The energy needed for this process is provided by light, which is absorbed by pigments (primarily chlorophylls and carotenoids). Electrons for this conversion come from water, which is converted to oxygen and protons, and produce reducing power as reduced nicotinamide adenine dinucleotide phosphate (NADPH). For simplicity, photosynthesis can be divided in three major parts (Allen *et al*., 1998): (i) the electron transport and photophosphorylation reactions of the thylakoid membrane, (ii) the CO_2-fixation reactions of the Calvin cycle, and (iii) stomatal control of CO_2 supply (Figure 8.1). The activities of many components of photosynthesis are regulated by the rates of these three photosynthetic reactions and, therefore, examination of a single process or component, such as the carboxylation velocity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the quantum efficiency

of photosystem II (PSII) photochemistry or stomatal conductance, does not allow identification of the primary stress induced limitation in photosynthesis. For this reason, under different situations one may need to determine which components of photosynthesis are limiting.

Electron transport and photophosphorylation reactions of the thylakoid membrane

A simplified and adapted scheme of the process of absorption and transfer of energy is shown in Figure 8.2. Electron transport reactions occur in thylakoid membranes of the chloroplast, and the main reactions of this process are possible due to the presence of proteins and pigment complexes called reaction centers. In higher plants, there are two types of reaction centers: Photosystem I (PSI or P700) and Photosystem II (PSII or P680). Peak light absorption is 700 nm for PSI and 680 nm for PSII. The photosystems consist of an array of pigment-protein complexes on the thylakoid membranes and functions as an antenna for light energy absorption. Excitation energy is transferred from one chlorophyll molecule to another until the energy reaches the reaction centers of the photosystem. This causes one of the chlorophyll molecules within the reaction center to go into an excited state, releasing electrons. Electrons are transferred between these large complexes by small mobile molecules (the primary quinone electron acceptors of PSII are Q_A and Q_B, plastoquinone (PQ) and plastocyanin (PC) in higher plants). These small molecules transport electrons or hydrogen atoms across the photosynthetic membrane. PQ serves two key functions: transfer of electrons from the PSII reaction center to the cytochrome b6/f complex and carries protons across the photosynthetic membrane. The electrons are eventually transferred to the PSI reaction

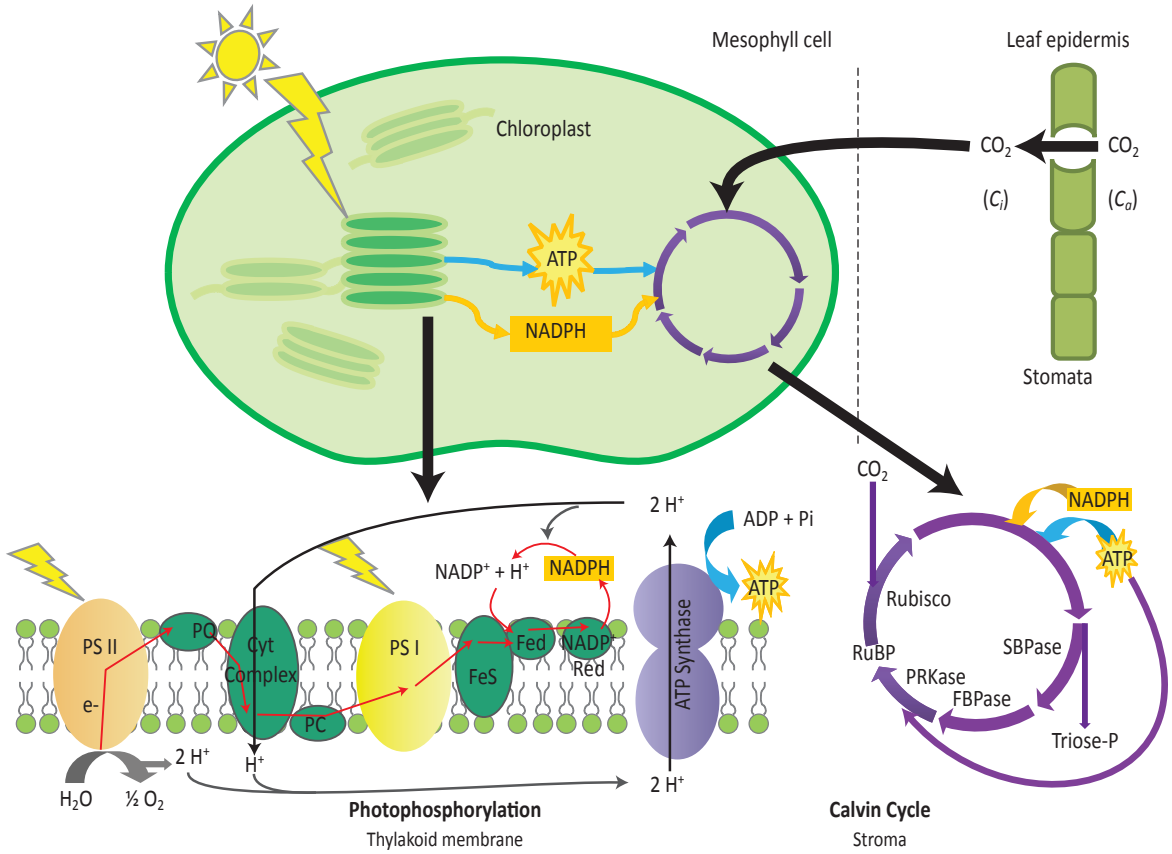

Figure 8.1. Representation of the main processes in photosynthesis of C3 plants showing electron transport and photophosphorylation, Calvin cycle and stomatal control of CO_2 supply. Photophosphorylation involves the absorption of light by photosystem II and I (PSII and PSI) which drives electron transport producing reducing power, NADPH and a proton H^+ gradient across the membrane that allows the production of ATP. The diffusion of atmospheric CO_2 (C_a) into the leaf and H_2O transpired is controlled by stomata. Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the assimilation (A) of intercellular CO_2 (C_i) with ribulose 1,5 bisphosphate (RuBP) in the carboxylation reaction of the Calvin cycle in the stroma of the chloroplast (adapted from Allen *et al.*, 1998).

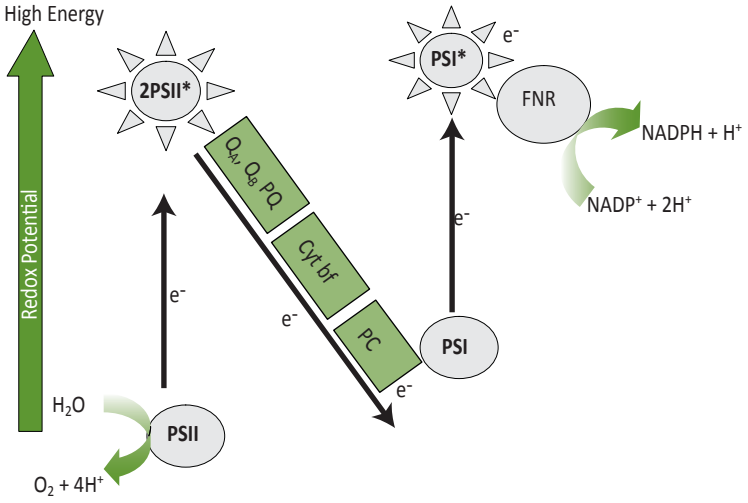

Figure 8.2. A schematic representation of the main processes of absorption and transfer of energy during the light reactions of photosynthesis, also known as the Z-scheme. Abbreviations used: PSII, photosystem II; PSII*, photosystem II in the excited state; PSI, photosystem I; PSI*, photosystem I in the excited state; FNR, ferredoxin-NADPH reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate (reducing power); ATP, adenosine triphosphate; O_2, oxygen; H^+, protons; Q_A and Q_B , primary quinone electron acceptors; PQ, plastoquinone molecule; PC, copper protein plastocyanin and; Cyt, b_6/f complex (now also called Cytochrome b_6/c or simply bf). (adapted from http://www.life.illinois.edu/govindjee/ZSchemeG.html).

center. The protons released into the inner space of the photosynthetic membrane contribute to the proton chemical free energy across the membrane. Electron transfer from the cytochrome b6/f complex to PSI is mediated by PC. Electron transfer from PSI to $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate) requires ferredoxin, a small FeS protein, and ferredoxin-NADP oxidoreductase (FNR), a peripheral flavoprotein that operates on the outer surface of the photosynthetic membrane. An excited electron in PSI is transferred to a molecule of NADP, along with an H^+, thereby reducing it to NADPH. Electrons leaving PSI are replaced by those that entered through the electron carriers from PSII and in order for this process to continue, the electron that was removed from PSII has to be replaced. This is achieved by the splitting of a H_2O molecule which yields electrons, H^+ and oxygen in the PSII. The transport of an electron between the two types of reaction centers results in the pumping of hydrogen ions (H^+) across the thylakoid membrane, thus forming a gradient with a high H^+ concentration inside the thylakoid compartments (i.e., lumen side) and lower relative concentration on the stroma side. The potential energy associated with this gradient is then used to form adenosine triphosphate (ATP) by a mechanism similar to that by which ATP is generated in mitochondria.

In summary, during the electron transport and the photophosphorylation reactions of the thylakoid membrane, two chemicals are produced; ATP and NADPH, which serves as a source of reducing power for subsequent reactions in the photosynthetic pathway.

The CO_2-fixation reactions of the Calvin cycle

The Calvin cycle (also known as Calvin-Benson-Bassham cycle or the reductive pentose phosphate pathway) is a metabolic pathway in which carbon in the form of CO_2 is fixed from the atmosphere and converted into sugars (triosephosphates). Melvin Calvin and co-workers, using ^{14}C, defined the main reactions involved in photosynthesis (Bassham *et al*., 1950). The cycle uses ATP as an energy source and consumes NADPH from the light reactions as reducing power for adding high energy electrons to make sugar. For simplicity, we can divide the Calvin cycle into three main steps: phase 1 (carbon fixation), phase 2 (reduction) and phase 3 (regeneration). In phase 1, CO_2 is incorporated into a five-carbon sugar named ribulose bisphosphate (RuBP). The enzyme which catalyzes this first step is ribulose-1,5-bisphosphate carboxylase/oxygenase, which is referred to as Rubisco. The product of the reaction is a six carbon intermediate which splits in half to form two molecules of 3-phosphoglycerate. In phase 2, ATP and NADPH from the light reactions are used to convert 3-phosphoglycerate to glyceraldehyde 3-phosphate, the three carbon carbohydrate precursor to glucose and other sugars. In phase 3, more ATP is used to convert some molecules of glyceraldehyde 3-phosphate back to RuBP, the acceptor of CO_2, thereby completing the cycle. For every three molecules of CO_2 that enter the cycle, the net output is one molecule of glyceraldehyde 3-phosphate (G3P). For each G3P synthesized, the cycle spends nine molecules of ATP and six molecules of NADPH. The regeneration of RuBP involves many enzymes, the most important are fructose 1,6-bisphosphate (FBPase), sedoheptulose

1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRKase), the first two of which, along with Rubisco, catalyze effectively irreversible reactions and, therefore, are important in regulating the rate of the cycle.

Stomatal control of CO_2 supply

Stomatal aperture and closure regulate the amount of CO_2 available at the Rubisco site and therefore limit photosynthesis as a consequence of stress conditions. Stomatal limitations to photosynthesis have been defined as the percentage decrease in light-saturated photosynthesis that is attributable to stomatal conductance (Farquhar and Sharkey, 1982). Analysis of the relationship between net CO_2 assimilation (A) and intercellular CO_2 concentration (C_i) allows separation of the relative limitations imposed by stomata, Rubisco carboxylation velocity and the capacity for regeneration of RuBP on leaf photosynthesis (Figure 8.1) according to the model of von Caemmerer and Farquhar (1981). A decrease in C_i indicates that the main cause of decline in leaf photosynthetic rate is a decrease in stomatal conductance. In contrast, an increase in C_i suggests that a decrease in photosynthetic activity of mesophyll cells is due to non-stomatal factors. Changes in C_i are the most important criterion for analysis of the stomatal limitation of photosynthesis, more so than the correlation between photosynthesis and stomatal conductance. Changes in stomatal conductance are mainly due to changes in guard cell turgor and are regulated by K^+ fluxes along an electrochemical gradient in the guard cell plasmalemma (Zeiger, 1983; Pandey *et al.,* 2007). Several environmental factors have important effects on stomatal conductance (including light, CO_2, humidity and temperature), and on internal factors such as tissue water status and the level of plant regulators such as abscisic acid (ABA) and cytokinins. Complex interactions often exist among these factors which make it difficult to distinguish the relative importance of individual factors such as light and CO_2 or water status and ABA.

Stomata control the CO_2 supply for photosynthesis and at the same time control water loss through transpiration (Cowan, 1977; reviewed in Jones, 1998). This leads to the concept of transpiration efficiency (defined here as the ratio of net CO_2 assimilation to transpiration rate) which is a function of both environmental and plant attributes related to resistances to CO_2 fixation by leaves (reviewed in Subbarao and Johansen, 2002).

Photorespiration and mitochondrial respiration

Photorespiration is the process by which, in the presence of light, plants consume O_2 and release CO_2 during photosynthesis (due to the oxygenase capacity of Rubisco), resulting in a decrease in the photosynthetic output, since no ATP is produced and carbon is inevitably lost (Zelitch and Day, 1968; reviewed in Foyer *et al*., 2009). Rubisco catalyzes either the carboxylation or the oxygenation of RuBP fixing CO_2 into a three carbon compound in photosynthesis and O_2 in photorespiration. O_2 competes successfully with CO_2 at ambient concentrations, leading to the formation of phosphoglycollate, which is broken down to release CO_2 in photorespiration, thereby reducing photosynthetic efficiency. Respiration, refers to mitochondrial respiration in plants regardless of light (i.e., whether light is present or absent to separate from photorespiration) in which O_2 is consumed and CO_2 released (Amthor, 1989). It has been shown that the rate of photorespiration in nature can vary between 10–30% of the photosynthetic rate (Sharkey, 1988). This percentage may grow dramatically under stress conditions or with age (Di Marco *et al*., 1994). In C3 plants, during gas exchange measurements, only net photosynthesis can be measured, which corresponds to the rate of carboxylation minus the release of CO_2 from photorespiration and mitochondrial respiration. Therefore, simple measurements of CO_2 exchange with an infrared gas analyzer (IRGA) give the values of leaf net photosynthesis (A_N) and this includes the difference between the gross amount of leaf photosynthesis (A_G, where CO_2 is consumed) minus the rates of respiration i.e., photorespiration (*PR*) and mitochondrial respiration (R_d, where CO_2 is released) as shown in Equation 8.1:

$$A_N = A_G - PR - R_d \qquad \text{Equation 8.1}$$

In the context of using leaf photosynthesis to screen germplasm, both photorespiration and mitochondrial respiration are, most of the time, difficult to measure. However, it should be kept in mind that photorespiration and mitochondrial respiration cannot be separated from the leaf photosynthetic rate measurements. This is especially important under high temperatures, in which photorespiration and mitochondrial respiration have been reported to increase substantially (Leegood, 2007; Bunce, 2007) and therefore may affect final photosynthesis measurements. Recent work on fruit crops such as tomato (Nunes-Nesi *et al*., 2005) shows that increases in the size of the sink are not only linked to an increase in CO_2 assimilation, but also to a reduction in dark respiration. This finding opens the discussion

on the importance of the respiratory pathways in photosynthetic metabolism. Related to this, the evaluation of discrepancies between theoretical and *in situ* measured values of respiratory coefficients, for example waste respiration (Amthor, 2000), suggests that targets for improvement may remain.

Measuring photosynthesis

There are two main systems to assess the photosynthetic capacity of leaves: gas exchange systems, which directly measure CO_2 and H_2O exchange, and the fluorometer which estimates photosynthesis by measuring chlorophyll fluorescence. Photosynthesis, defined as the net exchange of CO_2 between the leaf and air, can be measured directly with an IRGA and includes a balance between photosynthesis (CO_2 consumed) and photorespiration plus day respiration (Equation 8.1). At the same time, H_2O exchange is also measured and gives information on transpiration rates and stomatal conductance (Long, 1996; Long and Bernacchi, 2003). Changes in chlorophyll fluorescence induced by illumination of leaves are qualitatively correlated with changes in CO_2 assimilation and under some circumstances fluorescence emission could be related to their photosynthetic rates (Kautsky and Zedlitz, 1941; Kautsky *et al*., 1960; MacAllister and Myers, 1940). Now modulated chlorophyll fluorometers (Ogren and Baker, 1985; Schreiber, 2004) are available and provide the best estimations of chlorophyll fluorescence. Basic information on these two techniques (gas exchange and chlorophyll fluorescence) is given in the following sections.

Gas exchange measurements

Gas exchange basics

Gas exchange can now be measured due to the discovery that hetero-atomic gas molecules absorb radiation at specific infrared wave bands and each has a characteristic absorption spectrum. Gas molecules consisting of two identical atoms (e.g., O_2, N_2) do not absorb this long-wave infrared (IR) radiation, and thus do not interfere with the determination of the mole fraction of hetero-atomic molecules (reviewed in Long, 1996). Infrared gas analysis has been used for the measurement of a wide range of hetero-atomic gas molecules, including CO_2, H_2O, NH_3, CO, N_2O and gaseous hydrocarbons. The only gas normally present in air with an absorption spectrum overlapping that of CO_2 is water vapor; both molecules absorb IR radiation in the 2.7 µm region. Since water vapor is usually present in air at highly variable and much higher mole fractions than CO_2, this interference is significant. Most commercial leaf gas exchange systems incorporate a CO_2 and a water vapor IRGA which provide the gas exchange measurements that can be used for further calculating the rates of photosynthesis (*A*) and transpiration (*E*) based on Fick's law which measures the rate of diffusion of gases as follows:

A = (Air flux × ΔCO_2)/Leaf area Equation 8.2

E = (Air flux × ΔH_2O)/Leaf area Equation 8.3

The basic components of a gas exchange system are the chamber or cuvette, IRGAs, flow meters, gas lines, CO_2 and water vapor filters, power batteries and a console with keyboard, display and memory (Figure 8.3). Precise control of temperature, CO_2 concentration, humidity and light has to be achieved. An IRGA includes an infrared source that is directed through a gas sampling chamber and then focused on a detector. The energy received at the detector is the total entering the system minus the energy absorbed by the CO_2 in the sampling chamber. A major problem with IRGA performance is the discrimination between CO_2 and water vapor, since both gases absorb energy at similar wavelengths. To solve this problem, the gas sample chamber is dried to a certain water content specified by the user, using a desiccant, before measurements are made.

Two main types of systems for photosynthesis measurements can be found: closed and open systems (Figure 8.4). The term closed or open refers to whether or not the atmosphere of the leaf-enclosing chamber is renewed during the measurement. In a closed system, the leaf is enclosed in a sealed chamber that is not resupplied with fresh air. The CO_2 concentration in the chamber is decreased by leaf photosynthetic activity, while H_2O concentration increases. The change in CO_2 and H_2O concentrations per unit time are correlated with net photosynthesis and transpiration, respectively. In an open system, air flow with known CO_2 concentration is constantly passed through the leaf chamber. The objective is to supply a stable concentration of CO_2. As a result of photosynthesis, the air leaving the chamber (normally called the 'sample') will have a lower CO_2 concentration as well as a higher H_2O concentration than the air entering the chamber (normally called the 'reference'). A variation of the open system is the compensating system, where the CO_2 removed by photosynthesis is compensated by CO_2 injection until reaching equilibrium (called 'null balance'). At that

equilibrium point, photosynthesis rate is equal to the CO_2 injection. Old systems were closed mode types, but at present most photosynthesis systems are mainly open mode types (Figure 8.3). One strength of an open system is that the incoming air stream can be conditioned. That is, its humidity, CO_2 concentration, temperature, etc. can be established by some means prior to entering the system. The most recent open systems found on the market incorporate two IRGAs in the sensor head, compared to the traditional open system which incorporated them in the console (Figure 8.4). IRGA analyzers in the sensor head provide tight and rapid control response and eliminate plumbing-related time delays. Leaf dynamics are measured in real time because the return tubing between the leaf chamber and the console is eliminated. These systems also eliminate water absorption on the tubes between the chamber and the console, making measurements more accurate.

Gas exchange parameters

The most direct and fastest measurements that a gas exchange system can provide are net photosynthesis and transpiration rates in one instantaneous measurement. From these direct measurements, a number of mathematical parameters can be derived to calculate leaf conductance (g_s) and intracellular CO_2 concentration (C_i), among others. Dark respiration (R_D) can also be calculated, however, the plant must be in complete darkness during measurements. The ratio of net photosynthesis with transpiration rate or stomatal conductance provides an estimation of instantaneous water use efficiency. For a more detailed analysis of photosynthesis, response curves to different CO_2 concentrations (also known as A/C_i) and light intensities (A/PAR –Photosynthetic Active Radiation) can be established. These curves are time consuming but can provide a lot of information. The basic principle of these curves is changing CO_2 or PAR values inside the chamber and registering net photosynthesis at each concentration or light intensity (for more details see the accompanying volume, Chapter 13). The A/C_i response curve allows the *in vivo* calculation of parameters considered to represent the major limitations to light-saturated photosynthesis (Long and Bernacchi, 2003): maximum rates of Rubisco carboxylation ($V_{c,max}$), electron transport driving generation of *RuBP* (J_{max}),

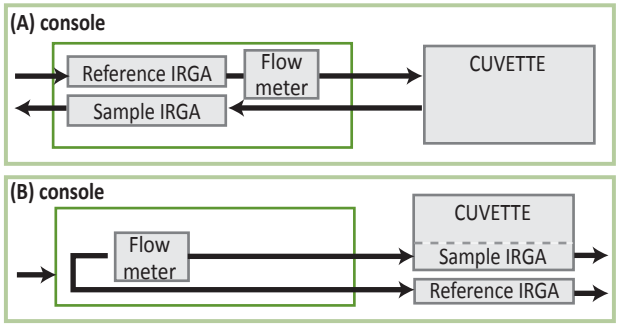

Figure 8.4. Schematic representation of the two main open photosynthesis systems: (A) traditional open system with reference and sample infrared gas analyzers (IRGA) inside the console; and (B) recent open system with reference and sample IRGAs near the cuvette.

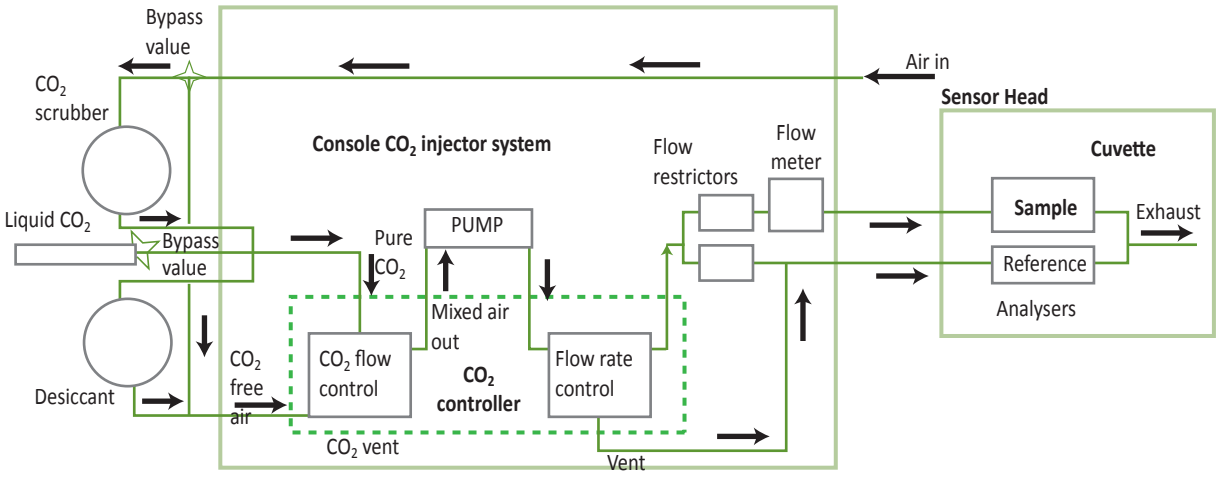

Figure 8.3. Schematic representation of an open gas exchange system. In the open mode, air-flow is moved through a controlled atmosphere surrounding a plant leaf enclosed in an assimilation chamber. Thus, the CO_2 level of the air is maintained steady state. Arrows indicate the direction of air/CO_2. (Adapted from http://www.licor.com/ see LI6400XT Brochure).

triose-phosphate utilization (TPU), R_d (day respiration) and g_m (mesophyll conductance). These parameters were mathematically defined by Farqhuar *et al.* (1980) and recently Sharkey *et al.* (2007) designed and published a user friendly excel file to directly calculate the parameters described above. The *A*/PAR response curves can give information about the maximum quantum yield (efficiency) of photosynthetic CO_2 uptake (ΦCO_2) or oxygen evolution (ΦO_2) using the initial slope of the *A*/PAR curve (Ziegler-Jons and Selinger, 1987; Murchie and Niyogi, 2011). The light saturation level of the leaf (A_{max}) can also be determined with light curves.

Chlorophyll fluorescence

Single chlorophyll fluorescence curves: the Kaustky effects

Kautsky and colleagues were the first to detect chlorophyll fluorescence (Kautsky *et al*., 1960) and only a brief summary of their findings is presented here. These authors found that, upon transferring leaves from the dark to the light, an increase in the yield of chlorophyll fluorescence was observed over a time period of less than 1 second, with a decrease thereafter (Figure 8.5A shows detection of chlorophyll fluorescence upon dark adaptation). The initial rise of the fluorescence intensity in Figure 8.5A (O-P) reflects a gradual increase in the yield of chlorophyll fluorescence as the rate of photochemistry declines, when the pool of Q_A, Q_B and PQ of the PSII reaction centers becomes increasingly reduced. Fluorescence then declines to a steady-state level which corresponds in time to the point at which steady-state CO_2 assimilation is attained. The emission of this fluorescence can be associated with the reduction of electron acceptors in the photosynthetic pathway, downstream of PSII. Once PSII absorbs light and the primary quinone acceptor of PSII has accepted an electron, it is not able to accept another until it has passed the first onto a subsequent electron carrier. During this period, the reaction center is said to be 'closed'. At any point in time, the presence of a proportion of closed reaction centers leads to an overall reduction in the efficiency of photochemistry and so to a corresponding increase in the yield of fluorescence (for further reading see Maxwell and Johnson (2000), Baker and Rosenqvist (2004) and Baker (2008)).

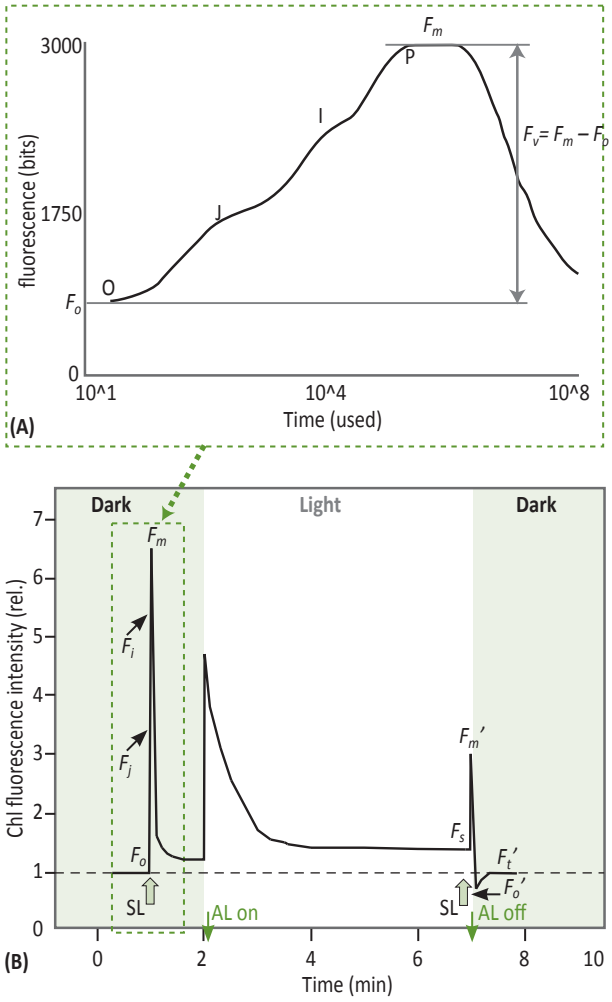

Figure 8.5. (A) Kautsky induction curve due to the reactions causing the different fluorescence kinetics that typically occur in a leaf in the first microseconds of illumination after transferring from the dark. The fluorescence peaks are denoted by the letter O, J, I and P and correspond to PSII fluorescence yield increases following triphasic kinetics (O–J, J–I and I–P). (Adapted from Maxwell and Johnson, 2000). **(B) Sequence of a typical modulated chlorophyll fluorescence curve.** The zero fluorescence level is measured (F_o) under dark conditions. Application of a saturating flash of light (SL) allows measurement of the maximum fluorescence level (F_m^o or simply F_m). A light to drive photosynthesis (AL) is then applied. After a period of time, another saturating light flash (SL) allows the maximum fluorescence in the light (F_m') to be measured. The level of fluorescence immediately before the saturating flash is termed F_t. Turning off the actinic light (AL), typically in the presence of far-red light, allows the zero level fluorescence 'in the light' to be estimated (F_o'). (Adapted from http://www.hansatech-instruments.com/fluorescenceGeneralPrinciples.htm).

Later, a simple model was developed by Butler (1978) who showed that in a leaf, absorbed light energy can have three possible fates:

(i) PSII photochemistry,

(ii) Processes of fluorescence, and

(iii) Heat loss for excitation energy in the pigment array of PSII.

This model mainly states that the concept of chlorophyll fluorescence is simply a consequence of the competition between these three processes when light hits a leaf. The amount of fluorescence decreases due to photochemistry as a result of photochemical quenching of fluorescence and also due to heat loss as non-photochemical quenching of fluorescence. This three-way model predicts that PSII fluorescence emission could be used to detect changes in photochemistry, provided that the rate of fluorescence and heat loss does not change. However, it is now well established that large changes can occur in the rate of heat loss from the PSII antenna (Kramer *et al*., 2004). Consequently, to estimate PSII photochemistry from fluorescence, it is essential to determine the fluorescence quenching which results from both photochemical and non-photochemical processes. Commercial fluorometers use weak modulated measuring beams in which phase and frequency decoding are used to detect fluorescence yield changes. This enables the routine, non-destructive, quantitative determination of photochemical and non-photochemical processes in leaves. These instruments use a brief (less than 1 second) saturating flash of light sufficiently intense (around 9000 µmol m^{-2} s^{-1}) so as to maximally reduce primary electron acceptors. In addition, a modulated chlorophyll fluorometer uses sophisticated electronics to separate chlorophyll fluorescence from ambient light. Actual systems achieve this using a rapid pulsing light that induces a corresponding pulsed fluorescence emission from leaves. The fluorometer uses a highly sensitive photodiode to detect and record the pulsed fluorescence signal and to ignore any non-pulsed signal. The value of the modulated technique is that it provides a continuous measure of the relative quantum yield of fluorescence (Schreiber, 2004). This technique was used to demonstrate that the quantum yield of PSII photochemistry of a leaf at a given actinic light intensity can be estimated from the modulated fluorescence yield prior to the application of the saturating flash and the maximum modulated fluorescence yield during the flash (Genty *et al*., 1989). The quantum yield of PSII photochemistry is directly related to the quantum yield of CO_2 assimilation by the leaf in the absence of photorespiration, which competes with CO_2 assimilation for the products of electron transport (Genty *et al*., 1989; Fryer *et al.,* 1998). Fluorescence, under certain conditions (where photorespiration is not particularly stimulated), provides a rapid, non-destructive probe of CO_2 assimilation.

Modulated chlorophyll fluorescence curves and derived parameters

The calculation of modulated chlorophyll fluorescence parameters is probably best explained by reference to a typical experimental curve (Figure 8.5B). The measurement is initiated by switching on the measuring light, giving a measure of the F_o (minimal) level of fluorescence. Many users do not take into account that the F_o level can change drastically under strong light illumination. This change in F_o will lead to irrelevant photochemical quenching (*qP*) values. To prevent such a mistake, it is recommended to measure F_o only after a saturating far red light pulse (Büchel and Wilhelm, 1993). A saturating flash of light is then applied, allowing the measurement of F_m in the dark-adapted state (F_m^o or simply F_m). Following on, an actinic light is applied and, at appropriate intervals, further saturating flashes are applied. From each of these, a value for F_m', the fluorescence maximum in the light, can be measured. Note that a prime (') notation used after a fluorescence parameter indicates that a leaf sample is exposed to light (actinic light) that will stimulate photosynthesis.

The steady-state value of fluorescence immediately prior to the flash is termed F_t. After a flash, removal of actinic light (preferably whilst simultaneously giving a far-red light) allows measurement of F_o'. Far-red light is important because it provides the transfer of electrons to PSI and allows the rapid re-oxidation of PSII. Under field conditions it is recommended to first determine light adapted parameters and afterwards the leaf should be adapted to the dark and determine dark adapted parameters. In the following subsections chlorophyll fluorescence parameters derived from the dark and light adapted states are shown.

Yield calculation, related equations and photochemical quenching

In the literature there are many fluorescence parameters defined; however, we will focus on the most commonly used by plant physiologists. One of

the most used parameters of chlorophyll fluorescence is a measurement of the overall efficiency of PSII reaction centers in the light, Φ_{PSII} (Genty *et al.,* 1989) and is calculated as:

$$\Phi_{PSII} = (F_m' - F_t)/ F_m' \quad \text{Equation 8.4}$$

This parameter measures the proportion of the light which is absorbed by chlorophyll associated with PSII that is used in photochemistry. With this parameter we can estimate the rate of photosynthetic electron transport (*J*) which provides an indication of overall photosynthetic capacity *in vivo* (Genty *et al.,* 1989) as described in the following equation:

$$J = \Phi_{PSII} \times \text{PPFDa} \times 0.5 \quad \text{Equation 8.5}$$

Where, PPFDa is absorbed light (µmol photon m^{-2} s^{-1}) measured using an integrating sphere and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI. Generally, it is not practical to measure the light absorbed by a leaf, however, provided that similar samples are being compared (i.e., that absorption of light is constant), relative changes in *J* can usually be monitored by simply multiplying PSII by incident light. A measurement can be made by simply pointing a fluorometer at a leaf and flashing it.

Another fluorescence parameter, measuring photochemistry, is 'photochemical quenching', *qP,* calculated as:

$$qP = (F_m' - F_t)/(F_m' - F_o') \quad \text{Equation 8.6}$$

Whilst Φ_{PSII} is the proportion of absorbed energy being used in photochemistry, *qP* gives an indication of the proportion of PSII reaction centers that are already open. Φ_{PSII} and *qP* can be interrelated by a third parameter, F_v'/F_m' (Andrews *et al*., 1993).

The PSII maximum efficiency (F_v'/F_m'), which varies between 0–1, can be used to provide an estimate of the maximum efficiency of PSII photochemistry at a given light intensity, which is the PSII operating efficiency if all the PSII centers were open (Q_A oxidized). F_v'/F_m' is calculated using the variable fluorescence ($F_v' = F_m' - F_o'$) and the maximal fluorescence from the light-adapted leaf (F_m').

Both *qP* and Φ_{PSII} are related as follows (Andrews *et al.*, 1993):

$$\Phi_{PSII} = F_v'/F_m' \times qP \quad \text{Equation 8.7}$$

The Φ_{PSII} is the product of the efficiency of excitation energy capture by 'open' PSII reaction centers, which is estimated by F_v'/F_m', and the proportion of PSII reaction centers that are open, which is estimated by *qP*. Decreases in F_v'/F_m' are associated with increases in excitation energy quenching in the PSII antennae and are considered indicative of 'down regulation' of electron transport (Horton *et al.*, 1996). On the other hand, decreases in *qP* are attributable to: (a) decreases in the rate of consumption of reductants and ATP; or (b) damage to PSII reaction centers.

The maximum quantum efficiency of PSII photochemistry (F_v/F_m) in a dark-adapted leaf (see the accompanying volume, Chapter 13) corresponds to the maximum efficiency at which light absorbed by PSII is used for reduction of Q_A and is calculated as:

$$F_v/F_m = (F_m - F_o)/F_m \quad \text{Equation 8.8}$$

As mentioned above, dark-adapted values of F_v/F_m are used as a sensitive indicator of plant photosynthetic performance, with optimal values of ≈0.83 independent of the plant species (Björkman and Demmig, 1987; Johnson *et al.,* 1993). A lower value may indicate that a proportion of PSII reaction centers is damaged and this phenomenon is known as photoinhibition (photoinhibition may occur when a plant is exposed to very severe drought stress conditions).

To estimate *qP*, F_v'/F_m' and F_v/F_m it is necessary to measure the value of F_o' (minimal fluorescence level of leaves in the light) and F_o (minimal fluorescence level from dark-adapted leaves) at the time of measurement. In the laboratory, determination of F_o is achieved by darkening the leaf as mentioned above and, more commonly, by applying far-red illumination (wavelength >680 nm) for a few seconds before and immediately after the end of illumination. The same far-red light is applied to a leaf exposed to the light in order to determine F_o'. Commercial portable fluorometers usually incorporate a far-red light source. Alternatively, an estimation of F_o' can be obtained using F_o without making direct measurements as proposed by Oxborough and Baker (1997) and shown in Equation 8.9. If using a calculated F_o' value, the error, compared to using a measured F_o', is smaller than if using F_o.

$$F_o' = F_o (F_v/F_m + F_o/F_m') \quad \text{Equation 8.9}$$

This method was developed specifically for use in fluorescence imaging techniques where estimation of F_o' is particularly problematic. Whilst this method seems to work well under specific laboratory conditions, it makes assumptions about the nature of the processes contributing to fluorescence quenching. As such, it does not necessarily apply under other conditions, especially when the plant is exposed to stress and where significant amounts of photoinhibition may occur.

Non-photochemical quenching

The most straightforward way of quantifying non-photochemical quenching (*NPQ*) is:

$$NPQ = (F_m – F_m')/F_m' \quad \text{Equation 8.10}$$

NPQ is linearly related to heat dissipation and lies on a scale 0–infinity. In a typical plant, values might be expected in the range 0.5–3.5 at saturating light intensities; however, this varies markedly between species and on the previous history of the plant (Bilger and Björkman, 1990). *NPQ* compares non-photochemical quenching from a dark-adapted leaf with a leaf exposed to actinic light and we can only use NPQ to compare leaves that have similar characteristics in the dark-adapted state, e.g., with similar F_v/F_m values.

A summary of all parameters presented here is shown in Table 8.1; however, many additional calculations could be added to this list (for example, see Baker and Rosenqvist, 2004; Baker, 2008).

Imaging of fluorescence

The development of instruments capable of imaging chlorophyll fluorescence has provided a powerful tool to resolve spatial heterogeneity of leaf photosynthetic performance generally observed when measuring only a part of a leaf when using commonly available cuvettes and fluorometers. Photosynthetic heterogeneity has been identified in many situations, e.g., during induction of photosynthesis, with changes in carbohydrate translocation, during senescence, in response to changes in leaf water status, chilling or ozone stresses, etc. Non-imaging fluorescence measurements would often not detect such heterogeneity. Perturbations of metabolic processes not directly involved in photosynthetic metabolism often induce changes in fluorescence parameters. The development of commercial fluorescence imaging instruments that can image areas greater than 100 cm^2 allows the screening of large numbers of plants simultaneously. For more details about imaging of fluorescence see Baker and Rosenqvist (2004).

Application of gas exchange and chlorophyll fluorescence in research and as a selection criterion

So far, gas exchange and chlorophyll measurements have been applied mainly to study the effects of major abiotic stresses over plants, like water and heat stress. Some examples are also available in the literature (though in minority) where these techniques have been applied to discriminate genotypes (Araus *et al*., 1998; Fischer *et al*., 1998; Reynolds *et al*., 2000; Richards, 2000). In this last subsection, we will describe how gas exchange and chlorophyll fluorescence have been successfully used to study the effects of different abiotic stresses on photosynthesis and, finally, how these techniques can be applied to screen for increased leaf photosynthesis performance.

Increasing yield potential

Theoretical considerations suggest that wheat yield potential could be increased by up to 50% (see Reynolds *et al.* this volume, Chapter 4; Reynolds *et al.,* 2011). It has been proven that increasing photosynthesis has the potential to increase crop yields, provided

Table 8.1. Commonly used chlorophyll fluorescence parameters in studies of photosystem II (PSII) photochemistry (Baker, 2008).

Parameter	Definition	Physiological relevance
F_o	Minimal fluorescence from dark-adapted leaf	Level of fluorescence when primary quinone electron
F_o'	Minimal fluorescence from light-adapted leaf	acceptor of PSII (Q_A) is maximally oxidized (PSII centers open).
F_m	Maximal fluorescence from dark-adapted leaf	Level of fluorescence when primary quinone electron acceptor
F_m'	Maximal fluorescence from light-adapted leaf	of PSII (Q_A) is maximally reduced (PSII centers closed).
F_v	Variable fluorescence from dark-adapted leaf	Demonstrates the ability of PSII to perform photochemistry
F_v'	Variable fluorescence from light-adapted leaf	(Q_A reduction).
F_v/F_m	Maximum quantum efficiency of PSII photochemistry	Maximum efficiency at which light absorbed by PSII is used for reduction of Q_A.
F_v'/F_m'	PSII maximum efficiency	Provides an estimate of the maximum efficiency of PSII photochemistry at a given PPFD, which is the PSII operating efficiency if all the PSII centers were 'open' (Q_A oxidized).
NPQ	Non-photochemical quenching	Estimates the non-photochemical quenching from F_m to F_m'. Monitors the apparent rate constant for heat loss from PSII.
qP	Photochemical quenching	Associated with the proportion of PSII reaction centers that are open.
Φ_{PSII}	Quantum yield of fluorescence	Number of fluorescent events for each photon absorbed.

other constraints do not become limiting (Kruger and Violin, 2006; Long *et al*., 2006) and ongoing research suggested that substantial improvements of total crop photosynthesis are theoretically possible (Parry *et al*., 2011). Long *et al*. (2006) summarize some of the aspects that can be changed in C3 crops to improve photosynthesis efficiency and capacity. These include increased RuBP regeneration, increased stomatal and mesophyll conductance, increased Rubisco specificity factor, decreased Rubisco oxygenase activity, optimized Rubisco regulation, CO_2 pump and CO_2 pump with Kranz anatomy. Kebeish *et al*. (2007) showed that by partially blocking photorespiration an increase in biomass production was observed in *Arabidopsis* while Parry *et al*. (2008) has proposed to improve Rubisco activase activity. To develop a cohesive portfolio of activities that will maximize the probability of impact in farmers' fields, a wheat yield consortium (WYC) was convened in 2009. WYC fosters linkage between ongoing research platforms with special emphasis on increasing photosynthetic capacity and efficiency. Undoubtedly, gas exchange and chlorophyll fluorescence will be crucial tools to screen a new generation of wheat crops with increased photosynthesis and possibly yields. Researchers are also becoming interested in exploiting new sources of variation, e.g., spike photosynthesis which intercept 25–30% of light during grain-filling and contribute substantially to grain yield (Tambussi *et al.*, 2007). In spite of results indicating highly significant genetic variation among cultivars (M. Reynolds *et al*., unpublished data), the extent and implications of this variation in spike photosynthesis has not been established. In this sense, gas exchange and chlorophyll fluorescence will be useful in screening for improved photosynthetic performance and will be important tools in the quest to raise the genetic yield potential of wheat (Parry *et al.,* 2011). For yield potential, almost all gas exchange and chlorophyll fluorescence parameters can be informative without limitations. Chlorophyll fluorescence measurements under yield potential can be useful to screen advanced germplasm, but also for chlorophyll mutants (Codrea *et al.,* 2010). Rates of A_{max} and g_s have been correlated with yield under potential conditions (Fischer *et al.,* 1998) and may be used to determine genotypic variation among a population. Under yield potential conditions, F_v/F_m is not a useful parameter to detect differences among genotypes (no genetic variance), but simple measurements of F_o, F_m and F_v may be valuable (Araus *et al.,* 1998).

Increasing heat tolerance

CO_2 assimilation rates are highly affected by high temperatures (Law and Crafts-Brandner, 1999). Increases in stomatal conductance have been observed in heat treated plants indicating that the reduction in CO_2 assimilation caused by high temperatures was not limited by stomatal closure but by alterations on mesophyll capacity, which depended on the activity of Rubisco and on the capacity of photosynthetic electron transport to regenerate Rubisco (Crafts-Brandner *et al*., 1997; Eckardt and Portis, 1997; Feller *et al*., 1998). Gas exchange has been applied successfully in wheat to detect heat tolerance cultivars or advanced lines (Reynolds *et al*., 2000).

It has been reported that heat stress on the plant triggers the inactivation of PSII and thylakoid disorganization. These changes have been followed by monitoring the sharp rise in F_o as a function of temperature that indicates the critical temperature for PSII inactivation (Havaux, 1993). Both the rise in F_o and a decrease in F_v/F_m have been used to determine differences in the response of photosynthesis to high temperatures in potato cultivars (Havaux, 1995) and species of birch (Ranney and Peet, 1994). As CO_2 assimilation and electron transport are decreased at high temperatures, measurements of F_v'/F_m' also have potential for use in screens to identify tolerance to high temperatures (Baker and Rosenqvist, 2004). The most common parameters used for stress measurement are F_v/F_m and Φ_{PSII}. Both parameters have been shown to correlate well with CO_2 fixation rates under most stress conditions. Their sensitivity to stress can vary with the type of stress and in some cases with the type of plant. Some types of stresses do not immediately affect PSII and therefore Φ_{PSII} and F_v/F_m do not normally detect these stresses until levels are severe or even at starvation levels. Other modulated parameters are valuable for stress measurement as the photosynthetic electron transport rate (*J*) and the various quenching parameters involved in photo-protection, state transitions and photoinhibition. In some cases they are more sensitive than Φ_{PSII} or F_v/F_m because different types of stresses can affect different mechanisms in PSII and in the electron transport chain.

Increasing drought tolerance

Drought results in a different plant response compared to the responses observed under heat stress. Drought induces decreases in the leaf water content and

increases stomatal closure, decreasing the supply of CO_2 to the mesophyll cells and, consequently, results in a decrease in the rate of leaf photosynthesis (Williams *et al*., 1999; Lawlor and Cornic, 2002). Such stomatal effects on photosynthesis will not impact on the efficiency of the primary photochemical events of PSII or modify the associated fluorescence induction parameters, such as F_v/F_m, as has been demonstrated in several species (Massacci and Jones, 1990; Nogués and Alegre, 2002; Lima *et al*., 2002). The stomatal limitations imposed on photosynthesis will be accompanied by a decrease in the rate of consumption of ATP and NADPH for CO_2 assimilation, which could result in decreases in the rate of linear electron transport. However, operation of the water-water cycle and an increase in photorespiration under stress conditions, in C3 plants, may maintain rates of electron transport similar to those observed in non-stressed leaves despite the decreases in the rate of CO_2 assimilation (Leegood and Edwards, 1996; Noctor *et al*., 2002). Sub-stomatal CO_2 concentration decreases as stomatal conductance becomes smaller, but increases again at small stomatal conductance (Flexas and Medrano, 2002). The analysis presented by Flexas and Medrano (2002) suggests that stomatal closure is the earliest response to drought and the dominant limitation to photosynthesis at mild to moderate drought. This was already shown by other authors (Chaves, 1991; Cornic, 2000; Cornic and Fresneau, 2002; Lawlor and Cornic, 2002). However, in parallel, progressive down-regulation or inhibition of metabolic processes leads to decreased RuBP content, which becomes the dominant limitation at severe drought, and thereby inhibits photosynthetic CO_2 assimilation (Flexas and Medrano, 2002). One of the main problems when measuring photosynthesis under drought is the occurrence of a phenomenon known as 'patchy' stomatal closure. Patchy stomatal closure or non-uniform stomatal closure across a leaf can be induced by changes in a range of environmental factors, such as water and salt stress, changes in light intensity, changes in ambient CO_2 partial pressure and low air humidity (Downton *et al*., 1988; Beyschlag and Pfanz, 1990; During, 1992; Mott *et al*., 1993; Beyschlag and Eckstien, 2001). This phenomenon is an important source of error when measuring photosynthesis under drought.

Under Mediterranean conditions F_o, F_m and F_v have been used successfully to detect differences across genotypes and showed high heritability (Araus et *al.*, 1998). Several examples exist in the literature showing F_v/F_m as a good predictor of drought tolerance in several species (Jiang and Huang, 2000) but very often this parameter only changes when stress levels are very severe, as explained above (Araus *et al*., 1998). Declines in the Φ_{PSII}, accompanied by decreases in F_v'/F_m' have also been observed. These decreases are associated with increases in excitation energy quenching in the PSII antennae and are generally considered indicative of down regulation of electron transport (Horton *et al*., 1996). Consequently, the decreases in F_v'/F_m' during water stress can be considered indicative of a physiological regulation of electron transport by increasing excitation energy quenching processes in the PSII antennae (Nogués and Baker, 2000). In summary, the most basic parameters, such as F_o, F_m and F_v, seem to be the most promising for phenotyping under stress; ratios derived from these basic values have lower heritability and only vary under extreme stress conditions.

Conclusions

Photosynthesis is a highly complex mechanism that involves the knowledge of many different components, requires expensive equipment, expertise, and is time consuming. Making this process useful to plant breeding is not an easy task, as large scale phenotyping requires fast, cheap and heritable traits. However, some successes have been reported in the literature and in this chapter we provided some theoretical basis for photosynthesis and the main techniques available to screen for increased photosynthetic performance. Photosynthesis is becoming one of the main targets to improve wheat yields further, and a better understanding of techniques like gas exchange and chlorophyll fluorescence is important. At the same time we show that photosynthetic parameters cannot be used indiscriminately. For example, gas exchange has been successfully applied to correlate photosynthesis with yield in wheat under well irrigated conditions. However, there are difficulties in accurately measuring stomatal conductance and photosynthetic performance under drought, where uneven stomata closure can produce high experimental errors; chlorophyll fluorescence has been successfully used under Mediterranean conditions, particularly using basic fluorescence parameters like F_o, F_m and F_v whilst F_v/F_m gives poor heritability and is only sensitive under very severe stress conditions. Φ_{PSII} and F_v'/F_m' may also have some utility, but there are still several difficulties in the field, like ensuring that all leaves in all plots are exposed to the same light intensity. Imaging of fluorescence will become a promising tool if portable systems are available as this will account for spatial variation within the leaf and plot.

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Chapter 9: Strategies to identify genetic diversity in root traits

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Abstract

The role that root traits may play in increasing grain yields has not been fully explored. Here, we review genotypic variation in root traits, focusing on traits that may increase drought tolerance and nutrient uptake. We also consider established and emerging methods used to screen for root traits, and we assess the opportunities for future progress by considering the suitability of the identified methods for high-throughput screening and the use of simulation models to identify which roots to screen. Genotypic variation in root traits has been reported in crops such as bread wheat, durum wheat, barley, and maize. As knowledge about root systems is fragmented, simulation models have been used as a strategy to integrate information, to reduce the number of traits to screen, and to target the most relevant ones for the response to a specific stress. Soil cores and minirhizotrons have so far been the methods most commonly used for field studies, whereas methods relying on hydroponics, moistened paper and petri dishes have been used for the high-throughput screening of root traits under controlled conditions. Recent developments in methods to measure root traits suggest that high-throughput simple screening techniques are also becoming available for field studies.

Introduction

Roots are essential for plant growth, survival, and fitness. Plant species differ in their temporal and spatial exploration of the soil and in their adaptation to biotic and abiotic stresses. Due to the heterogeneous nature of soil environments, root systems must respond to a wide spectrum of physical, biological, and chemical conditions, including resource availability, all of which show spatial and temporal variation. Consequently, root systems develop into a complex array of irregularly distributed roots. These root arrangements ultimately determine the ability of the plant to access soil resources (Robinson, 1994).

Quantifying the architecture of root systems is important because crop productivity is almost always influenced by the availability and accessibility of water and nutrients for plants. On the other hand, crops such as wheat show great plasticity in root growth, adjusting to nutrient and water availability (Cholick *et al.,* 1977). Therefore, any attempt to assess the relationship between physiological responses and root traits should first account for the plastic capability of roots.

In this chapter, we review genotypic variation in root traits, focusing on those that may increase drought tolerance and nutrient uptake. We also consider established and emerging methods used to screen for root traits, and we determine opportunities for future progress by considering the suitability of the identified methods for high-throughput screening and the use of simulation models to identify the most appropriate roots to screen.

Genotypic variation in root traits

Genetic diversity in root traits in wheat has been found in bread wheat (Mackay and Barber, 1986) and durum wheat (Motzo *et al.,* 1993). Such variation includes differences among wheat genotypes in the ability to establish a deep root system quickly (Siddique *et al.,* 1990), in root length density (Mian *et al.,* 1994), in root distribution (Ford *et al.,* 2006), in post-anthesis root growth (Ford *et al.,* 2006) and in the numbers of seminal roots (Robertson *et al.,* 1979) and total roots (Box and Johnson, 1987). Similarly, the root:shoot ratio was found to be highly influenced by genotypes (Sadhu and Bhaduri, 1984).

The effect of dwarfing genes on root traits was studied in particular detail. Bush and Evans (1988) found differences in root biomass, and Wojciechowski *et al.* (2009) reported a direct effect of dwarfing alleles on root growth during seedling establishment; whereas the total root length was altered by reduced height alleles, no effect was found on the root architecture (root diameter and lateral root:total root ratio).

Genotypic variation on root traits as related to drought tolerance

Manschadi *et al.* (2006) found a relation between the angular orientation of wheat seminal roots, root architecture and water uptake. They studied two contrasting spring wheat genotypes; the drought-tolerant one had a more compact root architecture and a greater root length at depth (Manschadi *et al.,* 2006). Genotypic differences in rooting depth and root angle (Nakamoto and Oyanagi, 1994) were also found in other studies. Under rain-fed conditions, the root length density of wheat has been found to be higher in dryer years (Hamblin *et al.,* 1990). Ekanayake *et al.* (1985a) identified a polygenetic system of inheritance for root length density, root diameter and root dry weight in upland rice. Although the root traits were screened under hydroponic conditions, they correlated well with the visual scores for drought tolerance in the field. In field studies, most drought tolerant semi-dwarf bread wheat genotypes (e.g., Pastor, Synthetic 2, Sujata, and Nesser) had higher root length density in deeper soil layers than the non-tolerant controls (Tevee 2, Pavon, and CRC) (Manske and Vlek, 2002).

Lopes and Reynolds (2010) demonstrated that differences in rooting depth expressed among near isogenic wheat lines explained superior adaptation to drought and Reynolds *et al.* (2007), showed that increased partitioning of root mass to deeper soil layers can be derived from wheat wild relatives and landraces. The differences in root length density and root mass among CIMMYT material were found in field trials using soil cores. Under drought conditions, deep rooting is more important when the crop depends on soil-stored water (Mian *et al.,* 1994) that is replenished annually, whereas in Mediterranean environments, higher root length at intermediate soil depths (0.15–0.60 m) is probably more important than deep roots for increasing water uptake (Gregory *et al.,* 2009). A strategy to reduce water movement beyond the reach of crop roots is to genetically extend the duration of the vegetative period while maintaining the flowering time due to frost/drought limitations later in the growing season (Richards *et al.,* 2007). Similarly, increases in shoot vigor showed a positive relation with root length, seminal root number and root branching (Richards and Lukacs, 2002). The ability of roots to grow through compacted soil layers is also associated with higher drought tolerance.

Genotypic variation on root traits as related to the uptake of nutrients

Studies in which vigorous and non-vigorous genotypes were compared for their ability to absorb nitrogen (N) were perhaps the most common type of studies in which root traits were studied with the objective to relate root traits to physiological process (Palta *et al.,* 2011). These studies showed that total N absorption and the absorption efficiency of N fertilizer (i.e., N absorbed/N applied) were greater in vigorous wheat genotypes than in other genotypes when rates equivalent to 50 kg N ha^{-1} were applied at seeding. The greater N absorption efficiency shown by vigorous genotypes was associated with greater root biomass and root length density in the top 0.7 m of the soil profile (Palta *et al.,* 2011). Also, the branching of the seminal roots started 5 to 7 days earlier and was more profuse in the vigorous wheat genotypes than in the non-vigorous ones (Palta *et al.,* 2011). Most of the root branching exhibited by the vigorous genotypes occurred in the top 0.7 m of the soil profile as a result of N application, leading to more root tips and 42–60% more N absorption at the stage of stem elongation. In addition, Greef and Kullman (1992) reported that the rate of N uptake per unit root length varies considerably between genotypes. Genetic differences in root dry weight could result from differences in the degree of adjustment to the onset of N stress; i.e., as a result of optimizing the functional equilibrium between roots and shoots (Vannoordwijk and Dewilligen, 1987) There could be other root traits that are beneficial for increasing N absorption, and the study of these traits may help us to better understand the interaction between N management and genotypes on the root system, including enhanced post-anthesis root longevity and rooting depth (Bengough *et al.,* 2006).

The root angle and the resulting orientation of the root system of common beans were influenced by the availability of soil P (Ge *et al.,* 2000). As a result, it was proposed that the more surface-oriented root system found in low-P soils was a positive adaptive response. A selection program for tolerance to acid soils, low phosphorus conditions, and aluminum toxicity resulted in semi-dwarf bread wheat genotypes with higher grain yield, phosphorus uptake and greater root length density (Manske and Vlek, 2002). Therefore, root branching (Gahoonia and Nielsen, 2004; Jones *et al.,* 1989), root hair length (Gahoonia and Nielsen, 2004) and root architecture traits were found to be important determinants of P uptake efficiency.

A major impediment to increasing the uptake of soil resources is a root system whose growth is limited by disease. Few efforts have been made to find genetic variation suitable to protect against root diseases. However, there has been some significant success in achieving resistance against the cereal cyst nematode (Ogbonnaya *et al.,* 2001). There is also good genetic resistance/tolerance to the root lesion nematode (Schmidt *et al.,* 2005) and crown rot (Collard *et al.,* 2005). However, there is no known resistance in wheat, barley, or triticale to root diseases such as take-all or rhizoctonia root rot. Resistance to take-all may one day be achieved by introducing genes to susceptible cereals from other resistant grass species (Lindelau *et al.,* 1973) or by transgenic insertion.

Generally, under conditions of low resource availability, higher root length density increases grain yield. In contrast, the benefit of this trait diminishes as the level of inputs increases. If the use of carbon by large root systems is not compensated for by the higher uptake of soil resources, the roots themselves may compete for assimilates with the grains and limit grain yield potential. On the other hand, variation in total root length may be valuable not only for resource uptake but also in escaping organisms that grow around the roots and have deleterious effects on their growth and functioning (Watt *et al.,* 2003). Different root traits may become important as a function of the level of inputs. Under high resource availability, root traits that decrease the occurrence of lodging such as the spread of the root plate (Berry *et al.,* 2007), root-clump weight (Thompson, 1968), and root pulling strength (Ortman *et al.,* 1968) may become important for increasing yield potential.

Although genotypic differences in root traits have been reported (Table 9.1), to date these differences have rarely been exploited in breeding programs. Gregory *et al.* (2009) attributed this to the difficulty of screening root traits and the large number of plants needed for such an undertaking.

Methods for measuring root traits

Root systems are inherently difficult to study due to their underground environment, the complexity of their dynamic interactions with their environment, and the diversity of their functions. An extensive list

Table 9.1. Examples of root traits with potential to increase grain yield. Compiled from Palta *et al.* (2011) and Richards *et al.* (2007).

Trait	Mechanism	Reference
Al resistance	Malate released from root tip protects the meristem	Fischer and Scott (1987) Sasaki *et al.* (2006)
Cereal cyst nematode resistance	Stops nematode infection	Ogbonnaya *et al.* (2001)
Root rot resistance	Stops infection by *Rhizoctonia solani*	Okubara *et al*. (2009)
Bacterial resistance	Bacterial populations in the rhizosphere differ among genotypes and specific populations suppress soilborne diseases	Neal *et al*. (1973) Miller *et al*. (1989) Mazzola *et al*. (2004)
Deep roots	Higher root density in deep soil layers increases water capture	Hurd (1968, 1974) O'Brien (1979) Manske and Vlek (2002)
Redistribution of roots	Higher root density in deep soil layers not associated with greater overall investment in roots	Reynolds *et al*. (2007)
Angle of seminal roots	Deep water capture	Nakamoto and Oyanagi (1994)
Larger intercellular air space in root tissue†	Reduces metabolic costs of root tissue, leaving extra energy to invest in grain	Zhu *et al*. (2010)
Reduced diameter of the xylem vessels of the seminal root	Conserves water for flowering and grain-filling	Richards and Passioura (1989)
Lodging resistance¶	Spreads the root plate	Berry *et al*. (2007) Pinthus (1967)
Increased root elongation	Increases phosphorus absorption	Gahoonia and Nielsen (2004)
Weak root gravitropism§	Shallower root distribution increases phosphorus absorption	Liao *et al*. (2004)
Overexpression of genes that synthesize alanine	Nitrogen in the amino acid alanine becomes available	Garnett *et al*. (2009)

Identified in: † maize; ¶ barley; § beans.

of methodologies for studying roots exists (e.g., see Neumann *et al.,* 2009), each with an inherent degree of artificiality. It is precisely the lack of an accurate and standardized methodology to detect roots that limits the understanding of the roles and functions of root systems (Pierret *et al.,* 2005). The combination of complexity and methodological problems has resulted in our knowledge of roots being limited and fragmented. However, it is still possible and necessary to study the root systems of plants, but it is important to consider carefully the suitability of the available methodologies to address the specific research questions.

Established methods

Harper *et al.* (1991) classified available methods to study roots into two groups: destructive and non-destructive methods. The main criterion determining the selection of a method to study roots depends on whether the focus is on changes in root traits over time or space. For the study of changes of root traits over time, with the exception of mass, non-destructive methods are recommended. Repeated non-destructive observations of roots avoid the overlap of spatial and temporal variabilities influencing root characteristics (Taylor *et al.,* 1990). Therefore, the decision of which technique to use should be made according to the root trait of interest. The methods that so far have been used for measuring root traits with potential to increase grain yield include:

• ***Monoliths:*** A cubic section of soil that contains roots (i.e., monolith) is dug out from the soil or obtained from a container in which the plant has been grown. Afterwards, the monolith is washed to remove soil and separate roots. Although the root system may be damaged during sampling (over 50% of the total root length of maize may be lost– McCully, 1999), a representative characterization of its morphology can be obtained.

• ***Soil cores:*** A soil core, which is small compared to the rooting volume (Pierret *et al.,* 2005), is taken from the rhizosphere. Samples can be processed in two ways: (i) the amount of roots can be estimated by breaking the soil core horizontally and counting the roots exposed on both faces of the breakage (Yamaguchi, 2002), or (ii) they can be processed similarly to the monoliths by washing the samples and recovering the roots (Kumar *et al.,* 1993). See the accompanying volume, Chapter 17.

The major advantage of this method is that it provides the most accurate measurements of root length and root mass and as a consequence leads to a better examination of the relation of these traits with nutrient and water uptake. Aside from collecting and washing the samples, this method involves removing roots from the soil, separating roots from organic debris, and storing and analyzing the samples. Although strategies to reduce the number of samples to manually process have been devised (Costa *et al.,* 2000), processing a high number of samples is time consuming. Sample processing can be partially accelerated by using a root washing machine (Smucker *et al.,* 1982). The biggest advantage of using such a machine is the consistency that it achieves in processing the samples. The limitations of this method include the impossibility of repeating the measurements on the same set of roots and the inability to determine the exact position of the roots. Root position is important because resource uptake is usually affected to a greater extent by the relative spatial distribution of roots and soil resources than by the size of the root system (de Kroon, 2007), and the relative arrangement of the roots will also influence processes such as root exudation (Li *et al.*, 2007). This method is also not recommended when the focus is on the dynamics of a certain root trait because the available area to sample roots is sequentially reduced and because the effect of time as a source of variability cannot be separated from the effect of space (i.e., sampling site). The latter may be critical, as root distributions differ between different locations.

• ***Mesh bags:*** The dynamics of root growth and root turnover can be studied by placing bags containing root-free soil in the field and removing them at regular intervals.

• ***Two-dimensional (2D) rhizotrons:*** Under this method, instead of growing the plant in the soil, it is grown in a flat container with side walls made of a transparent material such as glass. However, root growth is disturbed and forced to occur in only two dimensions, horizontally and vertically.

• ***Trench walls:*** The soil next to a plant is dug in such a way that the root systems become visible.

• ***Optical scanners:*** These can be used to process samples obtained by soil coring or by burying them in the soil to study roots in a similar way as with 2D rhizotrons (Dannoura *et al.,* 2008). The drawbacks of this technique are that there is only a partial measurement of the rhizosphere and that the root morphology is altered by the presence of an impenetrable object. On the other hand, this method provides data that can easily be analyzed by established software protocols.

• ***Minirhizotrons:*** Minirhizotrons are small-diameter transparent tubes inserted into the soil for the observation of root and soil processes (Figure 9.1). They belong in the category of non-destructive methods. The description of the basic technique, its variations, and applications were reviewed by Smit *et al.* (2000a). By permitting repeated observations of a large number of roots over time, transparent interfaces such as minirhizotrons constitute a unique method that provides detailed information on root production and mortality (Johnson *et al.,* 2001) in individual root segments in specific soil sites. Minirhizotrons minimize the potential of confounding spatial and temporal variation in root dynamics (Hendrick and Pregitzer, 1996). A disadvantage of minirhizotrons is that they have been reported to underestimate root density in the uppermost soil layers and to overestimate it in deeper layers (Pages and Bengough, 1997). Therefore, they may not be the most suitable method to assess root distribution.

Rooting parameters obtained from minirhizotron images are the number of intersections of root segments with different types of grids (Meyer and Barrs, 1991), the number of roots (Upchurch and Ritchie, 1983) and the length of roots (Beyrouty *et al.,* 1990). These parameters can be used directly as rooting indicators, or they can be transformed to root length density using theoretical (Merrill and Upchurch, 1994) or empirical relationships (Coleman *et al.,* 1996). The relationship between root length assessed using minirhizotrons and root length assessed using other methods, although significant, is weak (Mackie-Dawson and Atkinson, 1991). Minirhizotrons are perhaps also not the most appropriate tools for estimating root length density in the bulk soil. Soil coring methods are considered to be more representative for measuring the distribution and intensity of rooting (Hansson *et al.,* 1992). The length of roots on the minirhizotron surface could be influenced by the minirhizotron itself, as roots could grow along the minirhizotron at a different rate than in the bulk soil. It has been suggested that the number of roots arriving at the minirhizotron surface (Upchurch, 1987) or first intersections, as described in Mackie-Dawson *et al.* (1991), would show a better relationship with the rooting intensity in the bulk soil. The number of roots on the minirhizotron surface is probably less influenced by the conditions at the interface than length, and it is independent of any property expressed by the root after the root has reached the minirhizotron's surface (Smit *et al.*, 2000a; Box and Ramseur, 1993).

As in the case of 2D rhizotrons, the experimental setup does not necessarily represent a completely undisturbed system, and mechanical impedance, temperature, moisture distribution, solute concentrations and

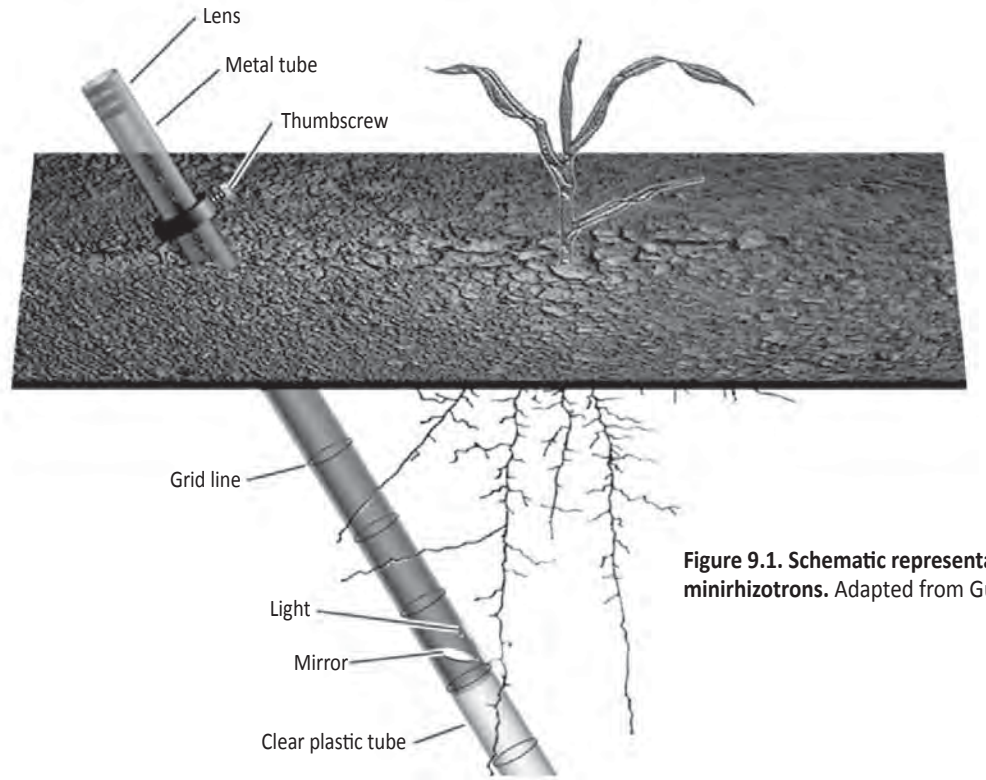

Figure 9.1. Schematic representation of the use of minirhizotrons. Adapted from Gurevitch *et al*., 2002.

redox conditions in the soil along the two-dimensional observation planes may differ from the conditions in undisturbed soils. However, the degree of interference is much smaller for minirhizotrons (i.e., transparent tubes inserted into the soil) than it is for rhizotrons (Taylor *et al.,* 1990). A common challenge for methods using minirhizotrons and 2D rhizotrons to reduce the sample analysis time is that the automatic analysis of the images is still limited by an insufficient ability of the software for background elimination. In spite of their limitations, minirhizotrons have made it possible to study a broad array of biological processes such as the following: (i) root development in the soil profile (Liedgens *et al.,* 2000b), (ii) root turnover (Pregitzer *et al.,* 2008), (iii) root parasitism (Eizenberg *et al.,* 2005), and (iv) proliferation of fungal hyphae (Vargas and Allen, 2008).

Root systems usually share the soil with intraspecific or interspecific neighbors. Because the presence of neighbors affect processes such as leaf orientation (Maddonni *et al.,* 2002), modifications in root architecture due to the presence of intraspecific or interspecific neighbors are also expected. However, most, if not all, of the studies on root architecture were conducted on plants grown alone. A recently developed method based on minirhizotrons allows for the distinguishing of roots belonging to different plants that are grown together (Faget *et al.,* 2009, 2010). This method is based on the use of genetically modified plants in which the constitutive expression of the green fluorescent protein (GFP) results in a green bioluminescence throughout the root extension (Figure 9.2). This green bioluminescence is not observed in non-genetically transformed plants and thus can be used as a visual marker to differentiate roots from intraspecific and interspecific neighbors.

• ***Electrical capacitance:*** This method was proposed to estimate root mass and is based on measuring the electrical capacitance of an equivalent parallel resistance-capacitance circuit formed by the interface between soil water and the plant root surface. It can

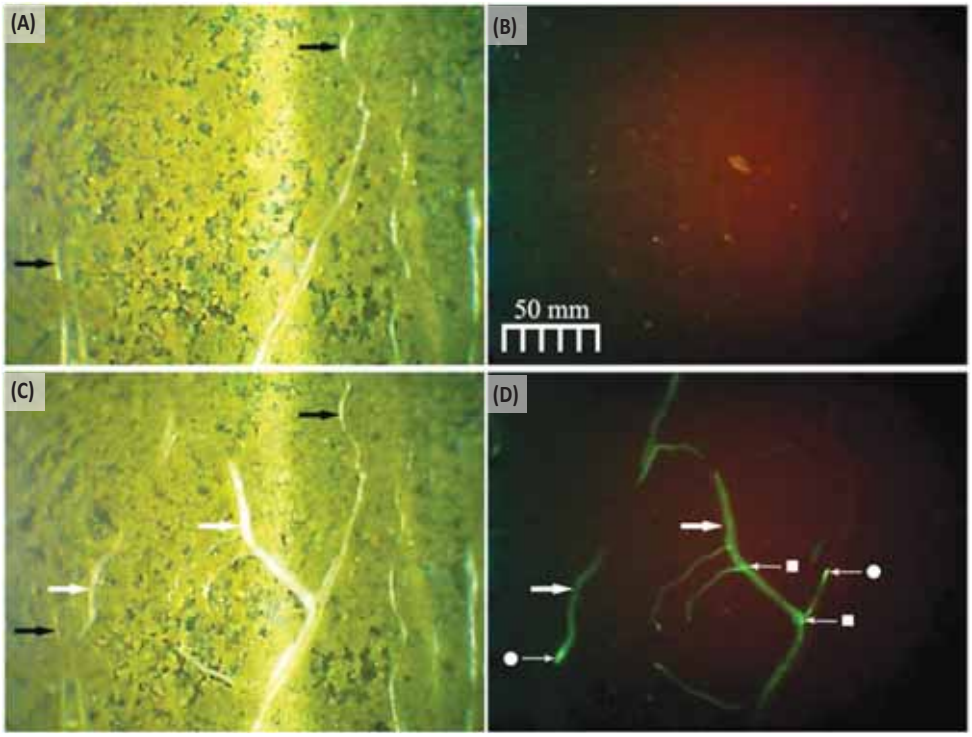

Figure 9.2. Minirhizotron images of mixed plant stands with the genetically transformed maize (*Zea mays* L.) genotype ETH-M72$_{GFP}$ and Italian ryegrass (*Lolium multiflorum* Lam.). The conventional minirhizotron images on the left-hand side were captured within the visible light spectrum at 37 (A) and 48 (C) days after sowing the maize. The fluorescent images in the right-hand side (C and D) were captured with a narrow-spectrum light source to trigger fluorescence on the same days as the corresponding conventional images. From the conventional images of the roots, it was not possible to assign roots to the plant type. Roots of Italian ryegrass (black arrows, A) can be recognized. Tips (○) and branch insertions (□) of the ETH-M72$_{GFP}$ roots (D) were particularly bright in the fluorescent images (L). Scale: 1 pixel = 40.6 µm. (Faget et al., 2009).

be used with several electrodes to generate maps of root biomass in the soil profile (Amato *et al.,* 2009). This approach has been used for taking measurements in barley (Chloupek *et al.,* 2006); however, in high tillering wheat, the use of the device is more difficult due to the high number of stems per plant. Good linear correlations between root capacitance and root mass were obtained for young plants (Chloupek *et al.,* 2006). However, there is still a poor understanding of how the capacitance readings are influenced by: (i) superficial, as compared to deep, roots, (ii) different types and orders of roots, and (iii) young, as compared to aged, roots. In addition, this technique is not suitable for measuring the spatial distribution of roots in the soil profile.

• ***Ground-penetrating radar:*** This method has been used successfully to study the root biomass of trees (Hruska *et al.,* 1999), but good correlations between readings from the radar and results obtained with soil cores still have to be found for cereals.

• ***X-ray, γ-ray, thermal neutron and magnetic-resonance tomography (computed tomography methods):*** These methods allow roots growing in the soil media to be imaged non-invasively (Tracy *et al.,* 2010). An advantage of these methods is that root growth and architecture can be screened simultaneously with soil processes such as water uptake (Oswald *et al.,* 2008; Tumlinson *et al.,* 2008) and root exudation (Raab and Vogel, 2004). Among the disadvantages of this method are the high costs, the small sample volume and the sterilization effect on soil microorganisms. This latter effect, combined with the fact that they are restricted to homogeneous soil densities and to non-swelling soils without clay minerals and paramagnetic elements (Fe, Mn, and Cu), limits the representation of the rhizospheric environment. An additional disadvantage is that because scanning the samples is time consuming, a smaller number of samples can be processed per day (less than 10) compared to methods such as 2D rhizotrons, which allow the processing of over 100 samples (Gregory *et al.,* 2009). These limitations prevent a more general use of these techniques in root growth analysis. On the other hand, the root can be completely measured and automatically analyzed with a computer. Due to the non-invasiveness, the roots are not damaged, and a continuous observation of the morphology is possible. This method allows angular distributions in three dimensions to be obtained in addition to root numbers and lengths.

Soil cores and minirhizotrons are the methods that have been used more frequently for field studies until now. In Table 9.2, these methods are compared according to six different criteria: (i) 'accuracy' considers the accuracy of the method to measure root length; (ii) 'work' describes the amount of work that has to be invested to collect the samples; (iii) 'analysis' judges the amount of work that has to be made to extract numerical data; (iv) 'dynamic' describes the suitability of the method to assess root traits over time; (v) 'cost' compares the methods in terms of the financial investment that they demand; and (vi) 'throughput' considers the suitability of the method to conduct high-throughput studies. Different methodologies were combined in facilities to relate root traits to processes occurring at the shoot or at the rhizosphere (Liedgens *et al.,* 2000a; Vandegeijn *et al.,* 1994). Although these facilities do not represent an undisturbed system, they combine controlled laboratory conditions with more field-orientated long-term observations. Similarly, these methods were followed in field studies and paired with modifications in the rhizosphere to test hypotheses related to the functional importance of root traits. For example, McKenzie *et al.* (2009) restricted the root penetration capacity of barley genotypes, burying mesh layers horizontally in the soil and Nakamoto and Oyanagi (1994) buried baskets in the field to evaluate root growth angles.

Table 9.2. Evaluation of conventional methods to measure roots according to six criteria on a scale from "---" (very low) to "+++" (very high).

Method	Accuracy	Work	Analysis	Dynamic	Cost	Throughput
Monoliths	+++	+++	+++	---	---	---
Soil cores	++	+++	+++	--	---	---
Trench walls	++	+++	++	-	---	---
Mesh bags	-	+	++	+	--	--
2D rhizotrons	+	+	+	++	+	+
Minirhizotrons	-	+	+	+++	+	+
Optical scanners	+	-	+	++	++	+
Electrical capacitance	--	---	-	+	-	++
Ground-penetrating radars	?	---	--	+++	++	++
Computed tomography methods	+++	--	++	++	+++	+

High-throughput strategies

Phenotyping root traits from a large number of genotypes is difficult because measuring root traits is time consuming and phenotyping usually requires screening a high number of plants. As a result, most attempts conducted to date have been done with young plants grown on homogeneous artificial media. For example, hydroponics media was used to screen for tolerance to Aluminum toxicity (Rajaram and Villegas, 1990; Rajaram *et al.,* 1991).

Screening for the number of root axis and traits such as the presence of root hairs has been possible for a large number of genotypes by growing them on hydroponic systems (Chen *et al.,* 2011), moistened paper (Hund *et al.,* 2009), petri dishes (Bengough *et al.,* 2004), or small-sized 2D rhizotrons (Devienne-Barret *et al.,* 2006) combined with image processing techniques. Petri dishes with agar have been employed to identify quantitative trait loci (QTL) that determine the root system architecture of doubled haploid barley lines (Forster *et al.,* 2007). Bengough *et al.* (2004) also used petri dishes with gel for the rapid screening of the number, angle, and length of seminal roots. This approach maintains the vertical and horizontal orientation of roots, enabling the sequential screening of the abovementioned root traits with suitable software. Recently, the principles of methods based on petri dishes with agar were used to develop a method in which the automatic screening and identification of root traits in 3D became possible (Iyer-Pascuzzi *et al.,* 2010). Methods based on agar allow differences in root length between genotypes to be identified in young seedlings; however, the rank order may not be the same when the same genotypes are grown in another substrate (Gregory *et al.,* 2009). To the best of our knowledge, the only strategy of screening root architectural traits in the field with a high-throughput was devised by Trachsel *et al.* (2011). With this strategy, ten different architectural traits of the root crown of field grown adult maize plants were visually scored. There is also a great potential to increase the efficiency of experiments to identify root traits by using innovative analytical methods such as morphometric (Grabov *et al.,* 2005) or fractal (Costa *et al.,* 2003) analysis.

Simulation of root traits

Because knowledge of root systems is fragmented, simulations can be used to obtain an integrated picture of the functioning of the entire system. Simulation models can also help to evaluate the potential impact of modifications in root architecture and improve the understanding of measured data. A fundamental and major difference in the modeling approach exists between those models that use architectural information to simulate the growth of individual roots within the root system and those that do not. Rarely are models employed merely to express the size and shape of the root system; rather, they are used to determine the surface available for the acquisition of water and nutrients from the soil.

Models that simulate root growth without considering root architecture

The most basic root models start with the assumption that the roots in each layer of soil take up the same amount of water and nutrients irrespective of the exact quantity of roots present. Consequently, they simulate only the advancing rooting front or the depth of the deepest root (Chapman *et al.,* 1993). The utility of this approach has often proved to be limited because roots may penetrate to deeper soil layers through cracks or worm holes without appreciably affecting resource acquisition. In addition, research has demonstrated that relationships exist between the root length density in a certain soil layer and resource absorption. Gerwitz and Page (1974) showed that it is possible to describe root growth and distribution in a manner analogous to the movement of solutes by diffusion, suggesting that roots grow from zones in which they are present in high concentration to zones of lower concentration. Their reports also showed that individual roots might be regarded as growing in random directions as a result of modular elongation. The consequence of the diffusion approach is the simulation of a root system in homogeneous soils, where root length density decreases exponentially with depth.

Many crop growth models simulate the exploration of the soil by the root system using two separate processes: (i) the downward growth of a single vertical axis, and (ii) the proliferation of roots in the soil layers throughout the achieved root depth (e.g., Jones *et al.,* 1991). Models in the CERES/DSSAT family simulate root growth based on this second principle. This modeling framework was further upgraded to allow for the simulation of the effects of several soil restrictions by incorporating a 'root stress factor'. For example, Asseng *et al.* (1997) included such a root stress factor to develop a model that is responsive to N levels in the soil in addition to soil

factors such as water, compaction, and aeration. These models made no attempt to describe the architecture of the root systems.

Models that simulate root growth considering root architecture

Many approaches have been used to model root architecture. Several reviews summarize these approaches in detail (Pierret *et al.,* 2007). With the improvement of the computational and graphical capacities of computers, it became possible for models such as 'Rootmap' (Diggle, 1988) to simulate three-dimensional roots with highly improved graphic representation. The graphic visualization was a key advancement for the comparison of real growth and simulated roots. Currently, the limitation of these models is that they simulate the growth of young plants and not at advanced growth stages such as grain-filling.

Conclusions

While root systems acquire the majority of the essential elements required for crop growth, the role that root trait selection might play in crop breeding programs has not been fully explored. Current breeding techniques rely on selection for above-ground characteristics. Although this process may indirectly be selecting for desirable rooting traits, given the large and varied functions that root systems accomplish, breeding objectives may be better met by directly targeting specific root traits.

The identification of root traits offers the potential to increase the grain yield of not only crops growing under limited soil resources but also crops growing under optimal water and nutrient supply by revealing physiological traits associated with the partitioning of dry matter between the crop's root system and the anchoring of the crop, which prevents lodging. The identification of optimal root traits under stress environments depends on targeting the probable stresses that the crop may face during the growing season. Simulation models can be useful to reduce the number of traits to screen and to target the most relevant ones for the response to a specific stress.

So far, soil cores and minirhizotrons have been the methods most commonly used for field studies. Methods featuring simple screening, the availability of molecular markers and the identification of environments in which these traits will provide advantages will be major challenges to incorporate these traits into breeding programs. Methods such as hydroponics, moistened paper and petri dishes have been used successfully for the high-throughput screening of root traits. These methods give valuable information about traits, such as the number of seminal roots and the root angle that are conserved across substrates. In contrast, for more plastic traits such as root length, methods using artificial substrates may not adequately reproduce root growth in the soil or the same ranking order of genotypes. Recent developments in methods to measure root traits suggest that high-throughput simple screening techniques are becoming viable. These will be fundamental for testing the field relevance of promising root traits that are identified under controlled conditions.

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Chapter 10: Wheat development: its role in phenotyping and improving crop adaptation

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Abstract

Developmental traits are critical for wheat breeding. This is because time to anthesis and the duration of each phase, determined by interactions between genetic and environmental factors, are responsible for adaptation and yield generation. This chapter provides a general description of the main developmental features of wheat, and their responses to photoperiod and temperature, as a basis for discussion on opportunities for improving adaptation and yield potential. Developmental traits are universally affected by temperature *per se* (all cultivars and all phases are sensitive to it). As developmental rate increases linearly with temperature up until heading, there is a single slope for the whole interval between base and optimum temperature, whose reciprocal is the thermal time. On the other hand, genotypes vary widely in their sensitivity to photoperiod and vernalization; from genotypes that are virtually insensitive through to those with strong quantitative (or even qualitative) responses. In addition, not all developmental phases are sensitive to these factors: vernalization sensitivity is expressed mainly in vegetative phases, while photoperiod sensitivity may influence development through both vegetative and reproductive phases up to anthesis. The rate of post-anthesis development towards maturity in wheat is insensitive to photoperiod and vernalization and only appears to respond positively to temperature *per se*. Genetic variation in development is also due to earliness *per se* (variation beyond sensitivities to photoperiod and vernalization). Finally, key developmental phases are identified and alternatives for improving adaptation and yield potential are discussed.

Introduction

Crop development is a continuous succession of changes during which initiation and growth of different organs occur to complete life cycle. Despite its recognized continuity, the time course of development, *viz.* ontogeny, in grain crops is frequently divided into component phases (mainly vegetative, reproductive and grain-filling phases).

The duration of each phase and, consequently, that of the whole life cycle, is determined by interactions between genetic and environmental factors. Accordingly, the number of primordia initiated in each particular phase is determined by the same interactions (if factors affecting the duration of the phase do not affect the rate of primordia initiation similarly, the number of organs initiated would be positively related to the duration of the phase). These responses largely determine the adaptability of a crop to a certain range of environmental conditions.

As wheat is cultivated throughout the world (from the southern regions of America and Oceania through the equator to the northern areas of America, Europe and Asia; from sea level to altitudes of about 3000 m), its developmental responses to environmental factors must be sufficiently complex to warrant such a wide adaptability. Adaptation of wheat to different regions has led to modifications in its pattern of development to suit each particular combination of environmental conditions, the key issue being that anthesis must occur when frost risks are small. Thus an important feature of the adaptability of wheat (and other crops) lies in its ability to sense the seasons so that development is accelerated or delayed depending on the environment.

There are different types of wheat which are termed in relation to the timing of their sowing or the condition in which they are grown; associated with the adaptation features required for good performance in these conditions. These types are: (i) spring wheats, (ii) winter wheats, and (iii) Mediterranean wheats (Bunting *et al.,* 1982).

(i) Spring wheats: many wheat crops in cold regions cannot survive the winter to produce a reasonable yield, and therefore, are normally sown in spring. These spring types sense the advancement of spring and accelerate their developmental rate accordingly. Day length (actually that of the night) is the best environmental factor to be sensed, as day length

invariably increases (and night length decreases) from the beginning of the winter to the beginning of the summer. Photoperiod sensitivity may help to delay anthesis in early sowings and accelerate development, to flower sooner, in late sowings.

(ii) **Winter wheats:** some temperate regions have winters that are quite severe, but not so cold as to prevent crops from surviving. Autumn sowing allows the crop to utilize a long growing season and to have a relatively early anthesis, having already produced a large biomass. In this case, plants must sense the season independent of day length. To allow autumn-sown plants not to initiate the reproductive periods until winter has ended, plants have evolved the requirement of a period of exposure to low temperatures before their development may proceed to reproductive phases. Winter types are capable of sensing such a condition (the exposure of the young plants to a period of low temperatures) and accelerate their developmental processes, a process known as vernalization. The adaptive role of this vernalization sensitivity is highlighted in these regions, as a prevention of inflorescence initiation in autumn, when photoperiod and temperatures are similar to spring.

(iii) **Mediterranean wheats:** in mild-winter temperate regions, such as those of Australia, Argentina and some Mediterranean areas of the northern hemisphere, wheat may be sown in winter, when soil temperatures are not so low as to prevent seedling emergence in a reasonable period after sowing and achieve normal crop establishment, even in the middle of winter (if soil moisture is adequate). In these circumstances, flowering has to be achieved in a period after sowing intermediate between the very long pre-anthesis period of winter wheats and the rather short period characteristic of spring wheats. This is achieved by having either strong sensitivity to photoperiod or slight sensitivity to vernalization, both characteristics guaranteeing crop flowering shortly after the onset of a period of low or no risk of frosts. Genotypes with slight vernalization requirements are frequently referred to as 'intermediate', 'facultative', 'semi-winter', or 'Mediterranean' wheats.

The aims of this chapter are to provide: (i) a general description of main developmental features of wheat, and (ii) to describe the main developmental responses of wheat to environmental factors, so that opportunities for improving adaptation to particular conditions are highlighted. In addition, the use of developmental responses of wheat, as an alternative avenue to further increase yield potential, is also discussed. Breeders can take advantage of this knowledge by using different factors to customize crops to fit within the targeted growing season. Regarding the nature of this manual, a summarized and simplified view of wheat phasic development is presented. A more comprehensive description, and wider discussion, can be found in Halloran (1977), Hay and Kirby (1991), Slafer and Rawson (1994a), Slafer and Miralles (1998), and Slafer *et al.* (2009).

Wheat adaptation to different environments

Environmental factors

Adaptation of the growing cycle to the best environmental conditions is based on the sensing of temperature and day length through the abovementioned vernalization and photoperiod sensitivities, with the additional regulation of flowering time given by temperature *per se* (Slafer and Rawson, 1994a).

Temperature affects wheat development in two markedly distinct aspects. Firstly, the rate of development is accelerated (and the time elapsed for a developmental phase is shortened) due to increased temperatures, at least in a wide range of thermal conditions. This is a general biological effect of temperature, likely through the activation of enzymatic processes. Due to the fact that in the range of sub-optimal temperatures the relationship between the rate of development and temperature is linear, progress towards flowering may be quantified in thermal time units. Secondly, wheat (as well as many other temperate crops) may accelerate its development by exposure to a period of relatively low temperatures. As the process is known as vernalization, these 'low' temperatures are referred to as vernalizing temperatures and are defined by their effect rather than as particular temperatures. There are variations in the literature on the ranges of temperatures at which vernalization is most effective. Although this variation may reflect methodological differences, it is likely reflecting genetic variation in those thermal thresholds for vernalization to

take place. The vernalizing stimulus may be perceived by seeds imbibed in the soil (immediately after sowing and before seedling emergence), by young green plants (during the vegetative stage) and even by developing grains in the spike of the mother plant, if exposed during grain-filling to temperatures sufficiently low. The most common characterization has been done for seedlings and a generalized pattern for the most effective temperatures in vernalization is represented in Figure 10.1. Thus, the maximum effectiveness has a lower threshold between 1 and 4°C and an upper threshold between 6 and 10°C (Figure 10.1). Temperatures higher than the latter, are still vernalizing, though with reduced effectiveness, up to temperatures as high as 18°C.

Day length is the most reliable environmental signal, as it changes invariably according to seasons. The actual day length for any particular site and day can be easily calculated by simply knowing the latitude of the site. In calculating the actual day length for plant responses, the length of the day includes the periods of civil twilight. This factor is markedly affected by latitude: the further North or South from the equator, the greater the range of variation of day length in the year.

Responses to environmental factors

Heading, the appearance of spikes out of the flag leaf sheaths, is the first unequivocal external sign that the plant is reproductive. Heading is also quite close to anthesis; therefore, the effects of environmental factors on time to heading are key determinants of wheat adaptability. These two reasons, together with the ease of its assessment, have made heading time the most popular variable for assessing the effect of genetic and environmental factors on wheat development.

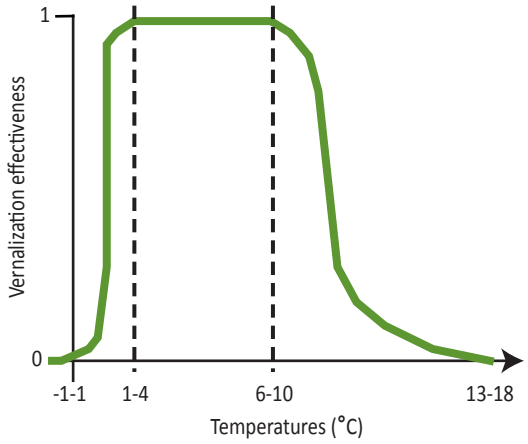

Figure 10.1. Generalized pattern of vernalization effectiveness of different temperatures. For each identified point in abscissa a range of values is provided, likely reflecting genetic variation in these parameters.

Sensitivity to temperature

Time from sowing to heading is universally affected by temperature *per se* (Angus *et al.,* 1981; Del Pozzo *et al.,* 1987; Porter *et al.,* 1987; Slafer and Savin, 1991; Slafer and Rawson, 1995a) and cultivars are sensitive to this factor at all phases of development (Slafer and Rawson, 1994a). It is widely recognized that time to heading is shortened in a curvilinear fashion, as temperature is increased (Figure 10.2A). However, the reduction in the time elapsed to reach heading is the result of the accelerated rate of development in response to increased temperatures. The relationship between this rate and temperature being almost invariably linear (Figure 10.2B; but see Slafer and Rawson, 1995a for a discussion on the topic).

Figure 10.2 is just a schematic example, but a substantial amount of published data can be used to confirm the generality of its shape (e.g., Gallagher, 1979; Angus *et al.,* 1981; Monteith, 1981; Rickman *et al.,* 1983; Morrison *et al.,* 1989; Slafer and Savin, 1991; Slafer and Rawson, 1995a). Thus, rates of progress towards heading increase linearly with temperature, from a theoretical threshold at which the rate is zero (it would take infinite time to reach heading at that temperature) to an optimum value at which the rate is maximized (and then time to heading is minimized), beyond which higher temperatures frequently reduce the rate of progress towards flowering (lengthening again the period to heading). These two thermal thresholds within which the rate of development increases linearly with temperature are the base and optimum temperatures (Figure 10.2). As the relationship within these thresholds is strongly linear, there is a single slope for the whole interval between base and optimum temperature. The reciprocal of the slope is the thermal time (degree days) needed to reach heading at the designated base temperature, and therefore, there is only one value of thermal time to heading for a particular genotype regardless of the temperature to which it is exposed, provided the plants are exposed to thermal regimes between the base and optimum temperatures and that photoperiod and vernalisation requirements are satisfied.

In practice, thermal time is simply calculated as the summation of daily effective temperatures (average minus base temperature, being the latter the abscissa intercept of the relationship; and generally

assumed to be 0°C in wheat). By means of thermal time, development events can be expressed fairly independently of fluctuations in temperature.

Sensitivity to vernalization and photoperiod
Wheat genotypes may vary widely in their sensitivity to photoperiod and vernalization. The range extends from genotypes that are virtually insensitive through to those with quantitative or qualitative responses (Figure 10.3A). Although all these responses are possible, most cultivars exhibit a quantitative type of response. The slope of the relationship (Figure 10.3B) indicates sensitivity to either vernalization or photoperiod, i.e., the magnitude of increase in rate of development (and then of reduction in time) due to a unit increase in either duration of the vernalization or photoperiod. This sensitivity is highly variable among genotypes and a likely reason for the wide adaptability of wheat to so many different climates.

Although, for the sake of clarity, the three examples in Figure 10.3A have the same optimum values of length of the vernalization exposure or photoperiod, there is genetic variation for them (see examples in Slafer and Rawson, 1994a). Similarly the example does not provide evidence for genetic variation in intrinsic earliness but, as discussed later, cultivars do differ in this trait.

In passing, it should be noted that the term 'optimum' is used in developmental studies in reference to the values of environmental factors that maximize the rate of development (optimum temperature, optimum vernalization and optimum photoperiod), which does not at all mean that these conditions optimize yield (Slafer, 1996). In fact plants growing under optimum thermal and photoperiodic conditions would likely yield very poorly as they would possess an excessively short growing period.

Which phases are sensitive to each factor?
Although development is a continuous succession of changes progressing towards maturity, in order to simplify understanding of the processes involved, it is frequently defined as a sequence of discrete phenological events controlled by external factors, each event making important changes in the morphology and/or function of some organs (Landsberg, 1977). Thus, key developmental events must be identified to mark the limits of the phenophases. The most accepted markers of developmental progress from sowing to maturity seem to be seedling emergence, floral initiation, terminal spikelet initiation and anthesis (Figure 10.4). These developmental stages delimit the phases of:

(i) Pre-emergence development:
when the crop is established. This phase is vegetative in nature: after seed imbibition the shoot apex re-starts the initiation of leaf primordia (which started during grain filling in the mother plant). When soil moisture is not limiting germination (and it would be hardly so as most farmers would attempt avoiding sowing in soil that is dry at the sowing depth) the rate of development depends only on temperature *per se*. Therefore, the length of the phase depends on the thermal conditions of the soil at the sowing depth, as well as the sowing depth (the deeper the sowing the longer the seedlings take to emerge at a particular temperature). There

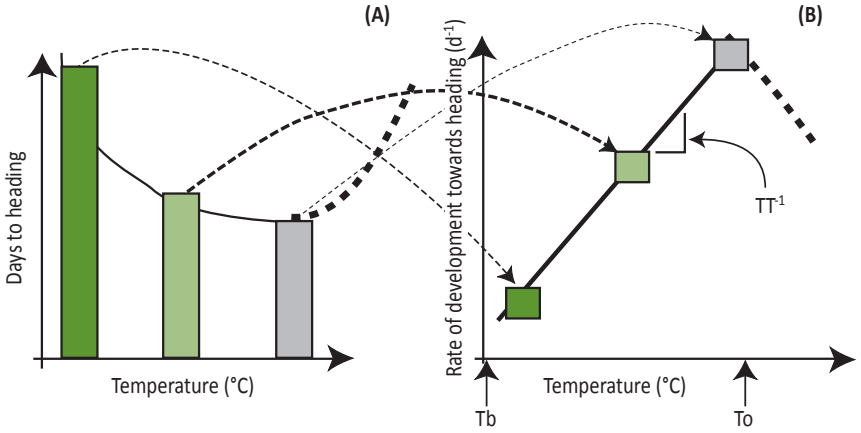

Figure 10.2. General model for the relationships between (A) calendar time to heading or (B) its reciprocal, the rate of development towards heading, and temperature. Figure 10.2B exhibits a strong linearity of the relationship between base (Tb) and optimum (To) temperatures, as implicit in the concept of thermal time. The slope ([°C d]$^{-1}$) represents the reciprocal of the thermal time (TT^{-1}) required for heading using the estimated Tb for any temperature between Tb and To. Heavy lines represent the most common trends for the sub-optimum (solid line) and supra-optimum (dashed line) temperatures.

is no evidence that vernalization affects the rate of development to seedling emergence and as day length is perceived by the leaves (and a signal transmitted to the apex; Evans, 1987), this factor does not affect the length of this initial phase.

(ii) Post-emergence vegetative development: when all remaining leaves (and potential tillers) on the main shoot are initiated through until the apex becomes reproductive (marking the end of the phase) and starts initiating spikelet primordia. During this phase leaves start to appear at a regular thermal interval (known as phyllochron) and tillering begins: the appearance of the first tiller coincides with the appearance of the fourth leaf, and the subsequent primary tillers appear at regular intervals of one phyllochron (e.g., Masle, 1985). This theoretical relationship between leaf and tiller appearance frequently holds for this phase (as plants are quite small, availability of resources normally matches the relatively low demands of the plant).

The rate of development in this phase is sensitive to all three major environmental factors. Although not strictly physiologically true (see examples in Slafer and Rawson, 1994a), in practice it may be assumed that vernalization requirements must be satisfied before a cultivar becomes responsive to photoperiod. While temperature similarly affects the rate of development towards floral initiation and the rate of leaf initiation, final number of leaves is hardly affected by temperature *per se* (e.g., Slafer and Rawson 1994b). On the other hand, vernalization and photoperiod affect the rate of development much more markedly than the rate of leaf initiation. Therefore, the longer the phase (due to lack of satisfaction of vernalization or photoperiod requirements) the greater the final number of leaves (e.g., Halloran, 1977; Kirby, 1992; Rawson, 1993; Rawson and Richards, 1993; Evans and Blundell, 1994; Slafer and Rawson, 1995b, 1995c).

(iii) Early reproductive development: when all the spikelets and many florets are initiated. During this phase leaves continue to appear and in most cases tillering will reach its maximum rate. However, this rate is not theoretically expected from the relationship with leaf appearance, because it is during this phase that the intra- and/or inter-plant competition for resources becomes strong, reducing the availability of assimilates for growth of all potential tillers. Depending on the length of the phase, and on agronomic conditions, the maximum number of tillers may be reached by the end of this phase.

The rate of development in this phase is also sensitive to all three major environmental factors. Although it is frequently assumed that vernalization affects only the length of the vegetative period (Halse and Weir, 1970; Flood and Halloran, 1986; Roberts *et al.,* 1988;

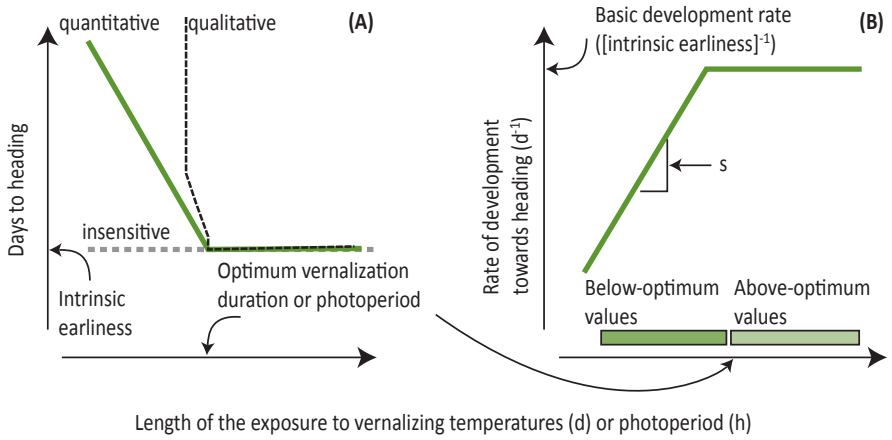

Figure 10.3. Schematic representation of the responses of wheat development to either the length of the period under vernalizing temperatures or the photoperiod. Figure 10.3A shows the possible responses of days to heading ranging from insensitive (dashed line) through quantitative (solid line) to qualitative (dotted line) type of response. Figure 10.3B shows the changes in rate of development for a quantitative response highlighting the parameters corresponding to the fastest rate or "basic development rate" (that determines the minimum duration or intrinsic earliness) under above optimum photoperiod and vernalization conditions. The green and brown bars indicate the values of below- and above-optimum photoperiod and vernalization treatments, respectively, while the slope of the relationship under below-optimum values represents the sensitivity (S) to these factors (Adapted from Slafer, 1996).

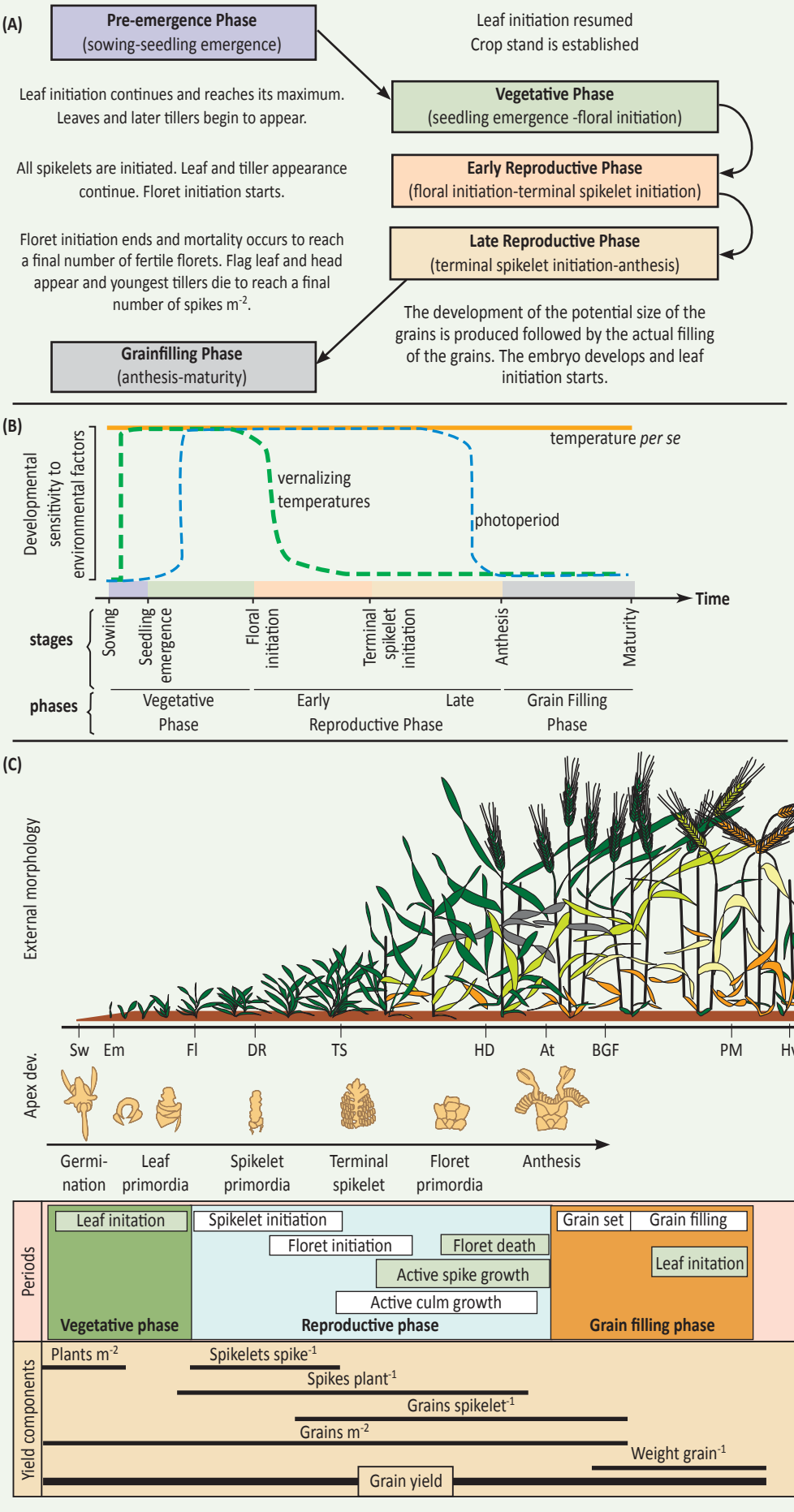

Figure 10.4. Flow diagram representing development progress in wheat from sowing to maturity in different phenophases, including: (A) some of the major events occurring at each phase, and (B) the relative sensitivity to vernalizing temperatures (dashed line), photoperiod (dotted line) and temperature *per se* (solid line) of different phenophases in a sensitive cultivar. The time scale is arbitrary.

Figure 10.4C. Schematic diagram of wheat growth and development adapted from Slafer and Rawson (1994a), showing the stages of sowing (Sw), emergence (Em), floral initiation (FI), first double ridge appearance (DR), terminal spikelet initiation (TS), heading (HD), anthesis (At), beginning of the grain-filling period (BGF), physiological maturity (PM), and harvest (Hv). Apex development is shown under each phenological stage. The periods of initiation or growth of specific organs and those of when different components of grain yield are produced are represented in the bottom boxes.

Ritchie, 1991), several authors (e.g., Halloran and Pennel, 1982; Fischer, 1984; Stapper, 1984; Masle *et al.,* 1989; Manupeerapan *et al.,* 1992; and more examples in Slafer and Rawson, 1994a) have recognized that vernalization could also affect the duration of the early reproductive development, though the impact is commonly lower than on the vegetative phase. Sensitivity to photoperiod and temperature *per se* are widely acknowledged in this phase (see many examples in Slafer and Rawson 1994a). In line with the discussion for the final number of leaves (see above), when the rate of development of this phase is accelerated by photoperiod and vernalization, the shorter period results in less spikelets being initiated, which is not necessarily true when the length of the phase is affected by temperature *per se*, as it also substantially affects the rate of spikelet initiation (Slafer and Rawson, 1994b).

(iv) Late reproductive development:
when the number of fertile florets is determined simultaneously along with the active growth of stems and spikes. During this phase leaves continue to appear until the last initiated leaf appears (known as the flag leaf). Stems first, and spikes later, grow actively during this phase dramatically increasing the demand for assimilates, which increases the competition for resources. It is during this phase that growth becomes most sensitive to changes in availability of resources and yield is reduced if the crop is exposed to stress. Therefore, the period from terminal spikelet initiation to anthesis is considered to be critical in determining yield potential. Due to the increased competition, the availability of resources becomes insufficient to maintain all the young tillers that have appeared. Consequently, some of these tillers die, normally in the reverse order in which they have appeared, and the number of tillers per m^2 is reduced from its maximum to the final number of spike-bearing tillers per m^2, normally determined at the time of heading. At the end of the early reproductive phase, when the terminal spikelet is initiated, the final number of spikelets per spike is determined and several floret primordia have been already initiated, particularly in the spikelets initiated in the middle third of the spike. From then on, initiation of florets continues to increase rapidly, until approximately booting (Kirby, 1988; González *et al.,* 2003b; 2005b) when they reach a maximum value, more or less coinciding with the full expansion of the flag leaf, and no further florets are initiated (Kirby, 1988; Miralles *et al.,* 1998). Afterwards, in the period from booting to heading/anthesis, many florets degenerate together with the active growth of the stems and spikes and only a few of the floret primordia become fertile and are fertilized at anthesis.

Most wheats requiring vernalization have by this time satisfied their requirements. Thus, even if the sensitivity to vernalization of this phase may be proven experimentally (Masle *et al.,* 1989; Slafer and Rawson 1994a; González *et al.,* 2002), it may be assumed that under reasonable agronomic conditions (e.g., avoiding sowing a wheat cultivar with strong winter habit in spring) the length of this phase is insensitive to vernalization. However, photoperiod may keep influencing the length of the late reproductive phase (Allison and Daynard, 1976; Rahman and Wilson, 1977; Masle *et al.,* 1989; Connor *et al.,* 1992; Manupeerapan *et al*., 1992; Slafer and Rawson, 1996; 1997; Slafer *et al.,* 2001), an effect that may be direct (Miralles *et al.,* 2000; Gonzalez *et al.,* 2003a; 2005a), rather than simply mediated by the increased final number of leaves due to exposure to short photoperiods during the vegetative phase (as described earlier). As discussed above, temperature *per se* affects the rate of development of all phases and the fact that time from terminal spikelet initiation to anthesis is reduced by increasing temperatures has been well documented (see examples in Slafer and Rawson, 1994a).

(v) Post-anthesis development:
or grain-filling phase, when the grains firstly develop their potential size and then grow to reach their maximum dry weight by maturity. The initial stage of this period is characterized by the development of most endosperm cells, and the generation of grain volume (maximum grain water content is achieved). During this latter stage, grains actively grow, gaining weight linearly with thermal time, until at the end of the phase, grain growth increasingly declines until grains reach their maximum dry weight. During this phase the embryo is formed and the shoot apex initiates the first (normally four) leaf primordia. Although the most common characterisation of post-flowering development divides the development into particular grain stages ("aqueous", "milky", "dough" and "hard" grain), a more quantitative characterisation may be achieved based in the water proportion of growing grains, as there is a steady reduction in grain water content from flowering to maturity and the water content at maturity is fairly constant across genotypes and conditions (e.g., Schnyder and Baum, 1992; Calderini *et al.,* 2000).

The rate of post-anthesis development towards maturity in wheat is insensitive to photoperiod and vernalization and only appears to respond positively to temperature *per se*. Thus, the length of the grain filling phase is quite conservative in terms of thermal time, unless a severe water stress occurs, that virtually ends grain-filling, regardless of how many degree days have elapsed since anthesis. As grain growth is most frequently limited by sink size (Slafer and Savin, 1994; Borrás *et al.,* 2004 and many references therein), accelerated development due to higher temperatures normally reduces the final weight of the grains (Sofield *et al.,* 1977; Chowdhury and Wardlaw 1978; Slafer and Miralles, 1992) much more than the total protein content (as nitrogen is mostly source limited). Thus, higher temperatures during grain-filling normally reduces yield and increases protein percentage.

Genetic factors controlling developmental responses

Although rate of development may respond markedly to the three major factors of vernalizing temperatures, day length and temperature *per se*, it appears that photoperiod and vernalization sensitivities account for most of the genetic variation. In other words, most of the differences between cultivars in time to heading (or in the length of any particular phase from seedling emergence to anthesis) would be ascribed to their differences in sensitivity to photoperiod and/or vernalization.

As temperature *per se* has a universal impact on the rates of wheat development (i.e., it affects the length of every phase in all cultivars), it has been assumed that there would not be genetic differences in sensitivity. However, there are frequently 'residual' differences among genotypes after their vernalization and photoperiod requirements are satisfied. Although these differences are commonly less than those derived from sensitivities to photoperiod and vernalization, they are still significant (both statistically and agronomically). These differences have been considered to reflect the impact of a third group of genetic factors determining differences in 'basic development rate' or 'intrinsic earliness', also termed earliness *per se* (e.g., Major, 1980; Flood and Halloran, 1984; Masle *et al.,* 1989; Worland *et al.,* 1994). Although some reasons have been put forward to consider these intrinsic earliness genes as responsive to temperature *per se*, and then differences in intrinsic earliness would be better defined as differences in sensitivity to temperature (e.g., Slafer, 1996; Appendino and Slafer, 2003), just for simplicity we will maintain the original term of intrinsic earliness in this chapter.

Genetic control of rate of development in wheat is sufficiently complex to guarantee that almost any pattern of development is possible when considering the duration of the period between seedling emergence and anthesis (Slafer and Rawson, 1994a), and that almost any length of time to heading can be obtained through genetic improvement. The three groups of genes (photoperiod-sensitive, vernalization-sensitive, and earliness *per se* genes; Worland, 1996) act coordinately to determine a particular time to anthesis for a specific environment.

Wheat is hexaploid and thus has three sets of genetic material ('A', 'B' and 'D' genomes), with seven homologous groups. While photoperiod-sensitive and vernalization-sensitive genes are major genes located in particular homologous groups, earliness *per se* genes are mainly minor genes and seem to be distributed among different homologous groups. Evidence for the location of these genes are:

(i) ***Photoperiod sensitivity genes*** are located on the short arms of the homologous group 2 chromosomes, the dominant alleles confer insensitivity while the recessive forms make a genotype sensitive (Welsh *et al.,* 1973; Scarth and Law, 1983; Sharp and Soltes-Rak, 1988). The major photoperiod sensitivity genes are termed *Ppd-D1* formerly *Ppd1* (chromosome 2D), *Ppd-B1* formerly *Ppd2* (chromosome 2B), and *Ppd-A1*, formerly *Ppd3* (chromosome 2A). *Ppd-D1* genes are believed to have the strongest effects (Worland and Law, 1985; Worland, 1999). Chromosomes of other groups (1, 3, 4, 6) may also be involved in the determination of photoperiod response (Law, 1987).

(ii) ***Vernalization sensitivity genes*** are located on the long arms of the homologous group 5. Similar to photoperiod-sensitive genes, sensitivity is provided by the recessive alleles and insensitivity is given by the presence of the dominant forms. They are located on chromosomes 5A (*Vrn-A1*, formerly *Vrn1*); 5B (*Vrn-B1*, formerly *Vrn2*) and 5D (*Vrn-D1*, formerly *Vrn3*) (Law *et al.,* 1975; Maystrenko 1980; Hoogendoorn 1985; Flood and Halloran, 1986; Snape *et al.,* 2001). Previously, the *Vrn2/vrn2* gene has also been termed *Vrn4/vrn4* (Snape, 1996). Furthermore,

a *Vrn5/vrn5* gene has also been reported to be located on chromosome 7B (Snape, 1996). Wheat with strong winter habits are reported to present the three recessive alleles, whereas spring wheats may present different combinations of recessive and dominant alleles (Pugsley, 1972) implying that some spring wheat may respond to vernalization (Slafer and Rawson 1994a).

(iii) ***Earliness per se genes*** have received much less attention than those for responses to photoperiod and vernalization. There is, however, evidence indicating that they are located on several chromosomes, including the long arms of homologous group 2 (Scarth and Law, 1983; Hoogendoorn, 1985). For example, the presence of these genetic factors in wheat has been reported on chromosomes 2B (Scarth and Law, 1983), 3A, 4B, 4D, 6B and 7B (Hoogendoorn, 1985), 2A and 5B (Major and Whelan, 1985) 7B (Flood and Halloran, 1983), 6D (Law, 1987) and 3A (Miura and Worland, 1994); and in barley they are distributed throughout the genome (Laurie *et al.,* 1995). A few of these earliness *per se* genes have been mapped as QTLs (Scarth and Law, 1984; Miura and Worland, 1994; Worland, 1996; Kato *et al.,* 2002; Toth *et al.,* 2003).

Identification of some key phenological phases

In order to visualize phenological changes occurring during plant development, accurate identification of the different stages is necessary (see the accompanying volume, Chapter 14). Although external morphological observations can give a general idea of phenology, microscopic observation of apex morphology is much more accurate in determining the stages of development.

In the field, wheat plot development can be monitored periodically, with plant samples being taken at random. The few stages described above as delimiters of phenological phases are mostly seen externally, with the exception of floral initiation and terminal spikelet initiation. The former cannot be unequivocally determined by simple observation (i.e., it is not marked by any clear morphological change in the apex) and is many times replaced by the observation of the first double ridge, which is the first sign that the plant is undoubtedly floral, and occurs slightly later than floral initiation (see Slafer and Rawson 1994a). The determination of both double ridge and terminal spikelet initiation requires dissection of the apex and use of microscopy (see Figure 10.5 for an illustration of the apex at different reproductive stages up to terminal spikelet initiation). A plant is taken and leaves are extracted by means of a sharp cutting instrument, then the apex can be observed under the latest formed leaves. The important events that mark the onset of phenological stages can be seen without major difficulty, after some practice has been gained. When apex dissection cannot be practiced, external morphological observation of important events can sometimes be useful. For example, the beginning of stem elongation (frequently coinciding with terminal spikelet initiation) can be determined by the "perception" of the first detectable node.

Improving adaptation

An important objective of crop adaptation is to match crop cycle, and critically the timing of heading, with the best possible environmental conditions. Selecting for improved adaptation appears to be relatively simple as it may be accomplished by including time to heading in the set of traits considered for selecting the progeny. If the program is local, aiming at a region where the breeding program is conducted, it is even simpler than when the program attempts to release cultivars to be grown in large areas (Figure 10.6). In the former case, the mechanisms controlling the rate of development towards heading may be disregarded, as the only feature of importance is to obtain cultivars that reach heading in a particular time, and that once released are not intended to be distributed beyond an area with similar

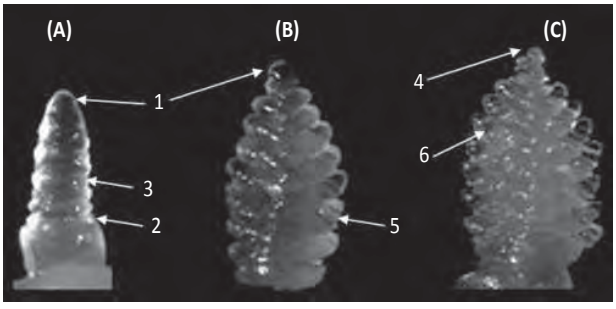

Figure 10.5. Stages of apex development. Panels (A), (B), and (C) show different reproductive stages. Panel (A) shows the apex somewhat after floral initiation, when spikelet initiation starts, when the firsts double ridges can be microscopically seen, (B) intermediate stages, and (C) floret initiation within central spikelets while the terminal spikelet is formed. Numbers within the panels stand for: shoot apex (1); leaf primordium (2); double ridge (3); terminal spikelet (4); spikelet primordium (5); and floret primordium (6). Photographs from Ariel Ferrante, University of Lleida.

characteristics to those of the breeding program (not only environmentally but also agronomically influencing time to heading, such as sowing time). Indeed, there would also be little interest in choosing the parents regarding specific sensitivities. If a second generation per year is obtained by growing the plots in an inappropriate season or a completely different environment, we would recommend the minimum selection pressure for time to heading that it would be possible for the program: as the selection environment would be too different from the one that is targeted and variation in time to heading in this condition may have no relationship with that in the proper growing season. Just as a simple example, consider a case when a vernalization-sensitive line provides an optimum timing of heading when sown in the normal growing season: if the second generation is conducted in warmer conditions than those found in the normal growing season, this line may appear unsuitably long, and may be insensibly discarded.

When the program aims to release cultivars for a large growing area, selection for adaptation must consider that the lines should be adapted to most of the environments they will be exposed to. Empirically, it may be done by running the program simultaneously in many sites representing most of the range of environmental conditions under which the released cultivars will be grown. In this case, it may be sensible, when choosing the parents, to consider not only their time to heading in particular circumstances but also their genetic sensitivities to major environmental factors governing rate of development (together with the ranges of these environmental factors in the region of interest). It may be possible that for some areas the requirements of plasticity given by vernalization- or photoperiod-sensitive genes could be predicted, and in those cases choosing the parents with the required genetic information may help in increasing the likelihood of obtaining a reasonable number of well adapted lines, that could be selected for yield or other targeted characteristics to provide the best possible cultivar.

Can developmental traits be manipulated to further increase yield potential?

Crop development has long been used as the most powerful trait to genetically improve adaptation, and through improving this adaptation, yield is improved for a particular area. However, there has not been clear evidence of whether wheat development could be manipulated to further increase yield potential in traditional wheat growing regions, where timing of anthesis has already been optimized.

There are some physiological traits that may help breeders substantially increase yield potential, breaking the barriers that have become apparent lately (Reynolds *et al.,* 2009). Regarding development, we are not fully certain whether phasic development could be manipulated to increase yield potential independent of changes in timing of anthesis, but have some hypotheses, on which we have been working, that may prove useful. A detailed explanation has been outlined earlier (Slafer *et al.,* 2001; Miralles and Slafer, 2007) and only a simplified summary is included herein.

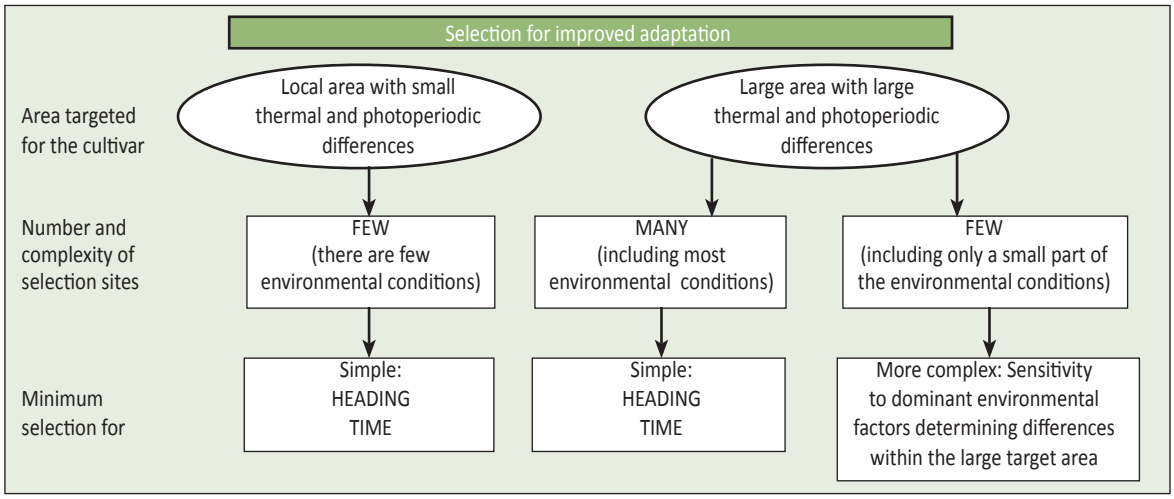

Figure 10.6. Alternatives for selection to improve adaptation depending on the diversity of the targeted area and selection sites.

Many authors have suggested that there are associations between particular developmental stages and yield components, see Figure 10.4B (e.g., Rawson, 1970, 1971; Rawson and Bagga, 1979), and associations between the duration of phases and absolute yield (e.g., Rawson, 1988a, 1988b; Craufurd and Cartwright, 1989). Therefore, it may be important to be able to manipulate the duration of these phases in order to indirectly manipulate yield components (Slafer *et al.,* 1996; Miralles and Slafer, 2007). In addition, it appears that a growing season of a determined length can be achieved with different durations of component phases (Slafer and Rawson, 1994a). Thus, it is hypothesized that manipulating development, with no major modification of the length of the whole growing period, could bring about increases in yield potential (Slafer, 2003).

There are clear indications that reductions in crop growth before the onset of stem elongation has negligible effects on the final number of grains, whilst crop growth reductions during stem elongation are directly related to the number of grains produced (Fischer, 1985; Savin and Slafer, 1991; Demotes-Mainard and Jeuffroy, 2004). In wheat, where grain-filling is mostly sink limited (Borrás *et al.,* 2004), reductions in grain number cannot be

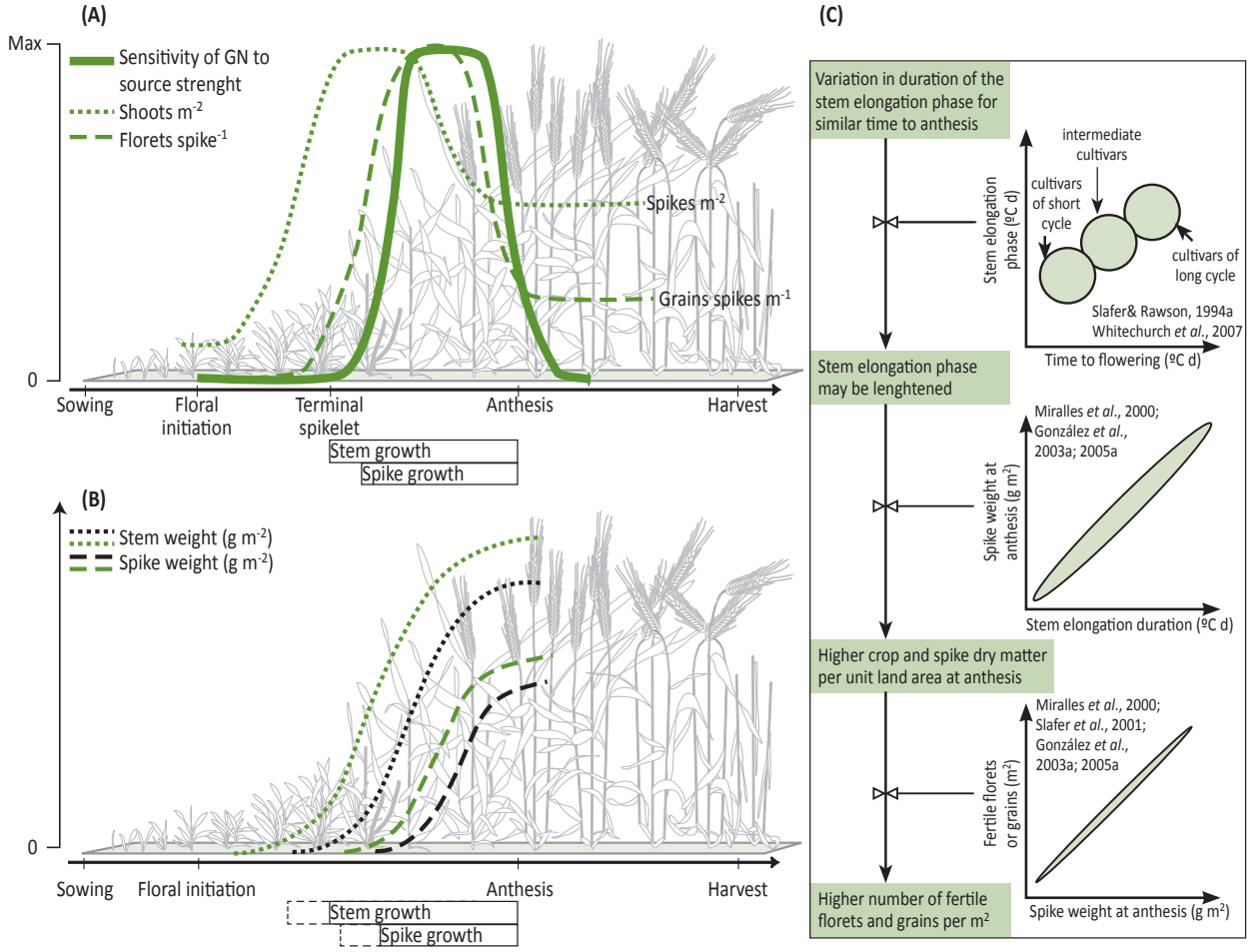

Figure 10.7. Panel (A) is a diagram of wheat development, showing the relative sensitivity of grains per m² to source-strength at different stages throughout the growing season (thick line), together with the total number of shoots per m². The curve ends with the number of spikes per m² (dotted line) and the total number of floret primordia per spike becoming fertile florets and then grains (dashed line), in both cases in relative scales from zero to their maximum values. Below the abscissa two boxes show the length of the period of stem and spike growth. Adapted from Slafer and Savin (2006). **Panel (B) illustrates the hypothesis proposed: lengthening the duration of the stem elongation phase at the expense of the duration of earlier phases such that time to anthesis is unaffected.** Adapted from Miralles and Slafer (2007). **Panel (C) shows a scheme for improvement in grain number through manipulating the proportion of developmental time allocated to the stem elongation phase, describing some reported relationships supporting the hypothesis.** Figures A and B adapted from Reynolds *et al.* (2009), Figure C adapted from Slafer *et al.* (2005).

compensated by increases in grain weight, except for small compensations that may occur if the decrease in grain number brings about increases in carpel size, concomitantly greater grain weight potential (Calderini *et al.,* 2001; Ugarte *et al.,* 2007). Therefore, it has been hypothesised (Slafer *et al.,* 2001; Miralles and Slafer, 2007) that a longer late reproductive phase would increase the amount of biomass accumulated during stem elongation, and the final number of grains would likely be increased, as the proportion of either floret abortion or tiller death (or both) would be reduced. This would result in an increased number of grains per unit land area to be filled (Figure 10.7). This is because spike growth takes place only during a few days immediately prior to anthesis (Figure 10.7A), in parallel and competing with stem growth (Kirby, 1988). Thus, if stem elongation were longer without compensations in dry matter partitioning, accumulated spike dry matter at anthesis would be greater (Figure 10.7B), resulting in an improved number of grains per m^2 (Figure 10.7C).

For this hypothesis to be realistic there must be genetic variation in the length of the late reproductive phase independent of that in duration of sowing to the onset of stem elongation; which has been shown in screenings addressed to search for such variability (Figure 10.7C; Whitechurch *et al.,* 2007); and has also been shown in barley (Kernich *et al.,* 1997), even within specific populations (Borràs *et al.,* 2009). This genetic variation may be controlled by photoperiod-sensitive and earliness *per se* genes. As the responses to photoperiod during pre- and post-terminal spikelet initiation phases appear to be independent, and there is clear evidence of substantial genetic variation in this response (e.g., Slafer and Rawson, 1996; Miralles *et al.,* 2000), it seems that attempting to lengthen the late reproductive phase at the expense of the length of either the vegetative or the early reproductive phase may be possible. If the control of the intrinsic earliness during a particular phase is independent of that for other phases (which appears to be likely; see Halloran and Pennel, 1982; Slafer, 1996) this would be another developmental possibility for changing the partitioning of a particular time to anthesis among vegetative, early reproductive and late reproductive phases.

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Chapter 11: Phenotyping in controlled environments vs. field conditions

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Abstract

The term phenotyping refers to the assessment of plant appearance and characteristics related to plant function and performance. The environment where these evaluations are conducted may condition the interpretation of the observed plant responses. For example, the control of the intensity, uniformity, and repeatability of factors affecting plant growth and development and/or experimental treatments is maximal in growth chambers and laboratory experiments, intermediate in glasshouses, and more limited in field experiments. In addition, controlled environments offer multiple possibilities for automatic and non-destructive evaluation of physiological traits. However, and despite the practical advantages of controlled environments, several limiting factors have been identified, including soil temperatures, rates of soil drying, uniformity of moisture in pots, volume and depth for root growth, and availability of nutrients. These 'artifacts' compromise extrapolation of results from controlled environments to field situations. Advantages and disadvantages of controlled environments versus field environments in relation to some physiological traits are also described in this chapter.

Introduction

The observable characteristics of an organism are defined as its phenotype. This phenotype results from the expression of genes (genotype), environmental effects, and their interaction. Phenomics is a relatively new term (Schilling *et al.,* 1999; Finkel, 2009) which in plant studies refers to the plant appearance, and its relation to plant function and performance. The application of phenomic tools allows physiologists to determine the basis for a particular trait from a cross-disciplinary approach. Phenotyping techniques, especially those performed in glasshouses or other controlled environments, are an essential part of current efforts to identify genetic loci associated with physiological traits (Earl, 2003). However, some special considerations must be made when phenotyping is conducted under controlled environments. These considerations, in addition to the 'pros and cons' of phenotyping under controlled environments vs. field conditions, are reviewed in this chapter.

Controlled environments vs. field experiments

The principal advantage of controlled environments vs. field experiments is that the intensity, uniformity, and repeatability of environmental factors affecting plant growth can be managed and more easily monitored. These include factors such as light, photoperiod, temperature, relative humidity, soil water, and nutrients. In addition, special treatments such as elevated CO_2 or extreme temperatures can be applied.

Practical advantages of working in laboratories and glasshouses are recognized when compared to field experiments. For example, pre-planned activities can be run in controlled environments as scheduled, independently of climatic conditions or other external factors. The access to lab or glasshouse facilities is generally easier and faster than reaching field stations. Highly controlled environments also permit the use of special equipment to measure specific traits and also to automatically, continuously and non-destructively record plant phenotypic traits while confounding variables can be easily controlled.

An additional advantage of contained units is the possibility of growing transgenic plants, for which open field liberations may be restricted. Out-of-season cropping can also be achieved without difficulties in glasshouses.

Tables 11.1 and 11.2 describe some of the advantages and disadvantages of studies conducted in the field and confined facilities, in relation to the control of environmental variables and the implementation of protocols for measuring physiological traits.

In spite of these benefits, field experiments are more realistic with regards to the ecosystem and the many possible interactions with environmental factors.

Differences in plant responses in confined facilities may be due to inherent differences in the responsiveness of individual plants and not representative of performance in field plots (Cox and Cochran, 1946). Therefore, the extrapolation of conclusions from controlled environments to field situations is only reliable if, as Passioura (2006) stated, 'some precautions are taken to minimize probable, often unrecognized, artifacts'.

Limitations under controlled environments

Despite the practical advantages of controlled environments, several limiting factors have been identified, including soil temperatures, rates of soil drying, uniformity of moisture in pots, volume and depth for root growth, and availability of nutrients (Townend and Dickinson, 1995). These 'artifacts' compromise extrapolations to field situations.

Plant containers: pot size, temperature, color, and growing media

Pot size has a large effect on both plant morphology and physiology. The reduction in growth associated with small containers has been reported in several studies, though the mechanisms involved are not yet well understood (Carmi, 1993; Fiscus *et al.,* 2007). Nutrient use efficiency and photosynthesis rates could also be affected by pot size. Responses to reduced soil volume may, however, vary from crop to crop and even between cultivars. One of the main reasons for the differences observed in small pots is the modification of the root environment, with negative consequences on root growth, morphology, and function (NeSmith and Duval, 1998). In addition to the volume limitation, the root system of container-grown plants often experiences extreme temperature fluctuations, low oxygen (hypoxia) and inadequate soil–water relationships (Passioura, 2006). Columnar containers (polyvinyl chloride–PVC–tubes) are preferable to pots for deep root growth studies (Figure 11.1). Root access to deep soil water and studies on soil moisture dynamics relative to root growth patterns can be more realistically addressed using these tubes which provide a soil depth that is more representative of the field conditions. Many tubes can be arranged within a glasshouse area, due to the small surface area to depth ratio of the tubes. This maximizes the glasshouse space use efficiency and allows the imposition of several treatments or replications in a given glasshouse area.

Temperatures within pots grown in glasshouses are generally higher than soil temperatures in the field and influenced by the container size and color. Roots in small containers are likely to also show significantly larger daily amplitude in temperature, principally due to direct exposure to solar radiation and the small soil mass

Table 11.1. Advantages and disadvantages of field and controlled environments (screen-houses, glasshouses and growth chambers).

	Field		Screen-house		Glasshouse		Growth chamber	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**
Temperature	Realistic	Uncontrolled	Less risk of low temp/ frost damage	High temperatures	Less risk of low temp/ frost damage	High temperatures	Controlled	--
Air relative humidity	Realistic	Uncontrolled	Realistic	Uncontrolled	--	High	--	High
Water	Realistic soil profile	Uncontrolled	Controlled Realistic soil profile	--	Controlled	Pot experiment limitations	Controlled	Pot experiment limitations
Light	Realistic	--	Realistic	--	--	Shading	--	Low intensity Artificial
Photoperiod	Realistic	Uncontrolled	Realistic Potentially controlled	--	Potentially controlled	--	Controlled	--
Soil								
Uniformity	--	Heterogeneous	Generally uniform	--	Uniform	--	Uniform	--
Properties	Realistic	Uncontrolled	Realistic	Difficult to control	Controlled	Unrealistic (pot experiments)	Controlled	Unrealistic (pot experiments)

to buffer temperature changes (Mathers *et al.,* 2007). Daily maximum mean temperature at the center of the container was not only higher but also higher earlier in the day in smaller pots compared to larger ones (Fiscus *et al.,* 2007). As a general rule, temperatures in black or dark pots tend to be higher than temperatures in white or clear pots, especially in a glasshouse exposed to sunlight, since black absorbs almost all radiation and reflects only a small proportion. In addition, non-porous plastic containers also prevent evaporative cooling from the walls (Mathers *et al.,* 2007). These factors may negatively impact both root and shoot growth, as high temperature stress can inhibit enzymatic and membrane processes, respiration rate and stomatal conductance, which would decrease plant performance. Furthermore, cold water added to warm pots could also cause an extra stress to the root system and, finally, inhibit plant growth (Passioura, 2006).

Table 11.2. Advantages and disadvantages of field versus controlled environments in relation to some physiological traits.

	Field		Controlled facilities	
Traits to study	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**
Treatments	Realistic	Less uniform	Control of the intensity, uniformity, timing and repeatability of treatments	Unrealistic
		Dependence on environmental/ seasonal factors	Out-of-season experiments are possible	
		Unpredicted interactions	Interactions between factors can be controlled	Variation in the glasshouse environment and handling of materials
			Particular variables (radiation, ozone, etc) can be manipulated and monitored	
Responses to drought	Realistic drying cycles	Co-occurrence of additional stresses (heat, low temperature)	Control of environmental factors	Unrealistic (rapid) drying cycles
	Realistic interactions with environmental factors	Less control over treatments	Control of water applied	Confounded by plant growth rate and differences in water status
	Realistic soil profile for root development	Confounding factors (toxicities, salinity)		Pot experiment limitations on root growth
Osmotic adjustment		Confounded by root depth and differences in soil water potential	Control of root depth	Unrealistic (rapid) drying/ rehydration cycles
			Equal soil water potential by growing all genotypes in the same pot	
Transpiration efficiency		Water fluxes can't be controlled	Precise control of water fluxes	
Canopy temperature	Integrative measurement, scoring the entire canopy of many plants	Measurements must be taken when the sky is clear and there is little or no wind	Control of external factors	Only single plant/small groups of plants can be screened
	Related to the capacity of the plants to extract water from deeper soil profiles			Not related to the capacity to extract water from deeper soil profiles –unless special pots are used
Root growth studies (biomass, length, growth rate, etc)	Realistic soil profile	Heterogeneity	Complete root systems are collected	Pot size, temperature, salinity, and hypoxia limiting root growth
		High sampling variance	Uniform sampling	
Adaptation to harsh soil	Realistic	Soil properties difficult to manipulate	Soil properties can be manipulated	Unrealistic
Phenotyping transgenic plants	Realistic	Risk of pollen flow	Low risk of pollen flow	Pot experiment limitations
		Strict regulations and protocols	Less/easier regulations	

Potting mixes used to fill the pots have some advantages and disadvantages linked to the objectives of the experiments. A pot substrate should be salt and pest free, inexpensive, uniform, lightweight, have high cation-exchange capacity and suitable physical and chemical properties (Mathers *et al.,* 2007). Commercial mixes are generally prepared to confer optimum aeration and drainage in small pots. Special mixtures can also be used to facilitate the recovery of clean roots. However, these conditions, although ideal for pot experiment goals, are generally different to real soil structure, making it difficult to extrapolate results to field situations. Even when using field soil in pots, results can still differ from field conditions because all soil structural properties are lost, which affects not only physical but also chemical and microbiological soil properties.

Physical properties of pot substrates (i.e., porosity, water holding capacity, percentage of fine particles and bulk density) differ from field soils, affecting root growth, function and morphology. Air pockets, especially around the walls of the pots, are not desirable, considering that roots tend to grow through areas of less mechanical resistance. Roots could then be exposed to rapid desiccation if soil mixtures shrink away from the pot walls when drying. Potting mixes usually have large pores that protect the roots from hypoxia. However, sometimes it is difficult to have adequate aeration, which negatively affects microbial activity. Cultural practices, such as the selection of an adequate container type and both irrigation intensity and frequency, need to be also planned taking into consideration the physical properties of the pot substrate.

Chemical properties of pot substrates, such as pH, cation-exchange capacity and salt content, can also be altered in containers (Mathers *et al.,* 2007). An associated aspect that has received little attention in experiments in small containers is the increase in the soil solution concentration when soil rapidly dries (Townend and Dickinson, 1995). Understanding these properties is especially important in studies of plant responses to fertilization, since the capacity of roots to acquire and use nutrients is strongly influenced by chemical variables. The soil pH, for example, affects the availability and solubility of some nutrients. Under high-pH substrates, the availability of aluminum, iron and manganese decreases (Mathers *et al.,* 2007). Micronutrient deficiencies can generally be corrected by adjusting the substrate pH. Therefore, in fertilization

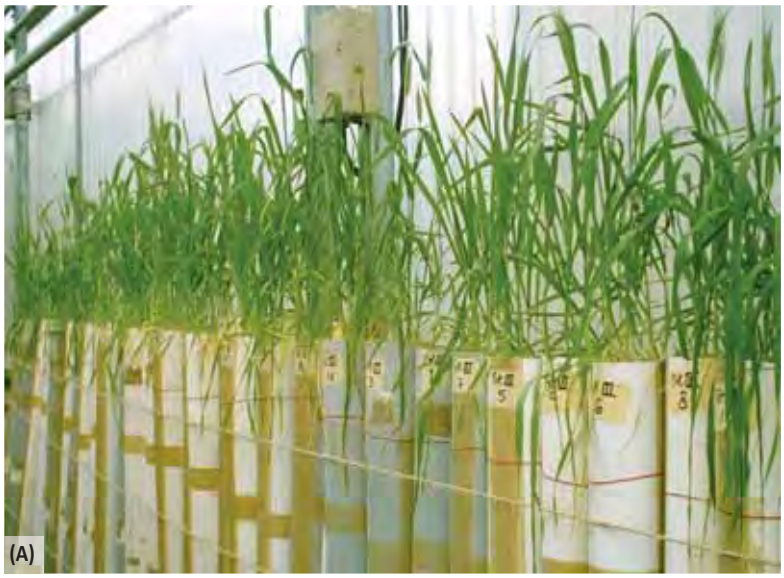

Figure 11.1. Root growth studies in long PVC tubes; showing (A) before, and (B) after root washing.

studies conducted in small pots, the selection of the fertilizer type, dose and method of application – according to the experimental goals– should be made taking into account the plant species, fertilizer cost, type of substrate, plant growth stages and irrigation practices (Mathers *et al.,* 2007). Slow-release fertilizers may be considered for decreasing nutrient runoff.

Variations in the glasshouse environment and handling of materials

One of the first steps towards increased accuracy in experiments conducted in controlled environments is to discover the principal causes of any variation observed in the glasshouse (Cox and Cochran, 1946). Usually, the major sources of variation are temperature and moisture gradients caused, for example, by proximity to ventilators and doors, shading from internal or external structures, methods of watering and air currents. Knowing the intensity and direction of these gradients helps to select the arrangement or experimental design that gives the most accuracy in accordance with the amount of time and labor required for its implementation.

One of the ways to account for environmental gradients is rotating the pots, with the intention of exposing all the pots to the different levels of the gradient. Another option is to place replications in blocks along the gradient. The main idea is to create homogeneous blocks in which the external factor is relatively constant. The pots in each block are then randomly assigned to the different treatments.

The different handling of materials and plants, preparation of experiments, application of treatments and measurement of variables, have also been identified as sources of error in glasshouse experiments (i.e., human error). When several activities common to all treatments are planned, experimental protocols should consider that a single person performs a given activity instead of several people working on the same task (Lawrence, 1955). Standardization of cultural and experimental techniques is essential for satisfactory results in experiments.

Differences in phenology between genotypes grown in pots

Plant phenology is the study of the timing, duration and abundance of recurrent biological phenomena, for example flowering. Several factors may trigger flowering, though there is no general rule: triggers often vary greatly among crops and even among cultivars. Therefore, different crops/cultivars may have different requirements in terms of photoperiod (day length), vernalization (wintering over) or a combination of both, to induce flowering. Drought and root growth restrictions may also trigger flowering, although responses, again, vary from crop to crop. Understanding these factors is especially important in experiments conducted in controlled environments.

Different durations of plant growth stages may result, for example, in differences in biomass production which could affect the interpretation of results. Comparisons between cultivars are difficult if their responses to a limiting factor or stress are tested at different phenological stages. The synchronization of growing stages in each experimental unit is particularly important when all genotypes are grown in the same pot, for example, when soil water potential must be standardized to estimate osmotic adjustment.

Methodology for applying water deficit stress in small pots

Different strategies have been used to simulate water deficit stress in experiments in controlled environments. However, some considerations should be made when plants are grown in small containers. Earl (2003) stated that the ideal approach for simulating drought in small pots should: (i) maintain uniform soil water content, (ii) control the rate at which stress develops, (iii) allow the imposition of different stress levels, and (iv) provide ways for quantifying the level of stress. Blum (2009) described the limitations of some of the methodologies commonly used in contained environments:

Stop irrigation and allow soil to dry

Although a simple methodology, the disadvantage is that plants in small pots are subjected to a rapid imposition of water stress, compared to field conditions. Responses to water stress could then be different in pots than in the field, since the rate at which stress progresses can trigger different patterns of gene expression (Talamè *et al.,* 2007). Long-term responses, rather than immediate ones, are more related to adaptation to real drought under field conditions. This constraint could be mitigated by using an adequate pot–plant size relationship, given large pots dry slower than small pots.

'Fixed' levels of drought stress

This method assumes a constant range of soil water potential, by weighing the pots and adding the required water. High soil water heterogeneity –i.e., higher water content at the top and lower in the bottom– may be observed because of the high frequency of small watering events. This may affect the normal root growth pattern. Additionally, plants are exposed to repeated stress–recovery cycles, which are not common under field conditions where stress is gradual.

Columnar containers

Such as PVC tubes, are preferable to pots when testing deep root growth and the ability of roots to access water in the soil profile. Reproducible levels of stress can be applied at specific developmental stages when plants are grown in these tubes (Salekdeh *et al.,* 2009). Water can be supplied from either the top or the bottom. The tubes can be set in containers with water which allows changes in the depth of the water table according to different treatments or during the growing season.

Effects of plant size in experiments evaluating water stress in small pots

The difference in leaf area –related to plant size and water demand– is an important factor when comparing plant responses to stress in pot experiments where water is the limiting factor. Larger plants use more water than smaller ones, and therefore, a large plant may show symptoms of stress before a smaller one when grown at limited water content (Ray and Sinclair, 1998; Blum, 2009) (Figure 11.2). Ray and Sinclair (1998) conducted an experiment where maize and soybean were grown in pots of different sizes. For each pot size, plants were divided into well-watered and water-deficit treatments. They reported a significant reduction of biomass and total transpiration with decreasing pot size, both for maize and soybean under both water treatments. The smaller plant size corresponded with lower cumulative transpiration among well watered plants grown in pots of differing sizes (Figure 11.2A). As expected, smaller pots had a smaller amount of total transpirable soil water (Figure 11.2B). Consequently,

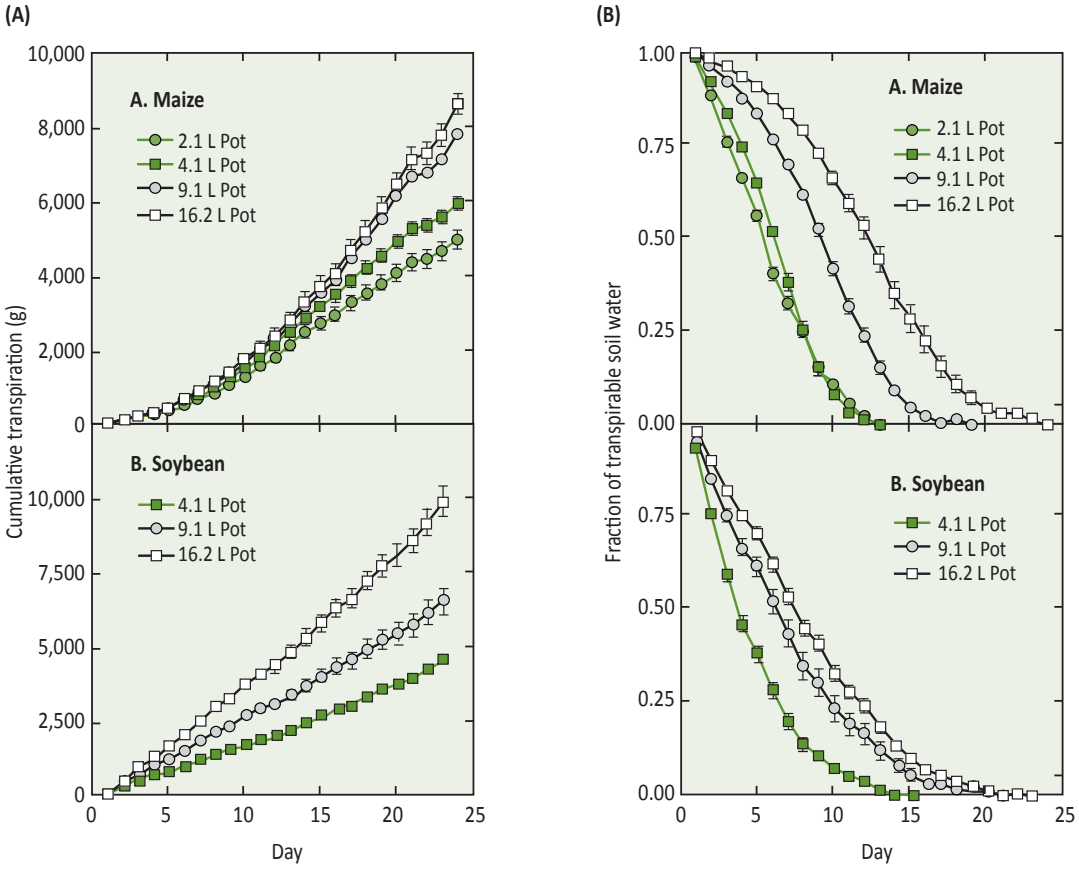

Figure 11.2. (A) Cumulative transpiration throughout time in well-watered maize and soybean experiments using different pot sizes. (B) Fraction of transpirable soil water throughout time, for maize and soybean plants under water deficit using different pot sizes. (Ray and Sinclair, 1998).

the smaller pots dried much more quickly than the bigger pots, and therefore, plants in small containers received a more rapid imposition of water-deficit stress. No significant effect of pot size on the fraction of transpirable soil water threshold at which transpiration begins to decline was detected for either maize or soybean.

One possible way to account for differences in soil water content is to grow all of the plants in the same pot, exposing all genotypes to the same soil water status. However, large plants may still express a lower leaf water status than small plants because of their greater demand for water (Blum, 2009). Planting the genotypes on different dates to obtain plants of comparable size has also been proposed as a strategy. Results, however, may be confounded by phenology.

Conclusions

Phenomics techniques play an important role in unlocking the information coded in plant genomes (Finkel, 2009). Controlled environments offer multiple possibilities for automatic and non-destructive evaluation of physiological traits. However, the characteristics of the environment where the evaluations are conducted need to be considered for both the interpretation of responses to abiotic stresses and the extrapolations of responses from controlled environment to field conditions (Passioura, 2006).

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Chapter 12: Field experimental designs in agriculture

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Abstract

Experiments are conducted to obtain information on the population of interest and this information can be used to draw inferences about parameters of interest, to make decisions about hypotheses and to plan future research. In general, experiments which have been improperly designed usually result in information that is irrelevant to the researcher. In agriculture the main objective of experimental design is to estimate the average response of a variety or an agronomic treatment, or the average differences between varieties (or treatments) as precisely as possible even when this response varies from environment to environment or from year to year. Any experimental program that has the objective to assess the value of a variety or an agronomic practice should be designed in such a way that provides an accurate and unbiased estimate of the average response of these varieties in each environment (site-year combination) and in a combination of circumstances in which that variety will be grown. In this chapter we provide a short definition of terms used in most agricultural experiments and some brief explanations and description of some common experimental designs used in agriculture.

Introduction

Experimental design is the study of strategies for efficient plans for the collection of data, which lead to proper estimates of parameters relevant to the researcher's objective. A properly designed experiment, for a particular research objective, is the basis of all successful experiments.

Terminology of experimental design

1. An ***experiment*** is a planned inquiry to obtain new knowledge or to confirm or deny previous results.
2. The ***population of inference*** is the set of ALL entities to which the researcher intends to have the results of the experiment be applicable.
3. The ***experimental unit*** is the smallest entity to which the treatment is applied and which is capable of being assigned a different treatment independently of other experimental units if the randomization was repeated. This is the most seriously misunderstood and incorrectly applied aspect of experimental design.
4. A ***factor*** is a procedure or condition whose effect is to be measured.
5. A ***level*** of a factor is a specific manifestation of the factor to be included in the experiment.
6. Experimental ***error*** is a measure of the variation which exists among experimental units treated alike.
7. A ***factor level*** is said to be replicated if it occurs in more than one experimental unit in the experiment.
8. ***Randomization*** in the application of factor levels to experimental units occurs only if each experimental unit has an equal and independent chance of receiving any factor level and if each experimental unit is subsequently handled independently.

Randomization

Randomization involves randomly allocating the experimental units across the **treatment groups**. For example, if the experiment compares new maize varieties against a check variety, which is used as a **control,** then new varieties are allocated to the plots, or experimental units, randomly. In the **design of experiments,** varieties are allocated to the experimental units, not experimental units to the varieties. In experimental design we consider the treatments, or varieties, as data values or levels of a factor (or combination of factors) that are in our controlled study. Randomization is the process by which experimental units (the basic objects upon which the study or experiment is carried out) are allocated to treatments; that is, by a random process and not by any subjective and hence possibly biased approach. The treatments or varieties should be allocated to units in such a way that each treatment is equally likely to be applied to each unit. Because it is generally extremely difficult to eliminate bias using only expert judgment, the use of **randomization** in experiments is common practice. In a randomized experimental design, objects or individuals are randomly assigned (by chance) to an experimental group. Use of randomization is the most reliable method of creating homogeneous treatment groups, without involving any potential biases or

judgments. There are several variations of randomized experimental designs, some of which are briefly discussed below.

Replication

Although randomization helps to ensure that treatment groups are as similar as possible, the results of a single experiment, applied to a small number of objects or subjects, should not be accepted without question. To improve the significance of an experimental result, ***replication***, the repetition of an experiment on a large group of treatments, is required. Replication reduces variability in experimental results, increasing their significance and the confidence level with which a researcher can draw conclusions about an experimental factor.

Control of local variability - by blocking

In the design of experiments, blocking is the arrangement of experimental units into groups (blocks) that are similar to one another. For example, if an experiment is designed to test 36 new maize varieties, the field in which the varieties are to be tested may not be uniform, having two fertility gradients – low and high soil fertility. The level of fertility may introduce a level of variability within the experiment; therefore, blocking is required to remove the effect of low and high fertility (Table 12.1). This reduces sources of variability and thus leads to greater precision. For randomized block designs, one factor or variable is of primary interest. However, there are also several other nuisance factors. Blocking is used to reduce or eliminate the contribution of nuisance factors to experimental error. The basic concept is to create homogeneous blocks in which the nuisance factors are held constant while the factor of interest is allowed to vary. Within blocks, it is possible to assess the effect of different levels of the factor of interest without having to worry about variations due to changes of the block factors, which are accounted for in the analysis.

Table 12.1. Differences between replicates and blocks

	Replicates	Blocks
Definition	More than one unit of the same treatment.	Group of units thought to be homogeneous.
Reasons	Estimate precision.	Increasing precision without increasing size of trial. Increase precision by increasing size of trial.
Number	May not be the same for all treatments.	Number of homogeneous groups.
Size		Size of homogeneous groups (may be unequal).

Important things to consider about experimental units

1. How to randomize treatments to experimental units.
2. What is the shape and the size of the experimental units?
3. Are the experimental units independent and are they homogeneous?
4. How to deal with experimental units if they are heterogeneous.

Functions of replication

1. To provide evidence of the repeatability of the results of the experiment.
2. To provide an estimate of experimental error.
3. To improve precision of the experiment by reducing the standard error of estimates of parameters involved in the experiment.
4. To facilitate extension of the results to a wider range of conditions.
5. To permit control of error variance.

Functions of randomization

1. To ensure applicability of results to the entire population of inference.
2. To reduce the chance of systematic (and often subconscious) bias affecting the accuracy of the estimates of the parameters of interest.
3. To validate the use of probability theory as a tool of inference based on experiment (i.e., sample) data.

The design process

The person conducting the experiment should go through a series of steps before an experiment is initiated. These steps are not meant as a “cookbook” but rather as a reminder that these aspects of the experiment should be accounted for before it is conducted.

1. The person conducting the experiment should clearly understand what facts or results he or she is attempting to establish. These objectives should be stated in terms of population parameters which can be estimated by response variables. Care should be taken to ensure that the response variable can be reliably measured.

2. The experimenter should clearly understand what treatment factors will be involved and what levels of these treatments will be used. They should also understand that there is a direct relationship between the quality of the experiment and the number of levels: the fewer the levels, the better the experiment.
3. The experimenter should clearly understand what experimental units will receive the various levels of the various treatment factors.
4. The experimenter should precisely define the intended population of inference. Care should be taken to make sure that the experimental units used are a representative cross-section of the population.
5. The experimenter should decide how many experimental units will be assigned to each factor level. Remember, to do this, one MUST know the following:
   (a) What is the magnitude of precision required? That is, how wide a confidence interval is permissible or how big a difference between levels is biologically meaningful?
   (b) What is the variance among experimental units? One must be familiar with existing literature in the area; good journals normally require publication of estimates of experimental error. If you are not so lucky, you will have to do a "pilot study" to estimate the variance.
   (c) What coefficient of confidence is considered tolerable? Journals tend to be arbitrary about this: it has not been a subject of a great deal of careful thought. You will find 90%, 95% and 99% to be standard figures, depending on the perceived seriousness of failing to include the parameter of interest. One of the more tenacious misconceptions about statisticians is that they can somehow decide how many experimental units the researcher needs without the above information.
   (d) A scheme to randomly assign experimental units to the various factor levels should be devised. Otherwise, systematic biases in the observations may occur or observations may not be truly independent, in either case destroying the validity of inference based on the data collected.
6. The experimenter should examine the experimental situation for any sources of potential variation in his or her data other than those identified as treatment factors or experimental error. If such sources of variation do in fact exist, experimental units can be grouped together in blocks (or pairs if only two treatment levels are being considered) so that the source of variation occurs between blocks rather than among blocks (i.e., blocking effects will not be confounded with treatment effects).
7. The person conducting the experiment should plan ahead so far as having an understanding of how the data are to be analyzed once collected. What parameters are to be estimated? How will they be estimated? What hypotheses will be tested? What procedures will be utilized to test these hypotheses? A useful practice here is to write out a "skeleton analysis", that is, an outline of the calculations to be performed and how they are to be interpreted. The importance of this step cannot be overstated. One of the most common errors in research is to conduct an experiment which cannot be analyzed.

Completely randomised designs (adapted from Lentner and Bishop, 1993)

A total of *n* experimental units (EU) are available for use in the experiment. These EU are as homogeneous as possible; that is no source of variation can be recognized among them under any grouping or arrangement. There is no basis for grouping the EU, as there is in some of the more complex designs studied in later sections. This does not rule out the existence of variation among the EU, we simply have no relevant information about it.

The experimental plan

Each treatment is randomly assigned to several EU in an unrestricted manner. Suppose the *i*-th treatment appears r_i times in the experiment; we usually describe this as r_i replications of the *i*-th treatment. The analysis of a completely randomised (CR) design is accomplished in a straightforward manner with equal or unequal replications of treatment. But from the standpoint of efficiency, all treatments should occur with the same frequency; that is, all $r_i = r$. To conduct a basic CR design, each treatment is assigned randomly to a number of EU. Random numbers are useful in making these assignments.

Design considerations – advantages and disadvantages of the CR design

One of the requirements for using a CR design is that the EU must be **homogeneous.** In what type of experimental situations does such a requirement hold? In some experiments, there may be so many design factors involved that non homogeneity would not be anticipated and becomes an issue that cannot be resolved adequately, the researcher must consider experimental designs other than the CR. In most of the field experiments conducted by agronomists and plant breeders, non homogeneity of EU is guaranteed due to soil variability. Thus, all the unrecognized variation and all extraneous variation are included in experimental errors (Exp. Error).

Randomised complete block designs (adapted from Lentner and Bishop, 1993)

In the CR designs, we assume that EU are homogeneous with respect to their potential effect on our response variable. For example, if plots of land are used as EU in a yield study of different wheat varieties, a CR design is appropriate only if all plots have the same degree of fertility. In reality this will never happen, thus the CR designs should never be used in field trials. Homogeneous effects of EU in the CR designs were assumed because we had no information of any consequence about the EU which could have been incorporated in the experimental plan. But what if we were told that a fertility gradient is present in the land that we wish to use for our wheat experiment? In this case we expect certain plots of land (the EU) to have a different impact on yield and growth responses. Adjacent plots of land should have about the same influence on these responses, whereas plots some distance apart likely have a different influence. Certain information about the EU can be used to identify groups of EU which will provide homogeneous influences on our response variable.

A block is a group of EU which provide homogeneous effects on a response variable. A complete block is a homogeneous group of EU upon which the *t* treatment appear equally often (usually only once). The notion of blocking refers to specific groupings (arrangements) of the EU in which subsets of homogeneous units are identified. Quite often the EU are naturally grouped by some criteria (age, location, initial height, and so on). At other times the grouping is done by the researcher on the basis of available information.

While most blocks contain only *t* EU, there is no reason why a block cannot contain 2*t* EU (in which case each treatment would appear twice) or 3*t* EU, and so on. Unless specified otherwise, we shall assume that blocks contain only *t* EU and will refer to these as basis randomised complete block (RCB) designs.

In a number of instances, homogeneous blocks of *t* EU cannot be obtained. When a block contains less than *t* EU, and therefore cannot contain all treatments, we have an incomplete block. Designs having incomplete blocks are covered later.

An RCB design is appropriate if we can arrange the EU into homogeneous blocks according to their effect on our response variable. When properly constructed, each block should consist of homogeneous EU, or at least EU that are as homogeneous as possible. In practice, perfect homogeneity is rarely, if ever, attained. Furthermore, blocks should be constructed so that EU of two different blocks are as heterogeneous as possible. As we achieve these two criteria to a greater degree, we increase the precision of the experiment. Blocking removes an identified source of variability from the experimental error variation of a CR design. Thus, we say that 'blocking is a form of error control'.

At this point, one might ask, "Why not always use an RCB design, even if we must randomly form blocks?" If we use *r* blocks, we will lose *r*–1 degrees of freedom from experimental error. Because of this, the EU need to be arranged in blocks so that the heterogeneity will cause a reduction in the experimental error sum of squares (Exp. Error *SS*) large enough to compensate for the loss in degrees of freedom. So if we block when all EU are homogeneous, we would inflate the experimental error and obtain less precise results than from the corresponding CR design. This is due to the reduction in degrees of freedom without a comparable reduction in the Exp. Error *SS*. Sometimes a researcher suspects heterogeneity among the EU but does not know specific sizes and locations of differences. The most effective blocking requires this knowledge. The ideal way of obtaining this information is from uniformity trials – a preliminary experiment in which all EU are subject to uniform conditions (a single treatment, the same management practices, environmental conditions, and so on). The effect of each EU can be determined and used in the formation of blocks. Note that the use of a common treatment removes any possibility of treatments playing a role in the blocking process. Blocking is a feature of the EU and not the treatments. A given set of blocks can be used with many different treatments.

The experimental plan

We assume an experiment is planned to investigate *t* treatments which may or may not be structured. Blocks containing *t* EU each are assumed to be available. The *t* treatments are randomly assigned to the EU within each block, with the randomization done independently for each block. This represents a restriction on the randomization process in that there is not complete freedom in allocating the treatments, as all treatments are forced to occur equally (usually once) within each block.

Because all *t* treatments appear in each complete block of homogeneous units, any contrast among treatments should reflect only treatment and extraneous components. When one factor contributes equally to all levels of a second factor, the first factor is said to be 'balanced out' of the second factor. Therefore, in an RCB design the block factor is balanced out of the treatment factor. If one or more observations cannot be obtained in a block design, some of the balance feature is lost. With no missing values, the RCB is a balanced design.

The complete block should be as homogeneous as possible in terms of soil and other environmental factors. The researcher may not know the direction of the soil gradient on the experimental area and may allocate the treatments in the wrong direction. However, it is clear that with the smaller the block size less chance exists of having heterogeneity of soil within the complete block. When the block size is large, the chances of having heterogeneity soil conditions within the block increase. As the number of treatments increases, the chances of finding a homogeneous complete block dramatically decreases and another design is required. It is recommended that the block (or replicates) be as compact as possible (Figure 12.1).

Design considerations – advantages and disadvantages of the RCB design

Some advantages of RCB designs are:

1. Straightforward analysis. Even with missing observations in some of the block, a meaningful analysis may be possible.
2. More accurate results. When significant blocking can be achieved, differences due to EU are eliminated from treatment contrasts.
3. Increased sensitivity. Variability due to heterogeneous groups of EU is removed from Exp. Error.
4. Flexibility. Subject to conditions for a balanced design and available resources, there is no limitation on the number of treatments and/or blocks.

Major disadvantages of the RCB designs are:

1. If it is large, homogeneous blocks may be difficult to set up. The more EU per block, the greater the chance of them being heterogeneous.
2. If block and treatment effects interact (that is, they are not additive). The RCB analysis is not appropriate.

Incomplete block designs – lattice (adapted from Cochran and Cox, 1957)

These designs are arranged in blocks that are smaller than a complete replication, in order to eliminate heterogeneity to a greater extent than is possible with randomized blocks; this reduction in the size of block was achieved by sacrificing all, or part of, the information on certain treatment comparisons. They may be 'balanced or partially balanced'.

Figure 12.2 shows the possible layout in the field of a randomized complete block design (RCBD) and the layout of an incomplete block design of four incomplete block sizes of size 4. The layout of the RCBD covers a band of the field that accounts for soil gradient on the upper and lower parts of the field. The two possible layouts of the 4 × 4 incomplete blocks will control local variability in a much more efficient manner.

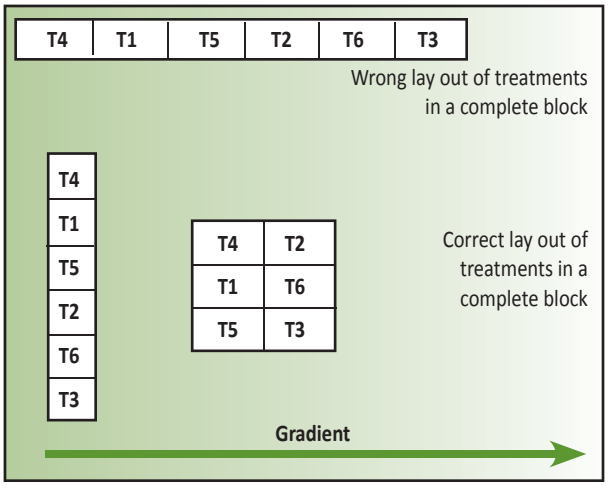

Figure 12.1. Figure showing different manners of laying out six treatments (T1–T6) in one replicate of a randomized complete block design. The soil gradient goes from the left of the figure to the right part of the figure.

Balanced incomplete block designs

The balanced designs will be illustrated first by simple examples of the experimental plans. Consider Table 12.2 which compares 9 treatments in incomplete blocks of 3 EU with 4 replications. Every pair of treatments will be found to occur once, and only once, in the same block.

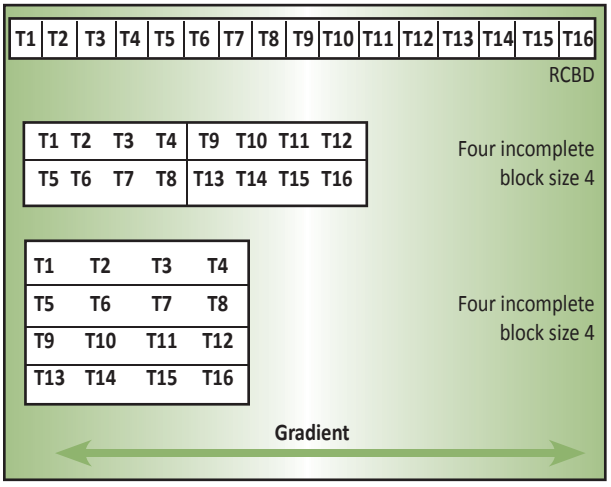

Figure 12.2. Figure showing the possible lay out in the field of a randomized complete block design (RCBD) and the layout of an incomplete block design of 4 incomplete block size of size 4.

For instance, treatment 1 occupies the same block with treatments 2 and 3 in the first replication, with treatments 4 and 7 in the second replication, treatments 5 and 9 in the third replication, and treatments 6 and 8 in the fourth replication. When the results are analyzed by the method of least squares, all pairs of treatments are compared with approximately the same precision, even though the differences among blocks may be large.

Table 12.2. Balanced design for 9 treatments in blocks of 3 units.

Block	Rep. I	Block	Rep. II	Block	Rep. III	Block	Rep. IV
(1)	1 2 3	(4)	1 4 7	(7)	1 5 9	(10)	1 8 6
(2)	4 5 6	(5)	2 5 8	(8)	7 2 6	(11)	4 2 9
(3)	7 8 9	(6)	3 6 9	(9)	4 8 3	(12)	7 5 3

This design belongs to the group known as 'balanced lattices', so-called because the plans are conveniently written down by drawing a square lattice, with the treatment numbers at the intersections of the lines. In the balanced lattices, the number of treatments must be an exact square while the number of units per block is the corresponding square root.

Balanced designs can be constructed for other numbers of treatments and units per block. Table 12.3 below shows 7 treatments arranged in blocks of 3 units.

Table 12.3. Balanced design for 7 treatments in blocks of 3 units.

Block	Rep. I	Block	Rep. II	Block	Rep. III	Block	Rep. IV
(1)	1 2 4	(3)	3 4 6	(5)	1 5 6	(7)	1 3 7
(2)	2 3 5	(4)	4 5 7	(6)	2 6 7		

Again every pair of treatments occurs once within some block. In this case, however, the blocks cannot be grouped in separate replications, since 7 is not divisible by 3. Designs of this type are known as 'balanced incomplete blocks'.

For certain numbers of treatments and units per block, both of the types above can be laid out in a kind of Latin square formation to allow the elimination of variation arising from the grouping. The appropriate rearrangement for the first example is shown in the Table 12.4.

Every pair of treatments now occurs once in the same row and also once in the same column. All comparisons between pairs of treatments are of nearly equal precision. This design is known as a 'lattice square'.

Partially balanced designs

Although a balanced design can be constructed with any number of treatments and any number of units per block, the minimum number of replications is fixed by these two variables. In most cases this number is too large for the usual conditions of experimentation and less replicates are used. Simple examples of such

Table 12.4. Balanced design for 9 treatments in 4 lattice squares.

Rep. I		Rep. II		Rep. III		Rep. IV	
	Columns						
Rows	(1) (2) (3)	Rows	(4) (5) (6)	Rows	(7) (8) (9)	Rows	(10) (11) (12)
(1)	1 2 3	(4)	1 4 7	(7)	1 6 8	(10)	1 9 5
(2)	4 5 6	(5)	2 5 8	(8)	9 2 4	(11)	6 2 7
(3)	7 8 9	(6)	3 6 9	(9)	5 7 3	(12)	8 4 3

designs are the lattices. These are constructed in the same manner as balanced lattices, except that there are fewer replications.

For example, the design with the first 2 replications for a balanced design with 9 treatments is called a 'simple lattice', and that with 3 replications a 'triple lattice' (Table 12.4). Similarly, with a lattice square, as in Table 12.3 above, may be used with less than the full number of replicates necessary for balance. For all these designs the number of treatments must be a perfect square.

Partially balanced designs are less suitable than balanced designs as the statistical analysis is more complicated. When the variation among blocks (or rows and columns) is large, some pairs of treatments are more precisely compared than others, and several different standard errors may have to be computed for tests of significance. These difficulties increase as the design departs more and more from the symmetry of the balanced design.

The simplest of the partially balanced designs are those with two associate classes. In these, some pairs of treatments occur in the same block λ_1 times, while other pairs occur in the same block λ_2 times, where λ_1 and λ_2 are whole numbers. In the lattice designs, which are of this type, some pairs of treatments never occur together in a block, i.e., $\lambda_1 = 0$; other pairs occur once in the same block ($\lambda_2 = 1$).

Comparison of incomplete block and randomized block designs

Incomplete block designs are no more difficult than randomized blocks. Some extra planning is involved in drawing up and randomizing the experimental plan, especially if care is taken to make the best possible grouping of the experimental units.

The gain in accuracy over randomized blocks depends on the type of experimental material and may be expected to increase as the number of treatments is increased. Most of the incomplete block designs cover the range from 6 to 200 treatments. If the experimental material is highly variable, yet lends itself to the formation of small groups which are homogeneous, the designs may be advantageous even with small numbers of treatments. From the results of varietal trials, a number of comparisons with randomized blocks indicated that an average gain in accuracy of the order of 25% was achieved by using incomplete block design.

There is one important property, possessed by many of the designs, which increases the attractiveness of the lattice designs relative to randomized blocks. The lattice designs are arranged in complete replications as well as in incomplete blocks. Such designs may be regarded as randomized block designs which have additional restrictions within each replication. It has been shown that these designs can be analyzed as if they were ordinary randomized blocks. This implies that the unadjusted treatment means give unbiased estimates of the true treatment effects, and that the F-tests do not lose their validity. Of course, this analysis will in general be less accurate than the complete analysis.

If there is any criterion for forming incomplete blocks, an incomplete block design is worth trialing in preference to a randomized block design which occupies the same set of replications. When the data have been collected, the experimenter may choose whether to analyze them as randomized blocks or to complete a full analysis, with the adjustments for incomplete block variations. In fact, if the variation among incomplete blocks is no greater than that within blocks, the complete statistical analysis reduces automatically to that for randomized blocks.

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WHEAT

Molecular markers and their application

Chapter 13: Genetic marker systems in wheat breeding

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Abstract

Genetic markers offer the possibility to adopt new biotechnology discoveries into current plant breeding strategies. They can be used to study the relationship between inherited traits and its genetic cause, and to uncover the phylogeny and population structure of diverse sets of crop germplasm including breeding parents. Marker-assisted selection (MAS) strategies can be designed to rapidly and efficiently generate fixed lines for a target gene or combination of genes. The practical value of a genetic marker depends on how successfully it can be integrated into a breeding program and how easily it can be applied on a large scale. Genetic markers are fragments of DNA sequence that are associated with a part of the genome and various types are available in wheat. Most marker types are based on polymerase chain reaction or hybridization and differ for a laboratory or breeding program, desirable qualities such as the abundance of the markers, methodology complexity, and costs. The principles, advantages and drawbacks of the most currently used marker types in wheat are described.

Introduction

A wide range of novel biotechnology based approaches have been developed and will yield major advances in future crop improvement. Amongst diverse established methodologies, such as genome sequencing, gene expression analyses or gene transformation, genetic marker systems, in particular, offer the possibility to adopt new biotechnology discoveries into current plant breeding strategies. With the assistance of genetic markers, trait based genes or transgenes can be followed through breeding generations enhancing the speed and effectiveness of breeding. Genetic markers are able to uncover the phylogeny and population structure of crop germplasm and support the characterization of parental germplasm. They are used to identify genes with as yet unknown functions via QTL or association mapping and they are applied in linking genomes of related species via comparative mapping for gene discovery and comparisons of gene functions. Genetic marker systems have been continuously developed since the late 1980s. It is evident that marker systems have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders.

Definition and understanding of genetic markers

A genetic marker can be defined as an identifier of a particular aspect in the genome, e.g., a nucleotide or short DNA sequence. It describes a variation, which can be observed and may have arisen due to mutation or alterations at the genomic loci. The inheritance of a genetic marker can easily be followed from generation to generation.

To be able to appropriately select and use genetic markers for use in plant breeding, it is important to understand how these markers are designed and how they are able to identify the specific aspects of the genome.

The key concepts in understanding genetic markers include:

- The basic structure of DNA,
- The organization of the DNA sequence, and
- The polymerase chain reaction (PCR).

Basic structure of DNA

Each DNA is composed of a string of nucleotides, which are formed from a pentose sugar, a phosphate group, and either the bases adenine, guanine, thymine or cytosine (abbreviated as A, G, T or C, respectively) (Figure 13.1). The shapes of the A and T molecules (and similarly for C and G) are 'complementary', a property which allows them to bind to each other. One molecule of sugar, phosphate, plus one of the four bases bound together is called a nucleotide, while the two nucleotides bound together are called a base pair (bp). The order of the nucleotides or bps in the DNA sequence of each individual organism is unique and forms a sequence. It carries all the genetic information needed for the organism to function. The entire string of nucleotides forms a double helix and is structured in chromosomes. Wheat has a total of 21 chromosomes. The genome is large and contains 16,000 mega bases (Mb), compared to maize with 2,500 Mb and rice with 430 Mb.

The organization of the sequence

Only parts (sometimes very small!) of the DNA sequence are composed of genes. The rest is non-coding sequence, including large amounts of repetitive sequences, microsatellites and transposons (Figure 13.2). In some species, the genetic fraction of the genome may be <10% of the total. Wheat contains a very high content, with 99% of repeated regions. In addition, wheat is allopolyploid, which means that the wheat genome is derived from different species. Bread wheat originated from natural hybridization of cultivated emmer wheat with the diploid goat grass *Aegilops tauschii*. About 10,000 years ago, cultivated emmer wheat was domesticated from its wild progenitor *Triticum dicoccoides* which was derived by hybridization of two diploid progenitors, *T. uratu* and an unconfirmed diploid species related to *Ae. speltoides.* Wheat, therefore, was derived from three diploid species and thus comprises three genomes (A, B and D), each represented by seven chromosomes. As the seven chromosomes are homeologous, their sequence organization is very similar.

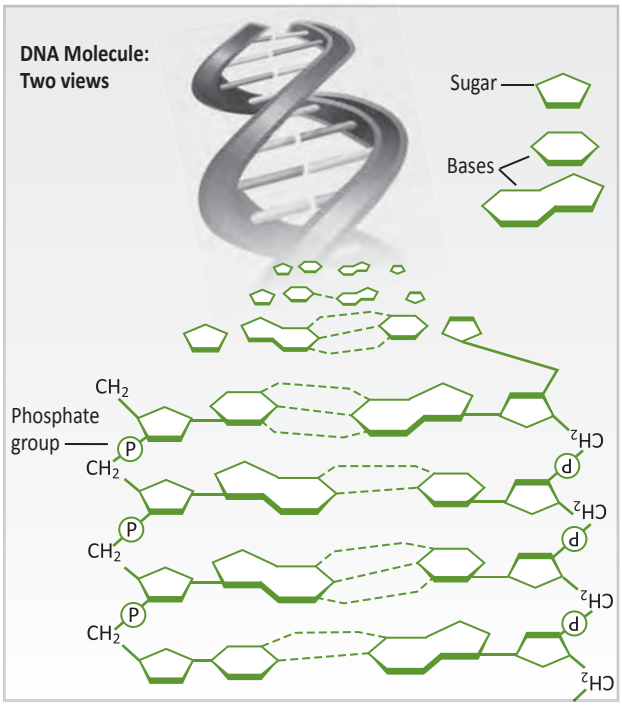


Figure 13.1. The structure of DNA. Adapted from http://www.cs.stedwards.edu.

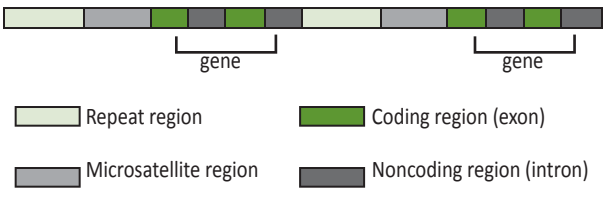


Figure 13.2. Sequence organization. Adapted from http://www.generationcp.org/mab/index.php?id=024.

The polymerase chain reaction (PCR) and electrophoresis

The vast majority of genetic marker systems rely on the use of PCR (Mullis *et al.,* 1986). This process mimics the natural way in which the cell replicates its DNA (Figure 13.3) and provides a quick, inexpensive way of making a large number of copies of a specific DNA segment. Genomic DNA with the target sequence is mixed with DNA polymerase, new nucleotides and short targeted priming sequences ("oligo sequences" or "primers"), then cycled through various temperatures to synthesise a copy of the target sequence between the two primers. Reiterating the cycle many times allows the new copies to serve as templates in the next round, resulting in an exponential increase in the number of copies of the target sequence.

The most important components of the PCR are the primers. They are short sequences of DNA (approximately 20 bps long) that help initiate the synthesis process and also to exactly determine the target DNA sequence which will be amplified. The design of primer sequences exploits the complementary property of the DNA molecule. In the example below, a target sequence to be amplified, and the possible primer sequences (in bold italic), are shown. A number of software programs can help to design your primers. A commonly used open source software is Primer3 (http://primer3.sourceforge.net/webif.php).

Primer development in wheat is somewhat complex due to the allopolyploid nature of the plant. The amplification of a target sequence from one of the three genomes requires the primer pair to be genome specific. Many primers, however, amplify the three different genomes and are difficult to interpret.

Example: Target sequence with primers.

TCCAACACTTTTGGC *(Primer1)*

5'

ACGTTGTGAAAACCGTACATGCCAATTCCGGAGTTTCAGTAACCTAGTCTTG

AAATGTCCCA 3'

3' TGCAACACTTTTGGCATGTACGGTTTCGGCCTCAAAGTCATTGGATCAG

AACTTTACAGGGT 5'

(Primer2) ***CTTGAAATGTCCCA***

Advantages of genetic markers

Genetic markers have several advantages over phenotypic, morphological or biochemical markers:

- They are not subject to environmental influence.
- They are unlimited in number.
- They are usually more objective since they can be distributed equally across the genome.
- They can be easier to analyze, and
- They may be less expensive than other types of markers (especially when they are deployed through a high-throughput approach).

Desirable properties of genetic markers

Different marker types have different characteristics. Desirable qualities of molecular markers include the following:

- Easy access.
- Highly polymorphic nature.
- High reproducibility.
- Inexpensive.
- Easy and fast assay.
- Co-dominant inheritance (determination of homozygous and heterozygous states of diploid organisms).
- Frequent occurrence in genome, and
- Selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices).

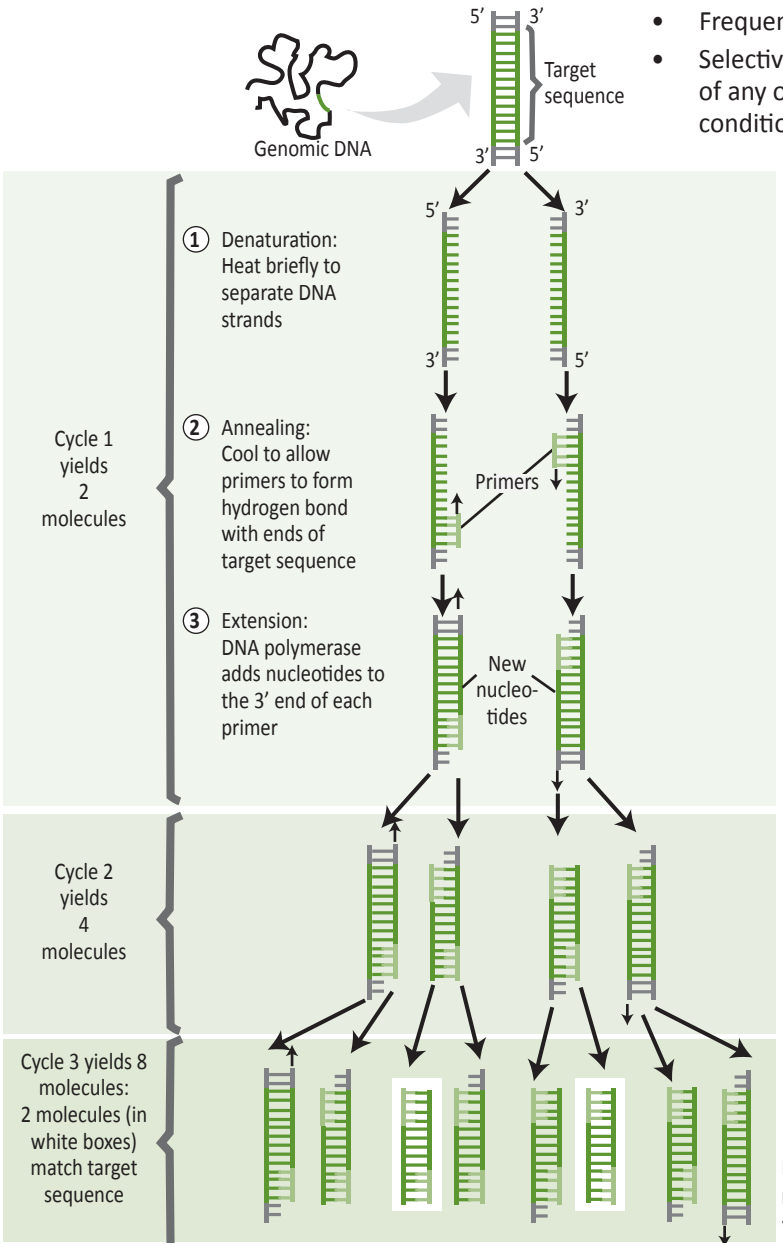


Figure 13.3. Polymerase chain reaction (PCR). Adapted from www.bio.miami.edu.

Types of genetic markers used in wheat breeding

PCR based markers

Microsatellites or simple sequence repeats (SSRs)
Microsatellites, or simple sequence repeats (SSRs), are tandem repeats dispersed throughout the genome (Grist *et al.,* 1993). These tandem repeats consist of 1 to 6 bp long monomer sequences that are repeated several times (Figure 13.4). The microsatellites can be amplified via PCR using primers that flank these regions. The tandem repeats are resistant to genomic changes during replication, which causes a high level of polymorphism. A very good relationship between the number of simple repeats and number of alleles detected has been observed. Thus, the larger the repeat number in the microsatellite DNA, the greater the number of alleles detected in a large population. Therefore, microsatellites form an ideal marker system. By simultaneously detecting multiple DNA loci, complex banding patterns can be created and the effectiveness of the marker system improved. Other prominent features of microsatellites are that they have co-dominant inheritance (homozygous and heterozygous loci can be distinguished and follow Mendelian inheritance) and they are equally distributed across a genome. In wheat, more than 3000 microsatellite markers have been developed which are specific for one of the three genomes. Information on these markers is compiled in the Grain Genes database (www.graingenes.org). The most commonly used microsatellites have been developed by Röder *et al.* (1998). They can be identified by the suffix 'GWM'. The Wheat Microsatellite Consortium (WMC) was a private effort coordinated by Dr. P. Isaac (IDnagenetics, Norwich, UK) and included 38 members. The majority of the WMC primer sequences were made publicly available in January 2004; the remainder of the WMC markers became available in January 2006. The BARC markers (Song *et al.,* 2002) were developed for the US Wheat and Barley Scab Initiative to map and characterize genes for *Fusarium* resistance. The CFA and CFD markers were kindly provided by Dr. P. Sourdille (INRA). More detailed protocols on how to amplify microsatellites via PCR are described in the CIMMYT laboratory manual (http://apps.cimmyt.org/english/docs/manual/protocols/labprotocols09.pdf).

Previously, microsatellite markers have been good platforms upon which to implement QTL mapping and subsequently marker-assisted selection (MAS) in breeding programs. Many linkage maps have been developed using microsatellite markers (http://wheat.pw.usda.gov/cmap/). One recent example of microsatellite markers being used for MAS is GWM192 and GWM165, the markers being closely linked (0.4 cM) to the slow rusting adult plant resistance gene *Lr67/Yr46* (Herrera-Foessil *et al.,* 2011).

Electrophoresis

All marker systems require a platform for visualization. The most common platforms for PCR-based markers are gel or capillary electrophoreses. Electrophoreses use an electric current to separate DNA fragments by size as they migrate through a porous sponge-like matrix. The matrix can be based on agarose or acrilamide. Smaller

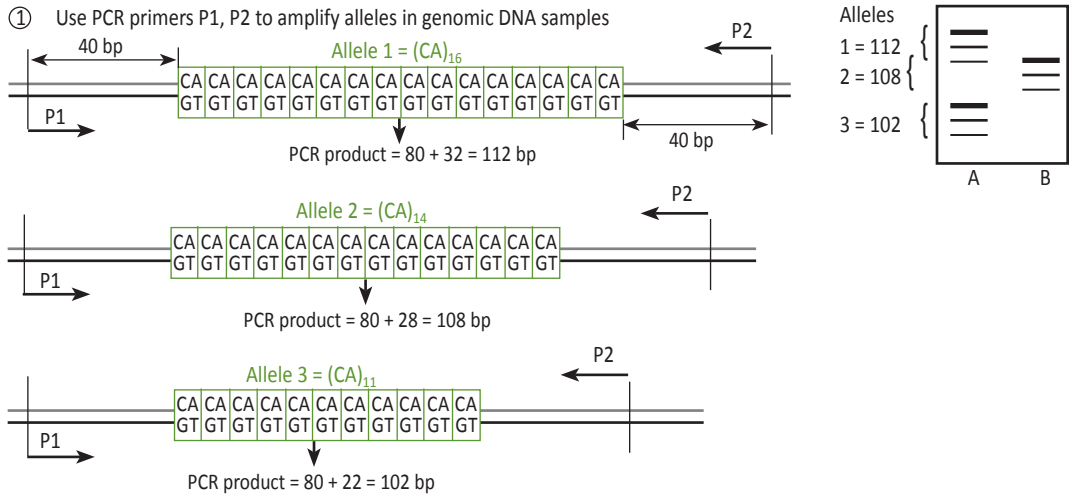


Figure 13.4. Microsatellite variation. Adapted from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=hmg&part=A551

molecules move more easily through the gel pores than larger molecules. After fragment separation, the DNA is stained, usually with chemicals (e.g., ethidium bromide or cybergreen) and visualized via UV light (Figure 13.5). Primers can also be fluorescent labelled and detected using specific cameras.

Sequence-tagged site (STS) markers

STS is a general term for markers that are developed with sequence-specific primers, usually for a particular genome region or type of region. The STS concept was introduced by Olson *et al.* (1989). In assessing the likely impact of PCR on human genome research, the authors recognized that single-copy DNA sequences of known map location could serve as markers for the genetic and physical mapping of important genes along the chromosomes. Cereal cDNAs, expressed sequence tags (ESTs) obtained from cDNA clones, cloned genes, and cloned PCR products constitute and are the basis for STS-based marker development. The markers have the advantage of producing a simple and highly reproducible pattern. They have two disadvantages: (i) they require suitable sequence data for each locus, and (ii) they are not as polymorphic as some other types of DNA markers, such as microsatellites. However, STS markers are an important application in breeding programs and germplasm management because they offer convenience and reliability for genomic analysis. Several STS markers in wheat have been developed during the last decade. One of the first examples was the validation and application of molecular markers for Cereal Cyst Nematode (CCN) resistance. Ogbonnaya *et al.* (2001) developed STS markers for the resistance genes *Cre1* and *Cre3* which expressed resistance in host roots against the Australian CCN pathotype Ha13. The biological assay traditionally used to select resistant lines in breeding programs is time consuming, not reliable on a single-plant basis, prone to inconsistencies, and relatively expensive. Using the STS markers is therefore a highly efficient alternative because it offers rapid and precise selection of the target genes. Another example is the detection of the point mutations responsible for the two major semi-dwarfing genes *Rht-B1b* (*Rht1*) and *Rht-D1b* (*Rht2*) in wheat (Ellis *et al.,* 2002). More examples of gene based STS markers are given in William *et al.,* 2007, http://wheat.pw.usda.gov/cmap/, and http://www.generationcp.org/sp5/?da=09148937).

Sequence characterized amplified regions (SCARs)

Michelmore *et al.* (1991) and Martin *et al.* (1991) introduced this technique wherein the RAPD (random amplified polymorphic DNA) marker termini are sequenced and longer primers are designed (22–24 bp long) for specific amplification of a particular locus (Figure 13.6). SCAR markers are more reproducible than RAPDs and are similar to STS markers in construction and application. SCARs are usually dominant markers.

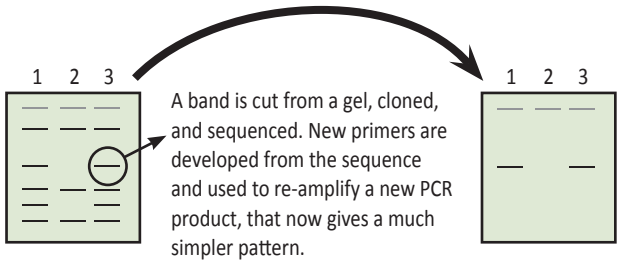


Figure 13.6. Development of SCAR markers. Adapted from De Vicente and Fulton (2004).

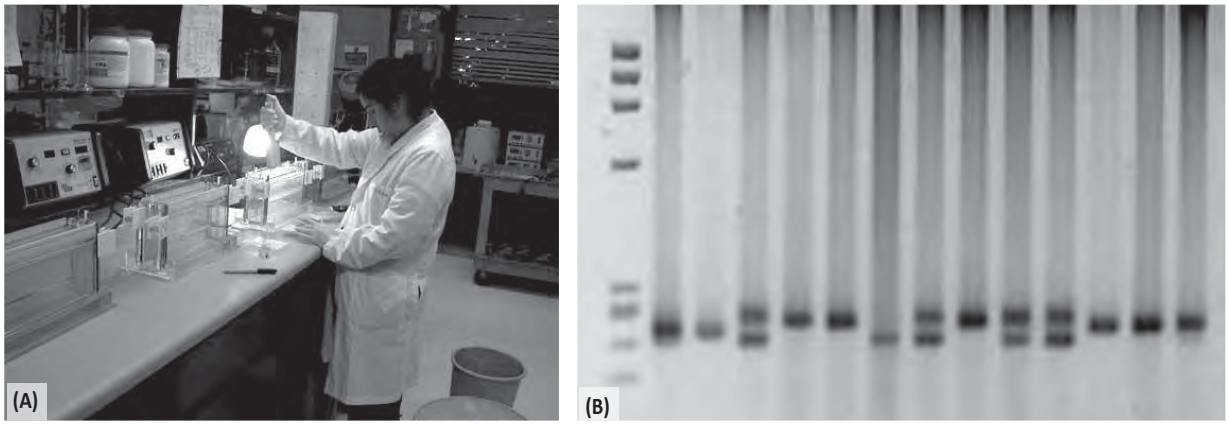
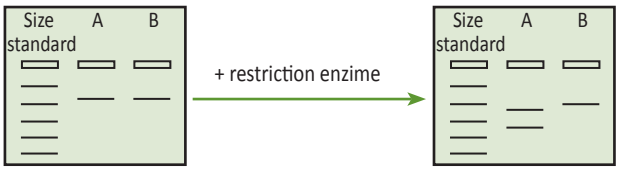


Figure 13.5. (A) Vertical and (B) horizontal gel electrophoresis.

The presence or absence of the band indicates variation in sequence. However, some of them can be converted into co-dominant markers by digesting them with restriction enzymes.

Cleaved amplified polymorphic sequences (CAPS)

These polymorphic patterns are generated by restriction enzyme digestion of PCR products. Such digests are compared according to their differential migration during electrophoresis (Figure 13.7). The PCR primer for this process can be synthesized based on the sequence information available in databanks of genomic or cDNA sequences or cloned RAPD bands (STS and SCAR markers). These markers are co-dominant in nature as either one or two alleles are digested. In general, some effort and expense is required to test the various restriction enzymes and to digest a larger number of samples. CAPS markers are an easy method to detect mutations such as Insertions/Deletions or single nucleotide polymorphisms (SNPs). Digestion with diverse restriction enzymes e.g., can be used to determine the allelic variation for grain texture in wheat which is mainly determined by the Hardness (Ha) locus consisting of genes Puroindoline a (Pina) and b (Pinb) (Lillemo and Ringlund, 2002).



In the first figure, no difference is seen in the PCR products of plant A and B, but after restriction digest a polymorphism can be identified.

Figure 13.7. Restriction of a SCAR marker to develop a CAPs marker. Adapted from http://www.generationcp.org/mab/index.php?id=034.

Nuclear binding site (NBS) profiling

In this method the conserved sequences in the nucleotide-binding sites of the nucleotide-binding site-leucine-rich repeat (NBS-LRR) class of disease resistance genes (R-genes) have been used for PCR-based R-gene isolation and subsequent development of molecular markers (van der Linden *et al.,* 2004) (Figure 13.8). NBS profiling is a newly developed method to probe functional diversity in and near disease resistance genes of the NBS-LRR type. NBS profiling can be used to produce markers tightly linked to R-genes and R-gene clusters for genomic mapping and positional cloning, and to mine for new alleles and new sources of disease resistance in available germplasm. NBS profiling has been used to determine genetic diversity in durum wheat (Mantovani *et al.,* 2006) and recently to detect a candidate gene for the leaf rust gene *Lr19* (Gennaro *et al.,* 2009).

Insertion based polymorphism (ISBP)

Transposable elements are known to be nested in large plant genomes, where they display unique insertion sites that are highly polymorphic between varieties. This feature has been exploited to develop PCR-based marker systems for genetic analysis in a range of cereal grass and grain legume species (for a review see Schulman *et al.,* 2004). Paux *et al.* (2006) exploited this method to identify the chromosomal location of BAC clones in hexaploid wheat, and confirmed its potential use for genetic mapping. ISBPs show unique amplification

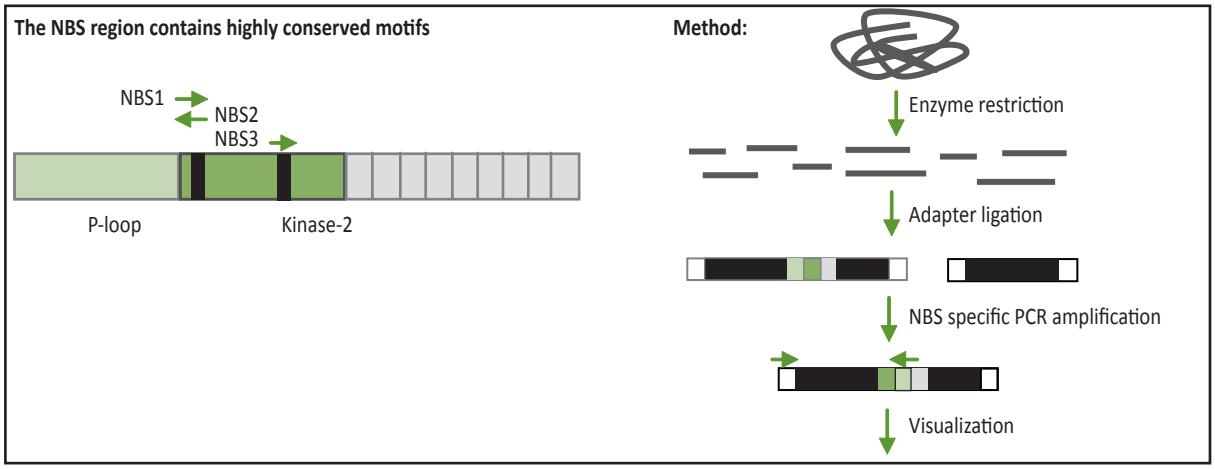


Figure 13.8. Nuclear binding site profiling.

products, are genome-specific and allelic. Therefore, the marker system represents a potential tool for phylogenetic and transposable elements evolution studies in wheat. Availability of markers is currently restricted to the 3BS chromosome.

Marker systems for high-throughput platforms

Diversity array technology (DArT)

Diversity array technology (DArT) detects single base changes and Insertion/Deletions (Indels) in any genome without relying on sequence information. DArT is an array, or chip-based marker system, of individualized fragments of one or various genomic representations of wheat or other crops (Figure 13.9). Individualized fragments are extracted from pools of genotypes that cover the genetic diversity of each species. Representations of the varieties to be genotyped are labelled and hybridized to the array. The polymorphisms scored are the presence versus absence of hybridization to individual array elements (Figure 13.10). They reflect DNA sequence variation that determines which genomic sequences are present in the genomic representations. DArT have a high multiplexing level and the array based nature of the marker systems ensures high-throughput and a low cost of analysis. DArT is, for that reason, an ideal marker system for genetic mapping and whole genome genotyping. DArT markers are in general less

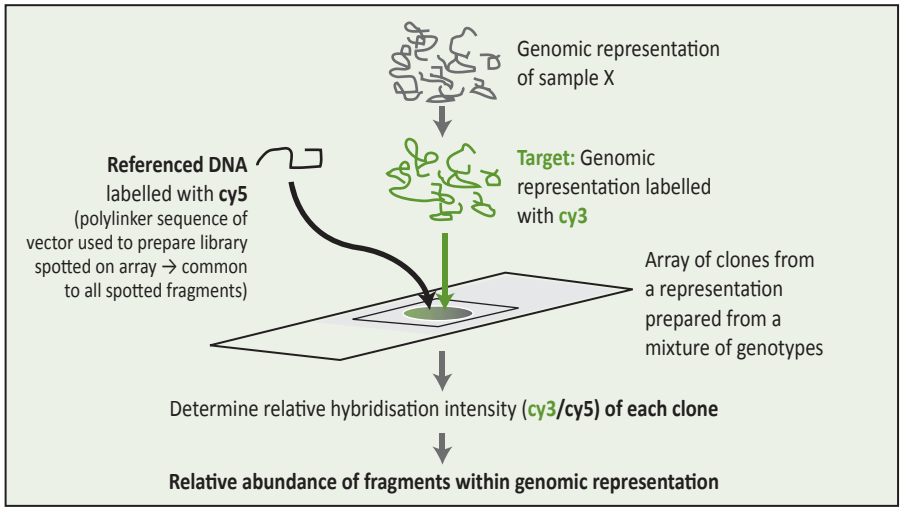


Figure 13.9. Generating the DArT array. Adapted from www.triticarte.com.au

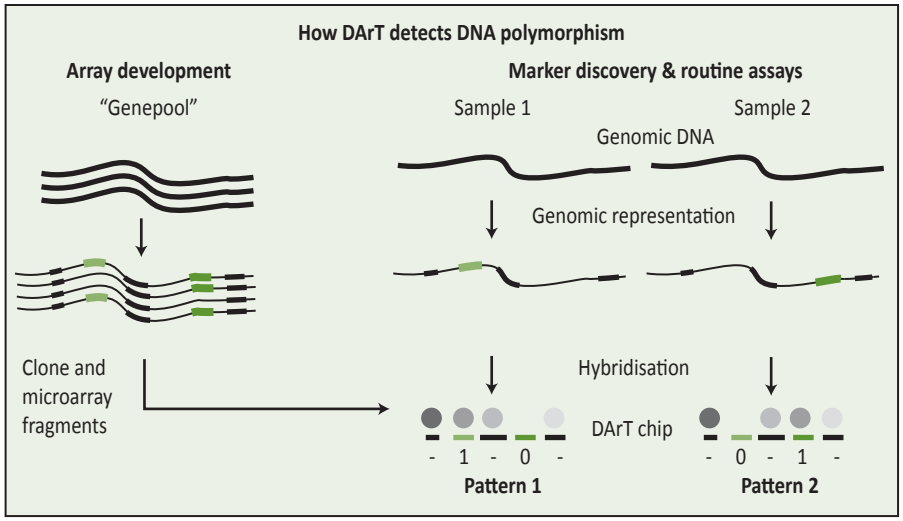


Figure 13.10. Genotyping two samples with DArT markers. Adapted from www.triticarte.com.au

polymorphic than SSR markers in wheat. However, their genome coverage and low cost compensate for this disadvantage. Generating a DArT array is highly complex and large efforts would be required for in-house development. Various arrays for wheat have been developed and are owned by the company Triticarte (www.triticarte.com.au), located in Canberra, Australia. The latest array is composed of 2,500 polymorphic markers within the genomic representation.

Single nucleotide polymorphism (SNP)

A single nucleotide polymporphism (SNP) is a single base pair mutation at a specific locus, usually consisting of two alleles. Because SNPs are evolutionarily conserved, they have been proposed as markers. Compared to other types of markers, SNPs have numerous detection systems with various throughput and multiplexing levels that are commercially available (Figure 13.11) (Syvaenen, 2005). Key technologies for SNP interrogation include hybridization-based methods, enzyme-based methods (PCR-amplification of the targeted sequence) and other post amplification methods based on the physical properties of DNA, such as single stranded DNA conformation. In addition, diverse platforms and software programs have been designed to further facilitate the high-throughput genotyping of SNPs using electrophoresis, fluorescent readouts, oligonucleotide microarrays, mass spectrometry or beads.

Currently, many efforts are ongoing, developing more genome specific SNPs. However, to date, in wheat, the observed frequency of SNPs in breeding populations is rather low (Ravel *et al.,* 2006).

Golden Gate and Infinitum SNP genotyping assays

Akunov *et al.* (2009) and a number of wheat scientists developed a Golden Gate and Infinitum SNP assay for a number wheat SNPS (1,536 and 9K SNP chips). The assays are highly multiplexed assays which means hundreds to thousands of SNPs can be analyzed at the same time. For Golden Gate assay, three primers are synthesized for each SNP: two allele specific primers that distinguish the SNP, and a locus specific primer just downstream of the SNP. Each primer sequence contains a target sequence for a set of universal primers (P1 to P3 in Figure 13.12). The locus specific primer also contains particular address sequences (the «illumicode»), complementary to sequences attached to beads. The allele specific primers and the locus specific primer querying a set of SNPs are synthesized and pooled by Illumina. To carry out the assay, the pooled primer set is hybridized simultaneously to genomic DNA, representing a single sample/reaction well. Following allele specific primer extension and ligation reactions, a set of fluorescently labelled universal primers (Cyanine dyes, Cy3 and Cy5 labelled P1 and P2, respectively) are added and PCR is carried out, generating multiple

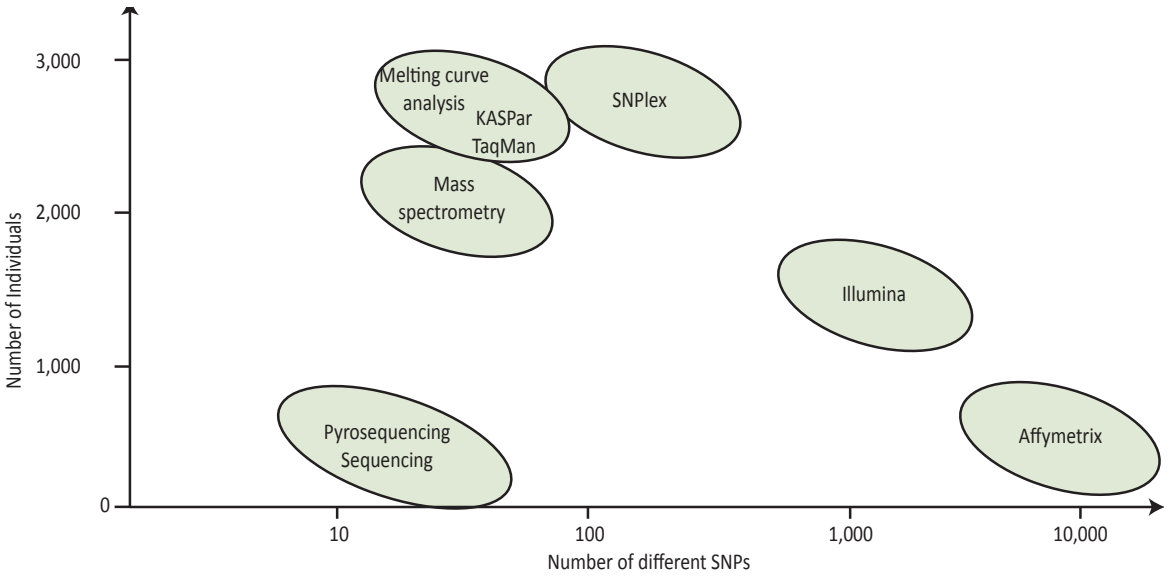


Figure 13.11. Examples of genotyping methods with the corresponding throughput with respect to the number of individuals and the number for different SNPs. Modified from Giancola *et al*., 2006.

labelled amplicons representing hundreds of different SNPs. These fluorescent products are then combined with Illumina beads, and data can be read. Various data readout options exist; e.g., the Bead Array, VeraCode or BeadXpress platform (see the Illumina web-site: http://www.illumina.com/). The address sequence within the PCR amplicons hybridize to their cognate sequence on the bead, and the fluorescence on each bead is quantified resulting in a signal associated with a particular address sequence. Each address translates to a particular locus, and the presence of Cy3, Cy5, or both signals on a given bead type indicates AA, BB or AB genotypes. The Golden Gate assay has a number of advantages for genotype analysis: it is robust and tolerates a variety of input DNA concentrations and integrities, no amplification of sequences containing the SNP is required (except for the final amplification with the universal primer set) and, apart from the custom primer pool, all materials and reagents are generic and can be applied to the analysis of a wide variety of organisms.

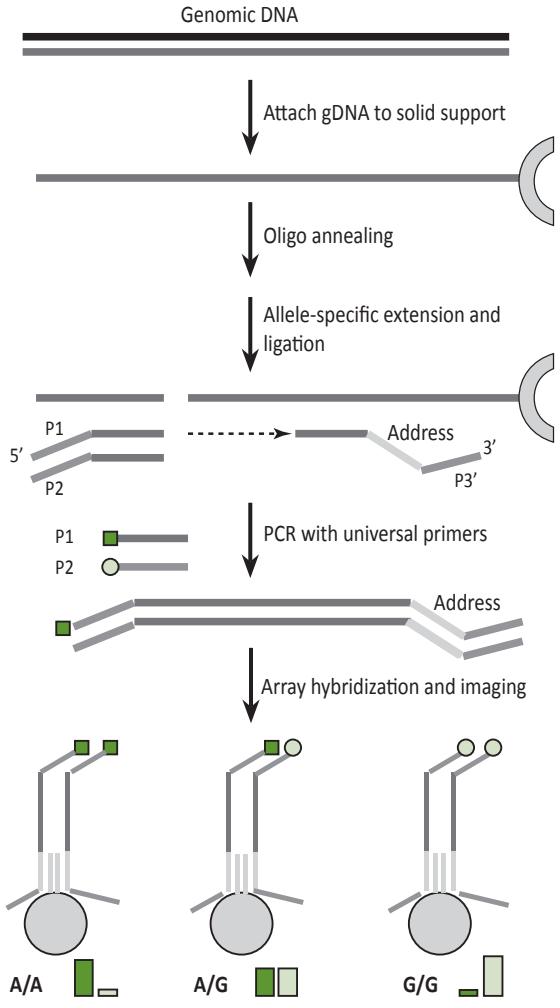


Figure 13.12. Golden Gate assay. Adapted from www.genomecenter.ucdavis.edu.

TaqMan and KASPar assays

The most widely used genotyping platform for the analysis of the low multiplex SNP assays are the TaqMan and KASPar assays provided by Applied Biosystems (Livak, 1999) and KBioscience (http://www.kbioscience.co.uk). TaqMan probes contain two dyes, a reporter dye and acceptor dye (quencher). The proximity of the quencher to the reporter in an intact TaqMan probe allows the quencher to suppress, or "quench" the fluorescence signal of the reporter dye. The TaqMan probes specifically anneal between the forward and reverse primer sites during PCR. The Taq polymerase cleaves the TaqMan probe between the reporter and the quencher only if the probe hybridizes to the target, so no SNP is present. The TaqMan probe fragments are displaced from the target, separating the reporter from the quencher and resulting in increased fluorescence of the reporter. The fluorescent signal is detected by RT-PCR and permits quantitative measurements of the accumulation of the product (Figure 13.13). A major drawback of the TaqMan assay is the relatively high cost of the assay design, since the assay requires two labelled probes per reaction. Lower cost assays such

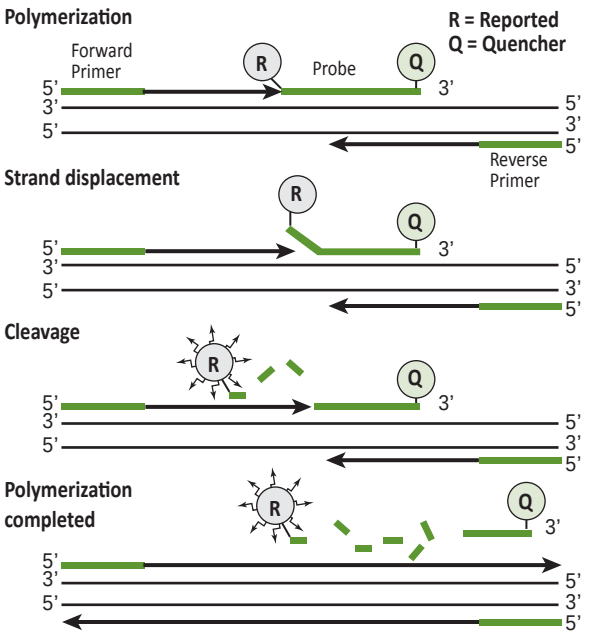


Figure 13.13. TaqMan Assay. Adapted from www.e-oligos.com.

as the KasPar assays (www.kbiosciences.co.uk) are becoming available and are facilitating low muliplex SNP application. A few reports of TaqMan assays for gene targets of marker-assisted selection in wheat are found in the literature. Helguera *et al.* (2003) reported a Taqman assay for the *Lr37-Sr38-Yr17* cluster of resistance genes located on a segment from *Triticum ventriosum,* translocated to the chromosome arm of 2AS of wheat. Dvorak *et al.* (2006) reported TaqMan assays for EST-derived markers linked to suppressors from *Aegilops speltoides* of the homoeologous pairing gene *Ph1*.

Considerations in selection of marker type

As described above, there are many types of molecular markers available. Which type to select to use for your project will depend on the following:

1. What is the goal of your project? You may wish to compare the entire wheat genome across various genotypes or you may wish to concentrate on a small part of the genome of a specific genotype.
2. How variable is your germplasm? More markers might be needed if your germplasm is highly related.
3. What level of resolution is needed? Studies of genetic diversity in general require a higher resolution as more alleles per locus might be observed.
4. Is there previous work you can take advantage of? For example, already observed marker-trait associations.
5. Which marker platforms are accessible or can be managed in your laboratory? For example, are agarose, acrylamide electrophoresis facilities, sequencing or SNP platforms available?

Applications of molecular markers in plant genome analysis and breeding

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of genes to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it.

Mapping and tagging of genes

Most marker systems in wheat were not developed from the genes themselves because the cloning of genes in wheat has been complicated by its allohexaploid nature and large genome size. Genetic linkage maps are the means to provide the position of markers and genes on chromosomes. Genetic linkage maps are based upon the frequency of recombination in segregating populations and provide breeders with choices of markers for tagging desired genes. Moreover, comparison of the chromosomal assignments and orders of marker loci common to several genetic linkage maps has shed light on ancestral chromosomal rearrangements and on evolutionary relationships between different chromosomes.

The very first genetic linkage maps in wheat were published by Hart *et al.* (1993). Once the framework maps are generated, a large number of markers derived from various techniques are used to saturate the maps as much as possible. Microsatellite markers have been found to be extremely useful in this regard. Owing to their quality of following clear Mendelian inheritance, they can be easily used in the construction of maps, which can provide an anchor or reference point for specific regions of the genome. Various genetic linkage maps in wheat have been developed (http://wheat.pw.usda.gov/cmap/).

The functionality of a mapped marker depends on the known linkage phase between the marker and target locus alleles. Functional markers are derived from polymorphic sites within genes that causally affect phenotypic trait variation. Quantitative trait loci (QTL) or association mapping analysis must be carried out to form segregating populations or particular germplasm panels and the linkage phases between the marker and QTL alleles can be assigned.

Marker assisted selection (MAS)

Once a marker is identified through linkage or association mapping analysis, its utility as an indirect selection tool must be validated in appropriate breeding populations. The practical value of a marker depends on how successfully it can be integrated into a breeding program (see Bonnett, this volume, Chapter 14), and must be easily applied on a large scale in modern breeding programs. A number of wheat breeding programs have begun to use the approach of MAS. The breeding strategies used depend on breeding objectives, resource availability and information from genetic characterization of different traits.

Simply inherited disease resistance is a common target for MAS, particularly where breeding programs do not have ready access to disease hot spots and where there is a need to pyramid resistance genes. In wheat, there are a number of inter-chromosomal translocations from related species that carry useful genes for which markers are available; these markers allow the translocated segment containing the target gene to be easily introduced into elite lines (http://maswheat.ucdavis.edu/; McIntosh *et al.,* 2003). Race specific disease resistance genes are more effective if deployed in combination. Reliably pyramiding these genes may not be possible without the use of markers. Marker-assisted selection strategies can also be designed to rapidly and efficiently generate fixed lines for a target gene or combination of genes. Considering the relatively high cost of DNA extraction and subsequent marker assays, it is important to identify the optimum points for MAS in the breeding process, to increase the efficiency and effectiveness of the breeding program. Molecular dissection of loci that contribute to complex traits such as yield and abiotic stress tolerance remains a considerable challenge, even with the newly available marker technologies

Phylogeny and diversity analysis

Most of the early theories of evolution were based on morphological and geographical variations between organisms. However, it is becoming more evident that the techniques from molecular biology hold promise of providing detailed information about the genetic structure of a natural population than what we have been able to achieve in the past. A number of PCR-based markers are being used extensively for reconstructing phylogenies. The techniques have provided path-breaking information regarding the fine time scale on which closely related species have diverged and genetic variations associated with species formation. One major conclusion which could be made in wheat was that the polyploidy of wheat has been able to compensate for diversity bottlenecks, caused by domestication, by capturing a relatively large proportion of the variability of its tetraploid wild progenitor (Dubcovsky and Dvorak, 2007).

The assessment of current genetic variation between cultivars is indispensable for plant breeding purposes since it allows breeders to make informed decisions in selecting parents for new crosses and provides a means for analyzing variation available in germplasm collections and wild populations. The range of genetic variation in a species is determined by many factors, including mutation, recombination, migration, selection, changes in population size, population subdivision, random genetic drift and others.

Molecular studies of regional breeding programs over a sustained period of time have provided new insights into the impact of plant breeding on crop genetic diversity. Some studies have suggested that the reduction in diversity which has accompanied plant improvement has been limited and observe rather a qualitative than quantitative shift in genetic diversity over time (Khan *et al.,* 2005). Other studies have demonstrated the reduction of allelic accounts in some improved gene pools of wheat (Fu *et al.,* 2006). A comprehensive characterization of bread wheat from CIMMYT from 1950 to 2003 with nearly 100 SSR markers observed a decrease of genetic diversity from landraces to improved CIMMYT lines in the 1980s, followed by an enhancement of genetic diversity to breeding lines in 2003 (Reif *et al.,* 2005). The significant increase in genetic diversity of recently developed CIMMYT breeding lines and cultivars could be explained by recent efforts by CIMMYT breeders to expand the genetic base of common wheat through the use of landraces, materials from other breeding programs, and synthetic wheat derived from wild species in the pedigrees of new advanced materials.

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Chapter 14: Optimizing marker-assisted selection (MAS) strategies for crop improvement

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Abstract

Markers linked to valuable traits are many and increasing in number. Together with decreasing costs of marker assays and the opportunity to outsource marker screening and avoid expensive setup costs, the potential to apply markers is becoming accessible to more and more breeding programs in developed and developing countries alike. This chapter outlines principles and provides formulas and tables to support breeders in designing crossing and selection methodologies incorporating marker-assisted selection. The focus is on inbreeding species or where inbreeding is used at certain stages of the breeding process as is common for hybrid crops. The best strategy will vary based on the objectives of the breeding program and the level and mix of resources available. The most efficient strategy can substantially reduce population sizes and cost needed to recover desirable genotypes or allow selection at the maximum number of loci for a given level of resource. Enriching rather than fixing alleles in early generations to avoid drift while minimizing population sizes is an important component of these strategies. However there are limits to how many desirable alleles can be combined in a single breeding cycle with even the most efficient strategy and this chapter allows a realistic assessment of what is possible.

Introduction

Markers are a tool that can be used by breeders to increase genetic gain but breeding will continue to depend heavily on phenotypic selection. Efficient integration of marker and phenotypic selection is vital to maximize overall gains. Every breeding program has a different set of breeding targets and also a different level and mix of resources at its disposal. These specific considerations mean that the best combination of marker and phenotypic selection will vary between programs. To date, use of MAS in wheat has been directed toward selection for alleles of large effect controlling traits with relatively simple genetic control. All of the markers listed on the MASWheat website (http://maswheat.ucdavis.edu/protocols/index.htm) fall into this class, with none currently listed for grain yield or drought tolerance Quantitative Trait Loci (QTL). Considering that such QTL are commonly population and environment specific, it is likely that techniques such as marker-assisted recurrent selection (MARS) or genomewide selection (GWS; Meuwissen *et al.,* 2001) – also referred to as whole genome selection (WGS) or genomic selection (GS) – will be necessary to identify and select useful regions for such traits in relevant populations and environments. This will require marker assays that provide good coverage of all chromosomes and capacity to screen large numbers of lines at low cost. Good phenotypic data on the lines to be genotyped and appropriate statistical algorithms will also be critical to success of such techniques. For more information on MARS and GWS see Bernardo and Yu (2007) and references therein. Currently, however, whole genome assays with sufficiently high-throughput and low cost do not exist for wheat and the technique is unproven even in experimental populations. Therefore it will not be considered further here.

This chapter will therefore aim to outline principles that should be useful in designing a breeding strategy integrating marker-assisted selection (MAS) with currently available technology. Examples will be given to illustrate these principles, and tables, formulas and useful reference papers provided to enable breeders to develop MAS strategies tailored to their own programs.

Limits on progress in one breeding cycle

In major crops like wheat, current varieties are elite combinations of alleles for yield, grain quality and tolerance to biotic and abiotic stresses that have been assembled over multiple cycles of crossing and selection. A cross made with the aim of producing a variety will probably have parents with many alleles in common controlling these characters. If parents have

a lower coancestry and differ for a greater number of alleles, even if they are phenotypically similar, it may be difficult to produce a line suitable for release as a variety from a simple biparental cross. In such crosses, prohibitively large population sizes would be needed to recover the most desirable alleles across large numbers of polymorphic loci even with the most efficient strategy (Figure 14.1). In these cases, or where one parent contributes only a small number of desirable attributes and the other contributes many more, one or more backcrosses may be necessary to recover a commercially viable line. It is an unavoidable fact that some desirable allelic combinations (genotypes) will require more than one breeding cycle to assemble.

Effective use of markers, or any selection tool, can make a large difference to the probability of recovering a desirable genotype or the population size (≈cost) needed to have a reasonable probability of recovering it. Cheaper, higher heritability and higher throughput selection screens should be used in earlier stages of selection to increase the frequency of the alleles under selection. Considering that DNA markers provide a more accurate means of selecting desirable alleles than most phenotypic screens, use of markers in early generations is attractive. The attraction of using markers in early generations will continue to increase with cheaper and higher throughput DNA extraction and screening techniques as well as greater numbers of markers available for use in screening. If markers are co-dominant it will even be possible to select homozygotes in early generations and avoid the need to do selection for any of these alleles in later generations. However, there are some important reasons, which will be discussed below, why selecting homozygotes in very early generations is only possible for a small number of loci. Trying to select homozygotes at greater numbers of loci in early generations dramatically increases the population size needed to recover a desirable genotype. This is illustrated in Figure 14.1 which shows the population sizes needed to recover a single individual with a particular desired homozygous allele combination. Each curve tracks the population size needed to recover a target homozygous genotype in populations segregating at increasing numbers of loci. Population sizes are based on assumptions of: (i) a biparental cross, (ii) development of inbred lines by single seed descent (SSD) or doubled haploidy, (iii) 95% probability of recovering at least one plant with the target genotype ($P = 0.05$), (iv) target loci are unlinked, and (v) complete linkage between markers and target alleles.

Figure 14.1 shows the population sizes needed to recover a target genotype in the F_2 generation. With only 4 segregating loci an F_2 population size of almost 800 is needed to recover an individual homozygous for all the desirable alleles. With no selection in F_2 and just a single generation of inbreeding, only 150 F_3 individuals (derived by SSD from 150 F_2s) are needed to recover one with the target combination at 4 loci. This is only around one fifth of what was required to do the same in F_2. Further reductions in population size are achieved with additional cycles of inbreeding as shown by the F_6 and DH (doubled haploid) lines. It is clear that high selection pressure to recover homozygotes is better in more inbred populations and allows recovery of desirable combinations of alleles across a greater number of loci or use of smaller population sizes. The reason for this is that there are many more individuals in the F_2 that are at least heterozygous for all of the target alleles than individuals homozygous for all target alleles. If there are *n* segregating loci in a biparental F_2 population, the frequency of homozygotes for the preferred

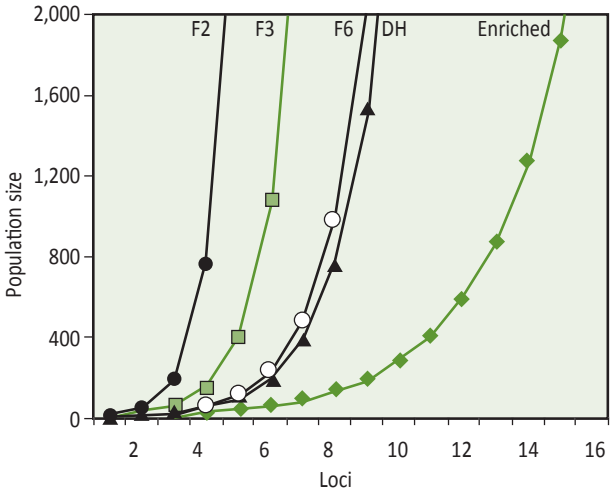

Figure 14.1. Population sizes needed to recover a target homozygous genotype in crosses segregating at increasing numbers of loci with different selection strategies. In all scenarios, except the enrichment strategy, selection is applied only at the generation indicated. In the enrichment strategy, selection is applied in F_2 and doubled haploid (DH) generations.

allele at those loci is $1/4^n$ compared with the frequency of individuals with at least one copy of all preferred alleles at $3/4^n$. If allowed to inbreed, the heterozygous individuals produce additional target homozygotes in subsequent generations increasing their frequency over successive generations from $1/4^n$ in F_2 to a maximum of $1/2^n$. As the number of heterozygotes is halved every generation, the greatest gains in frequency are in the first few generations with little gain subsequently. This can be seen in the relatively small difference between F_6 and a DH.

This illustration shows that applying a selection pressure that is too high in early generations, even with a highly heritable screen, is not the most efficient strategy because it does not take advantage of the much larger number of heterozygotes that will produce desirable homozygous progeny. Some individuals in early generations, however, do not carry desirable alleles and removing them from the population and allowing individuals with greater numbers of desirable alleles to produce more progeny can substantially increase the frequency of target homozygotes in subsequent generations. Examples of this type of allele enrichment strategy may include culling plants that are the tallest, most rust susceptible, or with the most extreme phenology in F_2 or F_3. The plants retained would include not only the best plants but those that may be slightly too tall, too early or late flowering or too rust susceptible in their own right, but would be better than the worst. These are likely to produce better progeny in subsequent generations as important alleles become homozygous. The potential of such an enrichment strategy can be seen in looking at the diamond line in Figure 14.1 (Enriched) – that is, the substantial reduction in population sizes needed to recover a target genotype and the potential to select for combinations of desirable alleles across many more loci. This assumes culling in F_2 of all individuals that are not at least heterozygous for all target alleles which is probably only possible with markers perfectly linked to all target alleles. Phenotypic screens will almost certainly be less accurate but provided sufficient individuals are retained and heritability is greater than zero, the probability of recovering a target individual should be greater than if no selection was applied. If heritability is zero, the frequency of target genotypes in subsequent generations should at least be no less than without selection, provided sufficient individuals are retained to avoid genetic drift.

Marker-assisted allele enrichment strategies

Enrichment of allele frequencies using markers is not fundamentally different to early generation phenotypic selection, but allows selection for a greater range of traits and will usually be more accurate. Although it will not be discussed in detail in this chapter, marker-assisted allele enrichment is commonly practiced among F_1s of topcross or complex cross populations to eliminate F_1s that do not carry essential alleles. This strategy is routinely applied in CIMMYT's rainfed breadwheat and durum programs (Y. Manes and K. Ammar, personal communcation). As with allele enrichment in F_2, this considerably increases allelic frequencies and reduces the population sizes necessary to recover a desired allele combination. If markers are going to be used, applying them in complex cross F_1s to select individuals carrying desirable, low frequency alleles or allele combinations is a highly desirable and efficient stage for their implementation. For example, desirable alleles coming from the non-recurrent or donor line will have a frequency of 1/4 in BC_1F_1 or TCF_1 populations and half of all F_1s will lack even a single copy of each allele. If there are desirable alleles from the donor at 2 loci, only 1/4 of all F_1s will carry both and therefore 3/4 of the F_1s have no chance of

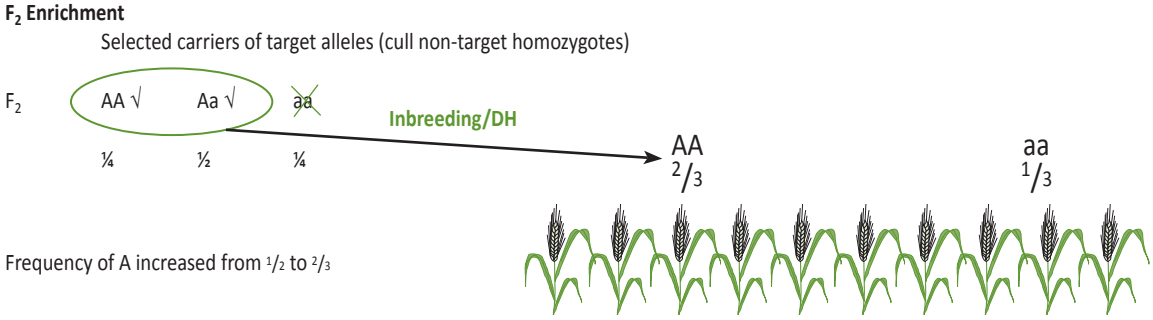

Figure 14.2. Schematic representation of an F_2 enrichment strategy.

producing progeny with the target genotype. Selection among BC_1 or TCF_1s will increase the frequency of target alleles from donors from 1/4 to 1/2 and ensure all F_1s carry a copy of all target marker alleles. If followed by F_2 enrichment, the frequency of donor alleles is increased from 1/4 to 2/3. For more information on application of allele enrichment in backcross and topcross populations at both F_1 and F_2 generations refer to Bonnett *et al.* (2005).

The principle of F_2 enrichment is illustrated in Figure 14.2. In the F_2 of a biparental cross, at every polymorphic locus, 3/4 of F_2 individuals will carry at least one copy of the preferred A allele. Both AA and Aa individuals will produce the preferred AA homozygous progeny and should be retained in the population. Individuals with the aa genotype cannot produce AA progeny and should be culled from the population. Culling aa and retaining both AA and Aa increases the frequency of the A allele from 1/2 to 2/3. If no further selection was applied and the population was progressed to homozygosity by inbreeding or production of DHs from selected F_2s, the frequency of AA genotypes in the final population would be 2/3 and the frequency of aa only 1/3. With greater numbers of polymorphic loci, the advantage of enrichment vs. direct selection of homozygotes becomes clearer (see Figure 14.1). With only one polymorphic locus the frequency of carriers (AA and Aa) of the preferred A allele is 3/4 and the frequency of AA homozygotes only 1/4. With greater numbers of polymorphic loci (B, C, D etc.) the difference in the frequencies and the population sizes needed to recover homozygotes vs. carriers of all desirable alleles becomes very large with only relatively small numbers of polymorphic loci (see Table 14.1). Even in a population segregating at just two loci, A and B, the frequency of the preferred AABB homozygote is much smaller at just 1/16 than the frequency of A-B- carriers (AABB, AABb, AaBB, AaBb) at 9/16. With *n* polymorphic loci the frequency of homozygotes in F_2 is $1/4^n$, the frequency of carriers is $3/4^n$. F_2 enrichment will increase the frequency of desirable homozygotes to $2/3^n$ in inbred or DH lines produced from the selected F_2s that carry at least one copy of the target allele at all loci. Each selected F_2 will need to produce several progeny to make up the required number of lines in subsequent generations. Each selected F_2 should contribute equal numbers of progeny to the subsequent population in order to avoid changes in allele frequencies due to genetic drift.

Further to Figure 14.1, Table 14.1 shows the population sizes needed to use F_2 enrichment in a biparental cross in the F_2 generation and in later generation populations derived from the selected F_2s. For comparison it also shows the population sizes needed to recover homozygotes in different generations when enrichment has not been applied. It should be noted that progeny testing may be needed to determine if a selected individual is homozygous or heterozygous if markers are dominant. Co-dominant markers or higher levels of homozygosity remove the need for progeny testing.

Table 14.1. Population sizes required for enrichment (enrich) vs. fixation (fix) of target alleles in biparental F_2 populations and to obtain at least one target homozygous genotype in later generation enriched (enrich) and non-enriched (rand) populations for different numbers of segregating loci (Bonnett *et al.*, 2005).

	Population required for fix vs. enrich ($P = 0.05$)		Population size required to obtain a target homozygote at all loci in non-rand and enrich populations ($P = 0.05$)									
Gen	F_2		F_3		F_4		F_5		F_6		DH	
Loci	fix	enrich	rand	enrich	rand	enrich	rand	enrich	rand	enrich	rand	Enrich
1	11	3	7	5	6	4	5	3	5	3	5	3
2	47	4	20	11	15	8	13	6	12	6	11	6
3	191	6	56	23	35	14	28	11	25	10	23	9
4	766	8	151	47	81	25	61	18	53	16	47	14
5	3067	11	403	95	186	43	131	30	111	26	95	22
6	12270	16	1076	191	426	75	281	49	231	40	191	33
7	49081	21	2872	382	975	129	601	79	478	63	382	50
8	196327	29	7659	766	2231	222	1284	128	988	98	766	76
9	785312	39	20427	1533	5100	382	2741	205	2040	152	1533	114
10	3141252	52	54473	3067	11660	656	5848	329	4213	236	3067	172

Partial enrichment strategies - balancing early and late generation, marker and phenotypic selection

In reality, markers or efficient phenotypic screens will rarely be available for alleles at all important loci segregating in a cross and it will not be possible to enrich frequencies of these alleles in early generations. In the case of complex traits like yield that require homogeneous lines, large seed quantities and expensive phenotypic screens to achieve acceptable heritabilities, population sizes and early generation selection strategies must be designed to retain important allelic variation until later stages of the breeding process. In such cases, the frequencies of some alleles will be enriched and others will remain at the original frequencies, provided population sizes are large enough at all stages to avoid changes in frequency, or in extreme cases complete loss of desirable alleles, through genetic drift.

Although partial enrichment requires larger population sizes than if markers were available for alleles at all important loci, substantial reductions in population sizes can still be achieved compared with not enriching the frequency of any alleles. This can be seen in Table 14.2 which compares population sizes required to recover a target individual by alternative methods ranging from selecting homozygotes in F_2, delaying selection until DH (or inbred) lines are produced, F_2 enrichment at all target loci and partial enrichment at 50% of the target loci. Additionally, Figure 14.3 shows population sizes required in a cross with 10 important polymorphic loci and application of enrichment at between 5 and 10 of those loci compared with no enrichment (analogous to delaying selection until inbred lines are developed). Of course, enrichment should be applied at as many loci as possible, that is all loci for which markers or a phenotypic screen are available.

Often, the number of important loci contributing variation to yield or other important traits in a cross will not be known and partial enrichment can be applied by estimating the number of important polymorphic loci or deciding on a certain number of inbred lines to retain for phenotypic selection (e.g., for low heritability traits like yield). For example, a breeder may want to have 50 lines for yield testing after selecting homozygotes for the desirable allele at each of 6 loci. The population size for a 95% probability of recovering one individual with the 6 marker alleles will, on average, include 3 lines with the target genotype. Therefore, to recover 50

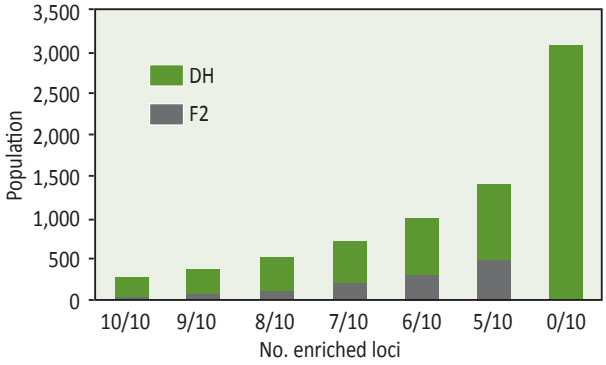

Figure 14.3. Population sizes (P = 0.05) to recover a target homozygote at 10 loci using F_2 enrichment at between 5 and 10 loci followed by DH production from selected F_2s. The population size for a non-enriched population (0/10) is shown for comparison. The lower part of the bar indicates the number of F_2s that must be screened and the upper part of the bar the number of DH lines that must be produced from the selected F_2s.

Table 14.2. Population sizes needed to recover a target genotype (P = 0.05) in biparental crosses segregating at different numbers of unlinked loci with different selection strategies (Passioura *et al.*, 2007).

Loci	A. Select homozygotes in F_2	B. Select only among inbreds	C. F_2 enrichment followed by selection among derived inbreds	D. F_2 enrichment at 50% of loci followed by selection among derived inbreds
2	46	10	6	8
4	765	46	17	24
6	12269	190	40	68
8	196327	765	92	184
10	3141251	3066	210	494
12	5.0×10^7	12269	475	1320
14	8.0×10^8	49081	1071	3523
16	1.3×10^{10}	196327	2413	9398
18	2.1×10^{11}	785312	5431	25066
20	3.3×10^{12}	3141251	12222	66845

such lines, this number can be multiplied by 50/3 (≈17) to recover 50 target individuals for yield testing. This multiplier must be applied at the F_2 enrichment stage and then through the rest of the breeding process. In this example the required population for F_2 enrichment at 6 loci with 95% probability of recovering one target individual is:

F_2 enrichment - 19 F_2s

DHs from selected F_2s - 40 DHs

On average 3 DHs with the target genotype will be recovered.

To recover 50 inbred lines for yield testing the numbers must be multiplied by 50 and divided by 3.

F_2 enrichment - 317 F_2s

DHs from selected F_2s - 667 DHs

On average 50 DHs with the target genotype at the 6 marker loci will be recovered.

This strategy can be compared with a partial enrichment strategy based on enrichment at 6 loci and estimated additional 4 important polymorphic loci for which markers are not available and enrichment cannot be applied. Population sizes required for 95% probability of recovering an individual homozygous for the desirable allele at all 10 loci is calculated as follows;

F_2 enrichment - 329 F_2s

DHs from selected F_2s - 668 DHs

On average, 3 DHs with the target genotype at all 10 loci will be recovered. Screening DHs with markers will result in selection of around 50 individuals with the target genotype at all marker loci. Phenotypic selection would then be needed to identify the individuals with the most desirable allele at the other 4 loci.

In the examples given here, the population sizes are almost the same. This illustrates that in the case where a breeder wants to recover 50 lines for yield testing, this population size would only retain sufficient variation to recover the most desirable combination at 4 additional loci. If more loci carried useful variation, the probability of recovering the ideal genotype from that cross would be relatively low.

The partial enrichment strategy described above was successfully applied in development of the Australian wheat variety 'Longreach Scout' (Plant Varieties Journal, 2009) combining low carbon isotope discrimination for high water-use efficiency through phenotypic selection in later generations following marker screening to enrich the frequency of key disease resistance and grain quality alleles in earlier generations. Population sizes were managed to retain variation for important alleles at unmapped loci. Another example of the use of partial enrichment is in the development of long coleoptile, *Rht8* germplasm lines through selecting at 6 marker loci and assuming a further 4 important polymorphic loci controlling variation in coleoptile length. Population sizes were managed to retain variation for phenotypic selection among inbreds (Wang *et al.,* 2009; Bonnett, unpublished). Coleoptile lengths as long as the donor line were recovered in BC_1 and biparental populations (Bonnett, unpublished). The estimate of numbers of important loci for coleoptile length and carbon isotope discrimination were taken from QTL mapping studies of three DH populations generated from common Australian varieties and breeding lines (Rebetzke *et al*., 2007, 2008a). Similar results were obtained for another water-use efficiency related trait, water-soluble carbohydrate concentration (Rebetzke *et al*., 2008b). In all of these studies, many of the loci differed between populations but around the same number accounted for roughly similar levels of variation in each. Therefore, although the identity of the loci segregating in any cross are likely to differ it seems that in crosses with roughly the same level of diversity between parents, the genetic architecture of the trait is often similar. This approach of estimating the number of important loci for a trait from QTL studies of similar populations could be applied for other complex traits such as yield or drought tolerance. The greater the number and relevance of populations and phenotyping environments, the greater the likely utility of this approach. If greater numbers of important loci are present in the population, it may not be possible to recover an individual with the most favorable allele at all loci but it may still be possible to make useful progress.

The above is a small set of examples to illustrate the principles and value of marker-assisted allele enrichment strategies that can be applied in breeding programs. In this section, tables and formulas are provided to allow breeders to calculate population sizes for a broader range of alternative strategies including those for backcross and topcross populations.

The frequency of a target genotype across multiple independent loci will be the product of frequencies of the target allele at each of the individual loci. This applies providing that all target alleles were present together in

at least the heterozygous state in F_1 and F_2. This condition may not be met if enrichment was not applied for donor (non-recurrent) alleles in backcross or topcross F_1s and only small numbers of F_1s were used as founders of the subsequent generations. If enrichment is not applied in the F_1 of such crosses, half of the F_1s will completely lack each target allele from the donor and the frequency of F_1s not carrying at least one copy of each donor allele will be high (for *n* loci the frequency of F_1s carrying the target alleles from the donor will be $1/2^n$. All carriers will be heterozygous for each donor allele. Therefore, special care needs to be taken to either enrich allelic frequencies by applying selection or keeping large populations to avoid substantial deviations from expected frequencies due to drift.

Table 14.3 shows frequencies of carriers and homozygotes for target alleles at single loci with a range of common initial allelic frequencies in different generations. This table can be used to calculate frequencies of carriers or homozygotes that can be selected in a desired generation. In populations with differing frequencies of target alleles at different polymorphic loci, the frequency of an individual with a particular genotype across all loci can be calculated by multiplying the individual frequencies at each locus. For example, in a biparental population in which F_2 enrichment has been applied for target alleles at 6 loci, the frequency of a genotype homozygous at all loci in the F_4 generation is $0.585^6 = 0.060$.

In a similar backcross population in which target alleles at 4 loci came from the recurrent parent and 2 from the donor with enrichment applied in the BC_1F_1 for donor alleles and in F_2 for donor and recurrent parent alleles the frequency of an individual in a DH population developed following F_2 enrichment would be 0.67^2 (donor alleles) × 0.857^4 (recurrent parent alleles) = 0.0494. This requires enrichment of the donor alleles in both BC_1F_1 (increasing frequency at each locus from 1/4 to 1/2) and subsequent enrichment in F_2 increasing the frequency of these donor alleles from 1/2 to 2/3. Enrichment of the recurrent parent alleles in BC_1F_2 increases their frequency from 3/4 to 7/8. In spite of the relatively high frequency of homozygotes for the recurrent parent alleles in a backcross, enrichment still requires smaller population sizes than selection of homozygotes (Bonnett *et al.,* 2005). Aside from requiring a larger population size, selection of homozygotes for recurrent parent alleles in BC_1F_1 or BC_1F_2 depends on availability of co-dominant markers.

Population sizes required to recover an individual with a target genotype are inversely related to the frequency of those individuals. A formula for calculating population size for any frequency and desired level of confidence of recovery is given in Bonnett *et al.* (2005). To achieve a commonly desired 95% probability of recovery, a useful rule of thumb is to multiply the inverse of the frequency by 3. For example with a frequency of 1/16, the population size needed for 95% probability of recovering the target genotype is 16 × 3 = 48. In other words:

Population size = (1/frequency of target genotype) × 3.

This formula applies regardless of whether the target genotype is homozygous or heterozygous.

Table 14.3. Frequencies of homozygotes (homo) and carriers of a target allele (*A*) for different allele frequencies and levels of inbreeding (Bonnett *et al.,* 2005).

Allelic frequency	1/4 (e.g., non-recurrent parent allele in BC1)		1/2 (e.g., biparental cross)		3/4 (e.g., recurrent parent allele in BC_1)		2/3 (e.g., following F_2 enrichment of biparental cross)		7/8 (e.g., following F_2 enrichment of recurrent parent allele in BC_1)	
Gen	Homo (AA)	Carrier (A-)	Homo (AA)	Carrier (A-)	Homo (AA)	Carrier (A-)	Homo (AA)	Carrier (A-)	Homo (AA)	Carrier (A-)
F_2	0.125	0.375	0.250	0.750	0.625	0.875	0.333	1.000	0.714	1.000
F_3	0.188	0.313	0.375	0.625	0.688	0.813	0.500	0.833	0.786	0.929
F_4	0.219	0.281	0.438	0.563	0.719	0.781	0.583	0.750	0.821	0.893
F_5	0.234	0.266	0.469	0.531	0.734	0.766	0.625	0.708	0.839	0.875
F_6	0.242	0.258	0.484	0.516	0.742	0.758	0.646	0.688	0.848	0.866
F_7	0.246	0.254	0.492	0.508	0.746	0.754	0.656	0.677	0.853	0.862
F_8	0.248	0.252	0.496	0.504	0.748	0.752	0.661	0.672	0.855	0.859
F_9	0.249	0.251	0.498	0.502	0.749	0.751	0.664	0.669	0.856	0.858
F_{10}	0.250	0.250	0.499	0.501	0.750	0.750	0.665	0.668	0.857	0.858
DH	0.250	0.250	0.500	0.500	0.750	0.750	0.667	0.667	0.857	0.857

Dominant and co-dominant markers

In all of the previous discussion, little mention has been made of whether markers used are dominant or co-dominant. Co-dominant markers have the advantage that it is possible to distinguish between homozygotes and heterozygotes. As has previously been discussed, trying to select only homozygotes in F_2 will substantially increase the population sizes needed so for F_2 enrichment dominant or co-dominant markers are almost equally useful. The advantages of co-dominant markers in enrichment are that they allow a more direct assessment of the frequencies of target alleles and if selected F_2s are homozygous for some target alleles, it may be possible to avoid screening their progeny for these alleles which could reduce marker screening costs. However, good tracking of individuals to their parent F_2 will be needed to allow this.

The other advantage of co-dominant markers is that they remove the need for progeny testing of selected later generation individuals (e.g., F_5 or F_6) to recover homozygotes. If progeny testing is not done, some selected individuals will be heterozygous for some of the target alleles. However, because the frequency of heterozygous individuals is halved with each generation of inbreeding, only relatively small numbers of selected F_6 individuals would be heterozygous at any of the target loci. Therefore, while co-dominant markers have some advantages they are usually not substantial.

Use of modified pedigree and bulk breeding methodologies

The previous discussion has assumed strict use of SSD or DHs, at least after selection of F_2s. This is a very extreme form of pedigree breeding methodology and may not be the most practical method in a breeding program. Nonetheless the same principles apply if other breeding methodologies are used. After enrichment in F_2 (and the F_1 of complex crosses), inbreeding by whatever system will ultimately produce the same frequency of target homozygotes as would be produced through SSD, providing selection for other traits does not affect frequencies of the target alleles through linkage or pleiotropy and population sizes remain large enough to avoid changes in allele frequencies due to drift. Bulk breeding methodologies may be a very efficient means of progressing populations to homozygosity while selecting for other traits and provided this selection does not cause changes in the frequencies of 'target' alleles following the enrichment step/s due to linkage, pleiotropy, or genetic drift, the expected frequencies of target genotypes should be similar to those predicted.

Linkage between target loci, imperfect linkage between markers and target alleles

With greater numbers of markers available for selection, it is inevitable at some point that a cross will involve target alleles that are linked. If they are linked in coupling they will behave more like a single gene and required population sizes will be smaller than if they were unlinked. If they are linked in repulsion and a crossover between the loci is necessary to bring the target alleles together on the same chromosome, required population sizes will be considerably larger. If target alleles are linked in repulsion it will usually be best to first recover a recombinant with the target alleles in coupling and then focus on combining the other alleles (Wang *et al.,* 2007).

If markers are not perfectly linked with target alleles, population sizes will need to be larger to allow for recombination between the marker and the target allele. If selection is only applied for homozygotes in later generations, the imperfect linkage does not affect the frequency of target individuals in the population, it just leads to some level of inaccuracy in identifying them. If used in F_2 enrichment, the change in allele frequency will be slightly less than if markers were perfect. In spite of a slight reduction in efficiency, use of imperfect markers still increases allele frequencies and is very worthwhile even with relatively high levels of recombination between marker and allele. In such cases flanking markers can be very useful. Table 14.4 shows the frequency of a target allele at one locus with different

Table 14.4. Allele frequency after selection with imperfect markers (Wang *et al.,* 2007).

Selection method	Marker type	Distance between marker and gene		
		1 cM	5 cM	10 cM
Homozygous selection in F_2	Single marker	0.991	0.954	0.910
	Flanking markers	1.000	0.998	0.990
Homozygous selection in F_{10}	Single marker	0.980	0.912	0.846
	Flanking markers	0.999	0.988	0.959
Enrichment selection in F_2, homozygous selection in F_{10}	Single marker	0.982	0.914	0.847
	Flanking markers	0.999	0.987	0.963

selection methodologies and levels of recombination between marker and target allele. With perfect markers the frequencies would all be 1. For more details on using imperfect markers refer to Wang *et al.,* (2007).

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WHEAT

Providing a basis for the development of sustainable cropping systems

Chapter 15: The principles of conservation agriculture

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Abstract

Today's global cultivated area has been strongly degraded. Agriculture should not only be high yielding, but also sustainable. Agriculture contributes significantly to greenhouse gas (GHG) emissions: carbon dioxide, methane and nitrous oxide. Promoting agricultural practices that mitigate climate change by reducing GHG emissions is important; but those same practices also have to improve farmer production and income and buffer the production system against changes in climate. Conservation agriculture (CA), based on minimal soil disturbance, permanent ground cover and crop rotations is a management system that achieves these goals. CA improves soil aggregation, infiltration is generally higher and runoff reduced, thus soil moisture is conserved and more water is available for crops in CA compared to conventional systems. Temperature fluctuations are smaller in CA. The residue retained on the soil surface provides residue-borne pathogens and beneficial soil micro-flora species with substrates for growth, and pathogens are at the soil surface, where spore release may occur. This can induce major changes in disease pressure in CA systems. However, functional and species diversity are also increased, creating more possibilities for integrated pest control. Water and wind erosion is also reduced by CA since the soil surface is protected and water runoff is lowered as more water enters the soil profile. CA-based systems drive on a set of principles that have to be adapted to each specific situation. It is unlikely that complex, multi-component technologies such as CA-based systems can be successfully scaled out through traditional linear models of research and extension: instead they require the development of innovative systems to adapt technologies to local conditions. Therefore, there is need for adaptive research and extension systems that install working examples of CA within the different agro-ecological areas and farming systems.

Introduction

Human efforts to produce ever-greater amounts of food leave their mark on our environment. Persistent use of conventional farming practices based on extensive tillage, especially when combined with removal or *in situ* burning of crop residues, have magnified soil erosion losses and the soil resource base has been steadily degraded (Montgomery, 2007). Many soils have been worn down to their nadir for most soil parameters essential for effective, stable and sustainable crop production, including soil physical, chemical and biological factors. Despite the availability of improved varieties with increased yield potential, the potential increase in production is not attained because of poor crop system management (Reynolds and Tuberosa, 2008). Another direct consequence of farmers' persistent use of traditional production practices is rapidly increasing production costs associated with the inefficient use of inputs whose costs continue to rise. In addition, any new, more sustainable management strategy must be compatible with emerging crop diversification policies that may evolve to meet new consumer or industrial requirements. All of this must be accomplished within a scenario of decreasing area available for crop production because of urbanization and industrial expansion and the recent dramatic increases in the use of land for biofuel and industrial crop production. Farmers concerned about the environmental and economical sustainability of their crop production systems have begun to adopt and adapt improved systems management practices that lead towards the ultimate vision of sustainable conservation agriculture (CA) based systems.

Conservation agriculture (CA)

The name CA has been used over the last seven years to distinguish this more sustainable agriculture from the narrowly-defined 'conservation tillage' (Wall, 2006). Conservation tillage is a widely used terminology to characterize the development of new crop production technologies that are normally associated with some degree of tillage reduction, for both pre-plant as well as in-season mechanical weed control operations that may result in some level of crop residue retention on the soil surface. The definition of conservation tillage does not specify any particular, optimum level of tillage, but it does stipulate that the residue coverage on the soil surface should be at least 30% (Jarecki and Lal, 2003). CA, however, removes the emphasis from the tillage component and addresses a more enhanced concept of

the complete agricultural system. CA is based on three principles: (i) minimal soil disturbance, (ii) soil cover with crop residues, and (iii) crop rotation. However, there is considerable misunderstanding as to what actually constitutes CA. There are those who advocate that "true CA" involves only the use of continuous zero till seeding in a narrow slit into untilled soils combined with permanent coverage of the soil surface with crop residues. CA practiced in this manner has been implemented successfully, particularly for rainfed production systems. Derpsch (2005) estimated that in 2005, there was over 96 million ha of zero till CA worldwide with over 90% of this area used primarily in rainfed production systems in five countries (USA, Brazil, Argentina, Canada and Australia). Thus, less than 10% of the zero till CA area occurs in the rest of the world.

It is, therefore, very apparent that there are many crop production systems in the world at large where the application of CA-based only on zero till seeding with permanent soil residue cover is not currently being used and may never be. However, there is a consensus developing that asserts that the best application or use of CA is defined by a set of principles (Kassam *et al.,* 2009) which can be applied essentially to all crop production systems and that these CA-based principles can provide the foundation to support most crop management/ improvement activities. These CA principles are applicable to a wide range of crop production systems from low-yielding, dry, rainfed conditions to high-yielding, irrigated conditions. However, techniques to apply the principles of CA will be very different in different situations, and will vary with biophysical and system management conditions and farmer circumstances. Specific and compatible management components (pest and weed control tactics, nutrient management strategies, rotation crops, appropriately-scaled implements etc.) will need to be identified through adaptive research with active farmer involvement.

Appropriate CA-based technologies encompass the following basic principles:

CA Principle 1 - Marked reductions in tillage

The objective is the application of zero tillage or controlled tillage seeding systems that normally do not disturb more than 20–25% of the soil surface (strip till or permanent raised bed planting systems, with only superficial reshaping in the furrows between the raised beds as needed before planting of each succeeding crop).

Zero till seeding *per se* may be a desired objective where practicable, however, in other systems some type of soil movement may be necessary. For example, under gravity-fed irrigated conditions, a permanent raised-bed system with furrow irrigation may be more suitable and sustainable than a reduced or zero tillage system on "the flat" to replace the widely used, conventionally tilled system of flood irrigation on flat land. Permanent raised beds are not tilled but only reshaped as needed between crop cycles. One to four rows are planted on top of the bed, depending on the bed width and crop, with irrigation. Figure 15.1 compares the land preparation associated with a conventional tillage system versus a furrow irrigated CA-based, permanent raised bed planting system using a single pass to reshape the beds (with no tillage disturbance on top of the bed), band basal fertilizer

Figure 15.1. (A) Conventional till system versus (B) permanent raised bed system.

and seed the subsequent crop – maize (*Zea mays* L.) in this case into retained wheat (*Triticum aestivum* L.) residues from the previous crop.

CA Principle 2 - Retention of adequate levels of crop residues on the soil surface

The objective is the retention of sufficient residue on the soil surface to protect the soil from water/wind erosion, water run-off and evaporation to improve water productivity and to enhance soil physical, chemical and biological properties associated with long term sustainable productivity.

To illustrate the importance of this principle, Figure 15.2 compares rain water runoff and associated potential erosion for raised beds following full tillage with full residue incorporation (left) versus permanent raised beds with full residue retention (right) after an intense rain event in the CIMMYT high rainfall humid (2,640 masl; 19.17°N, 99.33°W, silty clay loam soil of volcanic origin) experimental station in the central highlands of Mexico. The conventionally tilled field results in ponding water and a flooded crop at the lower end of the field; while in an adjacent CA field with permanent raised beds and residue retained on the soil surface the standing water is in the furrows, slowly infiltrating, resulting in no standing water at the lower end of the field.

Again, the importance of residue retention on the surface can be seen in the photos in Figure 15.3 for rainfed maize plots in the central highlands of Mexico. These plots were seeded with the same hybrid, on the

Figure 15.2. The result of a severe rainfall event (30 mm) in (A) a conventionally tilled field, and (B) an adjacent conservation agriculture field with permanent raised beds and crop residue retained on the soil surface in the CIMMYT high rainfall humid (2,640 masl; 19.17°N, 99.33°W, silty clay loam soil of volcanic origin) experimental station in the central highlands of Mexico. (Photographs: K.D. Sayre and F. Delgado).

Figure 15.3. (A) Permanent raised beds with full residue removal, and (B) full residue retention.

same day with the same crop management practices except for removal (left) and surface retention (right) of the previous wheat crop residues. These photographs were taken in late August 2009, following a period of very low rainfall from early July to late August (89 mm for this period in 2009 versus 233 mm for the long-term average) which, once again, very clearly demonstrates the value of surface residue retention and water use for the CA-based seeding system.

CA Principle 3 - Use of sensible crop rotations

The objective is to employ economically viable, diversified crop rotations to help moderate possible weed, disease, and pest problems; enhance soil biodiversity; take advantage of biological nitrogen fixation and other soil enhancing properties; reduce labor peaks; and provide farmers with new economic opportunities that can entail risk reductions through crop diversification.

Figure 15.4 illustrates a partial selection of crops that are being managed with the CA-based irrigated, permanent raised bed planting system in northwest Mexico.

Although irrigated spring wheat seeded during the winter cropping season in northwest Mexico is the main crop, many farmers realize that they can benefit if there are practicable economic opportunities to successfully grow other crops in their production systems. But to do this in a manner that will allow the application of the basic principles of CA across their production systems, relevant CA-based management practices for all crops including wheat must be developed as illustrated in the photographs in Figure 15.4.

Including an annual wheat crop in the system still remains the dominant practice in northwest Mexico, however, Figure 15.5 illustrates the potential positive effects associated with the diversification of the crop rotation for the irrigated wheat-based system on wheat

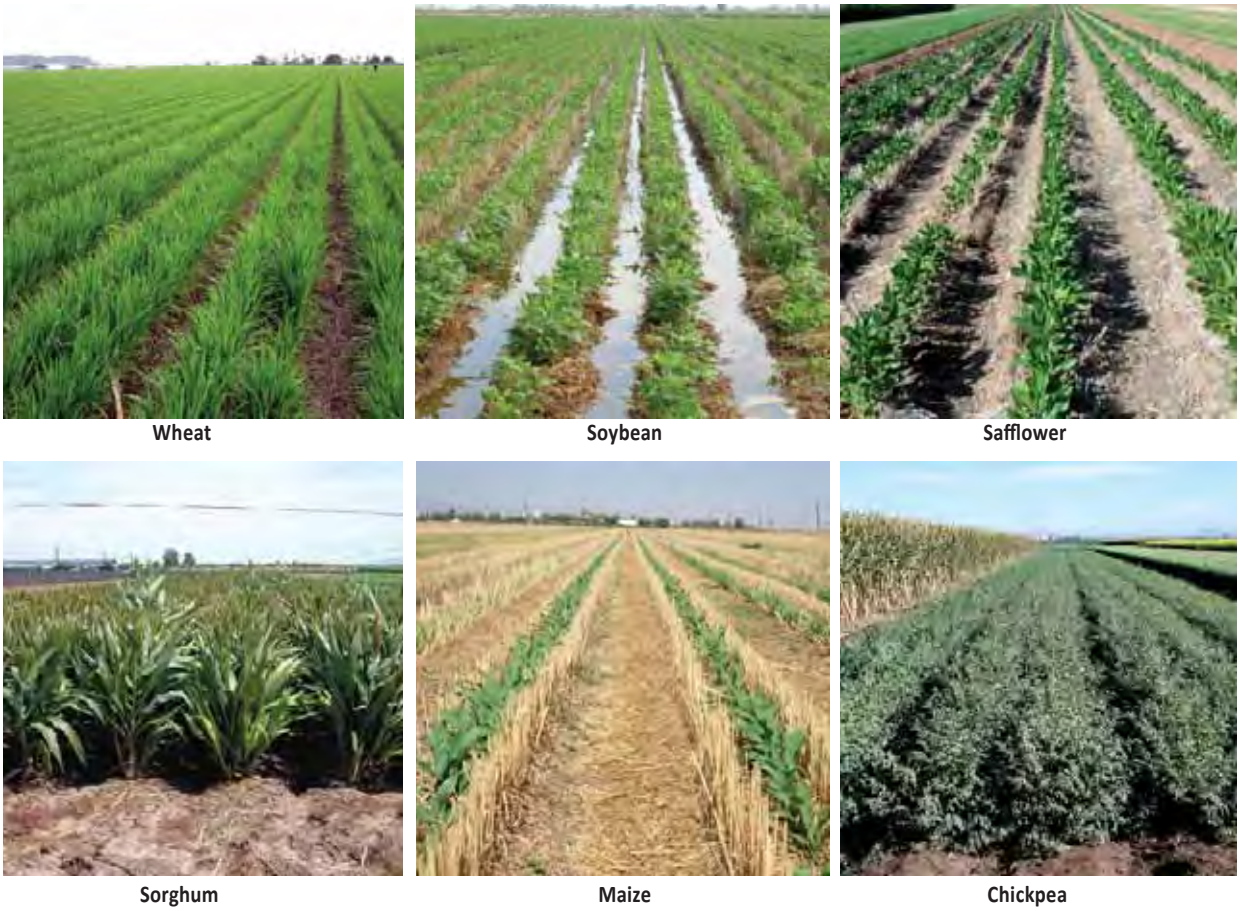
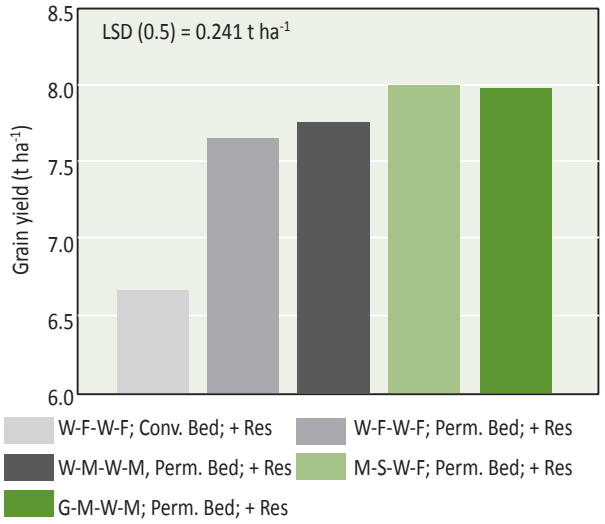


Figure 15.4. Examples of crops that are being used to diversify irrigated wheat-based cropping systems using permanent raised beds in northwest Mexico.

yields *per se* in northwest Mexico. Currently the most common rotation used by farmers is wheat followed by a summer fallow. As can be observed in Figure 15.5, wheat yields, averaged over four years, increase only slightly but significantly as the rotation is diversified from wheat–summer fallow to wheat–summer maize to wheat combined with several, alternative crops over a two year rotational sequence. Economic analyses are underway to determine the economic viability of the crops being used, with CA-based crop management practices, to diversify the rotation.

What is perhaps of more interest in Figure 15.5 is the marked yield advantage that is observed for wheat in the wheat–fallow rotation with permanent beds maintained with only occasional reshaping in the furrow between the beds with no tillage on top of the beds and with residues retained on the soil surface as compared with the common farmer practice of wheat–summer fallow using conventional tillage with full incorporation of the wheat residues. The marked reduction in tillage with surface residue retention with the permanent raised beds provides a distinct advantage over the current farmer practice that is still not clearly understood.



Where: W = wheat; F = fallow; M = maize; S = sorghum; G = chickpea. '+Res' = residue retention.

Figure 15.5. Effect of tillage, residue management and rotation on wheat grain yield averaged for 2006, 2007, 2008 and 2009 at CENEB, Yaqui Valley, Sonora, Mexico.

CA Principle - Farmer conviction of immediate economic benefits/livelihoods

The objective is to secure farm level economic viability and stability to enhance livelihoods by the adoption of innovative CA-based crop management technologies that are focused on the needs of farmers based on their various biophysical and socioeconomic conditions.

Since farmer adoption of CA-based crop management practices can involve several different changes (seeding equipment, residue management, weed control, disease and pest management, nutrient management, rotational crops etc.), farmers must closely participate in the development, testing, modification and demonstration/ extension (with as many of these efforts as possible performed directly in farmer fields) of new, CA-based production practices. This is by far the most effective manner for farmers to more fully understand how and why they should implement these technologies in their fields. It is also the most efficient way for farmers to better perceive the potential economic benefits that may be gained by adoption of these technologies. Farmers tend to believe other farmers more readily than they believe the results of many scientists and extension agents.

Figure 15.6 includes the wheat yields, estimated variable production costs and returns above variable costs from farmer modules in northwest Mexico, averaged over five cropping cycles. These results are derived from a large-

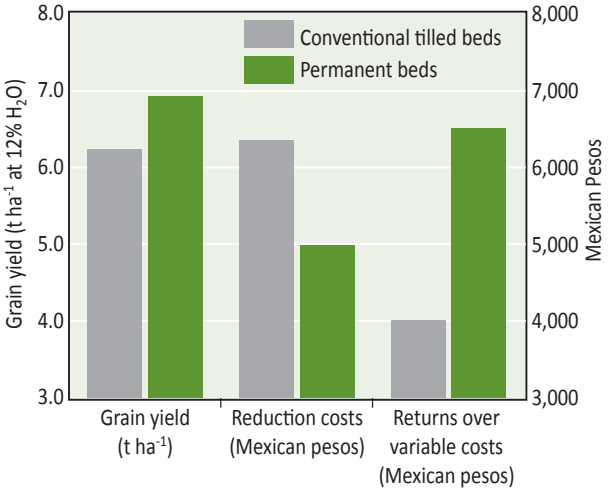


Figure 15.6. Comparison of average wheat grain yields, variable production costs and returns over variable costs of wheat produced with conventional tilled beds versus permanent raised beds. Conservation agriculture trial on irrigated wheat systems, Yaqui Valley, Sonora, Mexico, 1993–2006.

scale trial/farmer demonstration module where crops are planted when possible for each planting system (usually 7–10 days earlier for wheat in the permanent beds as compared to tilled beds due to faster turn-around between crops). As can be observed there is a moderate yet significant increase in wheat yield and a marked reduction in variable production costs which, together, have generated a markedly higher economic return over variable costs for the CA-based, permanent bed planting system as compared to the current farmer practice. The fact that the modules that generated this information were done with full involvement and participation of farmers in the region has had a large influence in encouraging farmers to adopt the permanent raised bed technology.

The basic principles of CA outlined above are not location or cropping system specific but provide the foundation to tailor and integrate needed tactical crop management practices/components (seeders/implements, crop residue management, cultivars, weed, disease and pest control practices, fertilizer and irrigation management etc.) that must be developed, tested and modified as needed for application to a given crop production system. The principles of CA-based technologies can provide the primary, tactical foundation for integrated actions applicable to essentially all crop production systems, from low-yielding, dry rainfed conditions to high-yielding irrigated conditions and which are relevant from large to small-scale farmers (Figure 15.7). What should be very clear is that CA should not be considered a separate "discipline" *per se* but a strategy based on the three CA-based principles outlined that can guide efforts towards the development of new, sustainable cropping practices.

CA results in sustainable cropping systems

The decision by many farmers (and scientists) to embark upon the development and use of CA-based technologies is largely justified by what is now known about the widespread issues of soil degradation related to the extensive use of intensive tillage, crop residue removal/burning and widespread mono-cropping. A list (but not an all inclusive list) of potential benefits that farmers may gain by adoption of sound CA-based crop management practices includes the following:

- Stabilize/reverse widespread soil erosion/degradation to improve sustainable use of natural resources land, water and air (Verhulst *et al.,* 2010).
- Enhance water use efficiency for both rainfed and irrigated crop production systems.
- Increase crop productivity by increasing time use (faster turn-around times between crops) and input use efficiency (Hobbs and Govaerts, 2010).
- Provide opportunities to better cope with and/or mitigate the potential adverse effects of climate change (Hobbs and Govaerts, 2010).

Figure 15.7. Use of CA-based seeders with 2-wheel tractors in Bangladesh for (A) strip till seeding of wheat after rice, and (B) zero till seeding of chickpea.

- Reduce production costs and increase cropping opportunities for farmers to better improve rural family livelihoods and reduce risk. Generally, the off-site public benefits of CA exceed the on-farm private benefits (Knowler and Bradshaw, 2007).

Verhulst *et al.* (2010) made an extensive review of the existing literature, summarizing the effects of CA for different systems on soil quality. A summary of some of the findings is presented in Table 15.1. The resulting improved soil quality and improved nutrient cycling will improve the resilience of crops to adapt to changes in local climates. The minimal soil disturbance and soil cover will protect the biological component of the soil and help with biological tillage, keeping pests and diseases under control through biological diversity processes and making nutrients available to plants for good growth.

Table 15.1. Overview of the system (conservation agriculture and conventional practices) with increases indicated for some key soil parameters in different cropping systems (Adapted from Verhulst *et al.*, 2010; Hobbs and Govaerts, 2010).

Conservation system (CA)	System with an increase for the selected parameter	Reference
Soil aggregation and structural stability		
Zero-tillage + residue	CA	Govaerts *et al*., 2009
Minimum-till + residues	CA = CT	Hulugalle *et al*., 2006
Minimum-till + residues	CA	Hulugalle *et al*., 2007
Zero-tillage + residue	CA	Kennedy and Schillinger, 2006
Permanent raised beds + residues	CA	Govaerts *et al*., 2007b
Permanent raised beds + residues	CA	Limon-Ortega *et al*., 2006
Zero-tillage + residue	CA	Mikha and Rice, 2004
Zero-tillage + residue	CA	Roldan *et al*., 2007
Zero-tillage + residue	CA	Franzluebbers, 2002
Soil water content		
Zero-tillage + residues or manure	CA	Anikwe *et al*., 2003
Zero-tillage + residue	CA	Govaerts *et al*., 2009
Permanent raised beds + residues	CA	Govaerts *et al*., 2007b
Minimum-till + residues + cotton/wheat	CA	Hulugalle *et al*., 2002
Zero-tillage + residue	CA = CT	Kennedy and Schillinger, 2006
Zero-tillage + residue	CA	Bescansa *et al*., 2006
Zero-tillage + residue	CA	Fabrizzi *et al*., 2005
Zero-tillage + residue	CA	Kemper and Derpsch, 1981
Zero-tillage + residue	CA	Azooz and Arshad, 1995
Zero-tillage + residue	CA	Johnson *et al*., 1984
Infiltration		
Zero-tillage + residue	CA	Govaerts *et al*., 2007a
Permanent raised beds + residues	CA	Govaerts *et al*., 2007b
Zero-tillage + residue	CA	McGarry *et al*., 2000
Zero-tillage + residue	CA	Zhang *et al*., 2007
Zero-tillage + residue	CA	Pikul and Aase, 1995
Zero-tillage + residue	CA	Cassel *et al*., 1995
Zero-tillage + residue	CA	Freebairn and Boughton, 1985
Zero-tillage + residue	CA	Thierfelder *et al*., 2005
Permanent raised beds + residues	CA	Verhulst *et al*., 2009
Erosion		
Zero-tillage + residue	CT	Cassel *et al*., 1995
Zero-tillage + residue	CT	Freebairn and Boughton, 1985
Zero-tillage + residue	CT	Thierfelder *et al*., 2005
Zero-tillage + residue	CT	Kemper and Derpsch, 1981
Permanent raised beds + residue	CT	Verhulst *et al*., 2009
Zero-tillage + residue	CT	Zhang *et al*., 2007
Zero-tillage + residue	CT	Schuller *et al*., 2007
Zero-tillage + residue	CT	Montgomery, 2007
Earthworm populations		
Zero-tillage + residue	CA	Kladivko, 2001
Zero-tillage + residue	CA	Barnes and Ellis, 1979
Zero-tillage + residue	CA	Gerard and Hay, 1979
Soil sodicity and salinity		
Permanent raised beds + residues	CT	Govaerts *et al*., 2007b
Minimum tillage	CT	Hulugalle and Entwistle, 1997
Permanent raised beds + residues	CT	Sayre, 2005
Zero-tillage + residue	CT = CA	Du Preez *et al*., 2001
Zero-tillage + residue	CT = CA	Franzluebbers and Hons, 1996
Fuel use		
Zero-tillage	CT	Erenstein *et al*., 2008
Zero-tillage + residue	CT	West and Marland, 2002
Zero-tillage + residue	CT	Wang and Dalal, 2006
Zero-tillage + residue	CT	Robertson *et al*., 2000

CA= Conservation agriculture; CT= Conventional tillage based system.

With the above outlined potential benefits from appropriate CA-based crop production practices, it is certainly difficult and problematic to understand why most scientists, who are working on the various facets of crop management, still continue to center their efforts to merely fine-tune crop management practices based on continued use of extensive tillage in lieu of following CA-based principles to better guide their efforts to develop the sustainable, more efficient technologies needed by farmers. It seems logical, effective and efficient if the principles of CA outlined above could be "mainstreamed" to provide the underlying motivation for essentially all efforts to improve crop management and crop improvement practices. This seems imminently logical, especially for agronomists, soil scientists and weed scientists.

CA for breeders and physiologists

One major question that remains acutely unresolved is how plant breeders may need to modify their breeding strategies and field selection methodologies to develop the new cultivars appropriate for farmers that already have or will soon adopt CA-based production practices, especially those based on reduced/zero till seeding systems with retention of crop residues on the soil surface. Nearly all plant breeders continue to conduct all their field selection/screening activities using conventional till field preparation and seeding and very few even test advanced lines under the CA-based seeding practices that many farmers in their targeted areas have already or will soon adopt. This is clearly a high priority issue that needs rapid resolution to better guide plant breeding efforts. Therefore, physiologists in collaboration with agronomists should guide the breeders and determine the agronomic and physiological basis for improved or different plant performance with CA as opposed to conventionally tilled systems. At CIMMYT in Mexico, wheat breeders, agronomists and physiologists are considering this breeding methodology issue.

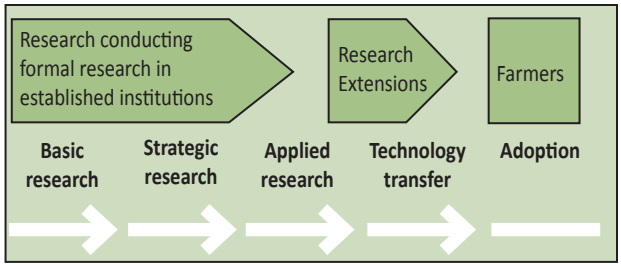


Figure 15.8. Traditional linear flow of knowledge/new technologies from researchers to farmers (Ekboir *et al.*, 2001).

Strategy for the integrated, multi/interdisciplinary development and delivery of CA-based crop management practices

The development, testing and extension to farmers of new crop production technologies has traditionally followed a linear process (Figure 15.8). This technology delivery progression has tended to function fairly well when new, usually single component technologies have been extended to famers for adoption (a new cultivar, herbicide or pesticide, etc.).

However, in recent years, farmers interested in improving their crop production systems in more sustainable and economically viable ways have begun to adopt and adapt improved crop management practices which focus more closely on their complete agricultural system which then may require the integrated and sometimes simultaneous application of a number of new technology components (new reduced/zero till seeding systems that may require new weed and nutrient management options and perhaps a different cultivar etc.) which will require the interaction of a number of partners working as cohorts to generate the needed integrated crop management practices. Therefore, the linear approach to extension is not very efficient in the case of CA. Farmers have to see for themselves and overcome their apprehensions before they are willing to adopt this new technology. A network of stakeholders has to be developed in order to address various issues that arise during adoption. Researchers and extension agents need to interact with farmers to address issues and problems that arise during the initial phases of CA adoption. Local manufacturers need to be actively involved with farmers to identify improvements to machinery that lead to better performance. Banks and credit agencies are needed to provide funds for farmers to buy equipment. Input agencies are needed to supply fertilizers and other inputs needed for good yields. These can be coordinated through public institutions or through public–private collaborations. However, it is clear that rather than a linear line of adoption an inter actor innovation process has to be promoted.

Therefore, a network of decentralized learning hubs within different farming systems and agro-ecological zones should be developed (Sayre and Govaerts, 2009). In those hubs, an intense contact and exchange of information is organized between the different partners in the research and extension process. Multiple actors within the production system (farmers, scientists, machine builders, decision makers, input suppliers, etc.), come together, work together, and learn together in order to multiply this effort in an intense extension and out-scaling process. Because of the multi-faceted nature of CA technology development and extension, activities should be concentrated in a few defined locations representative of certain farming systems rather than having lower intensity efforts on a wider scale. Through the research and training, regional CA networks are established to facilitate and encourage research and the extension of innovation systems and technologies. Research at the hubs also provides an example of the functionality of CA systems, helping to break down the culture of the plough. The hubs are linked to the strategic science platforms operated by international centers and national research institutes to synthesize a global understanding of CA, and its adaptability to different environments, cropping systems and farmers' circumstances. Innovative farmers are intensively involved and are the key factor for the build up and extension of a successful CA network that leads to a sustainable impact (Figure 15.9).

At CIMMYT in Mexico, CA-focused agronomists have currently developed three CA-based Research and Delivery Hubs which focus on irrigated wheat-based systems in northwest Mexico and on low rainfall, rainfed maize/small grain systems and high rainfall, rainfed systems in the central Mexican highlands. The hubs provide the needed structure at the field level, both on station if merited and in farmer fields. Scientists of all relevant disciplines have the opportunity to interact and test their proposed component technologies in an integrated manner, under CA-based circumstances, to identify their most relevant component technologies. These can then be combined together with relevant technologies coming from other disciplines in a long-term trial situation to determine potential, component technology interactions and to observe their medium to long term effects as compared to the common farmer practices in use. At the same time, the best bet CA-based technologies versus farmer practice in a number of CA-based delivery modules in farmer fields with active farmer participation in all hubs can be identified and tested. These hubs have allowed the evolution and practical application of the two most crucial aspects necessary to provide farmers with needed sustainable, CA-based technologies: (i) the opportunity/venue to encourage the needed multi/interdisciplinary collaboration between scientists, change agents and public and private

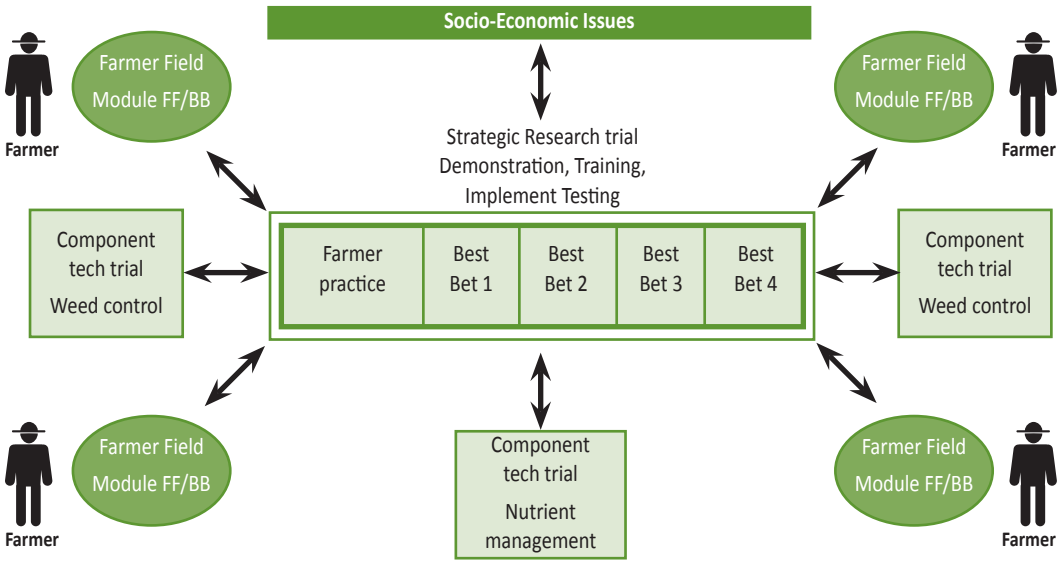


Figure 15.9. Structure and essential elements for a CA-based Research and Delivery Hub crucial to the development, testing and deployment of new, integrated CA-based, crop management practices with active farmer participation. FP/BB = Farmer Practice/Best Bet.

sector participants to generate integrated, holistic CA-based crop management practices that farmers need to confront their integrated farm level requirements, and (ii) the opportunity to insure that there is active farmer participation throughout all aspects of the hub activities to insure relevance. The approach is geared to make scientists better farmers, and farmers better scientists.

Conclusions

The consequences that will be faced to feed, clothe and fuel an ever increasing and demanding world population if new, more effective and integrated efforts to develop the higher yielding, sustainable and economically viable crop management practices needed by farmers are not implemented, will potentially be catastrophic. The issue of developing and then implementing the required strategies to elaborate and then deploy the needed crop management practices that will meet the production/yield requirements in an economically viable way while both stabilizing/reversing the widespread soil degradation that is occurring in most tillage-based production systems and providing better opportunities to mitigate climate change effects is crucial.

It seems completely logical that the application of the principles of CA as the foundation for the development of new, integrated crop management practices provides the way forward. Furthermore, the use of CA-based Research and Delivery Hubs focused on priority cropping/production systems can provide the necessary means to better insure that these crop management practices meet farmer needs since the farmers themselves are active participants in all hub activities.

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