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RESEARCH PAPER

Histological characterization of root-knot nematode resistance in cowpea and its relation to reactive oxygen species modulation

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Abstract

Root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites with a broad host range which includes economically important crop species. Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume grown in many regions where root-knot nematodes are a major problem in production fields. Several sources of resistance to root-knot nematode have been identified in cowpea, including the widely used *Rk* gene. As part of a study to elucidate the mechanism of *Rk*-mediated resistance, the histological response to avirulent *M. incognita* feeding of a resistant cowpea cultivar CB46 was compared with a susceptible near-isogenic line (in CB46 background). Most root-knot nematode resistance mechanisms in host plants that have been examined induced a hypersensitive response (HR). However, there was no typical HR in resistant cowpea roots and nematodes were able to develop normal feeding sites similar to those in susceptible roots up to 9–14 d post inoculation (dpi). From 14–21 dpi giant cell deterioration was observed and the female nematodes showed arrested development and deterioration. Nematodes failed to reach maturity and did not initiate egg laying in resistant roots. These results confirmed that the induction of resistance is relatively late in this system. Typically in pathogen resistance HR is closely associated with an oxidative burst (OB) in infected tissue. The level of reactive oxygen species release in both compatible and incompatible reactions during early and late stages of infection was also quantified. Following a basal OB during early infection in both susceptible and resistant roots, which was also observed in mechanically wounded root tissues, no significant OB was detected up to 14 dpi, a profile consistent with the histological observations of a delayed resistance response. These results will be useful to design gene expression experiments to dissect *Rk*-mediated resistance at the molecular level.

Key words: Cowpea, histology, hypersensitive response, *Meloidogyne incognita*, reactive oxygen species, root-knot nematode, *Vigna unguiculata*.

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a food and fodder legume of significant economic importance worldwide especially in semi-arid regions of Africa. It is also grown in North and South America, southern Europe, and Asia. Cowpea is cultivated in an estimated area of 12.5 million hectares with an annual production of three million tonnes of dry grains worldwide (Singh *et al*., 1997). In the United States, cowpea is a crop of minor interest grown on an area of about 80 000 hectares (Fery, 1985, 1990).

Root-knot nematodes (RKN) are one of the most important nematode pests of crop plants and have a diverse host range. RKN (*Meloidogyne* spp.) are sedentary root endoparasites and are involved in the development of specialized feeding structures known as giant cells. The infective stage of the nematode is the second-stage juvenile (J2). The J2 penetrate the roots and go through three successive moults to become adult females or males. Several of the most important root-knot nematode species,

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Abbreviations: CPS, counts per second; DCFH, dichlorofluoroscein; DPI, diphenylene-iodonium; HR, hypersensitive reaction; RKN, root-knot nematode; ROS, reactive oxygen species.

including *M. incognita*, reproduce by obligate mitotic parthenogenesis (Jung and Wyss, 1999).

The mechanism of feeding site development by root-knot nematodes is not well understood as it is a very dynamic and complex process involving genes from both the nematode and the host plant. The secretions from the oesophageal glands of the nematode are important in initiating the development of feeding structures (Davis *et al*., 2000; Williamson and Kumar, 2006). The first sign of giant cell induction by RKN is the formation of a binucleate cell. Rapid divisions of the nuclei continue in the absence of cytokinesis (acytokinetic mitosis) which gives rise to several large multinucleate cells. The surrounding cells divide to form the characteristic galls often known as 'root-knots' (Gheysen and Fenoll, 2002). The xylem parenchyma cells become transfer cells by forming finger-like wall invaginations (Jones and Northcote, 1972). This helps in water transport from the xylem to the feeding sites.

Root-knot nematodes are important pests of cowpea worldwide and host plant resistance is a preferred strategy for managing this problem in infested cowpea fields (Roberts *et al*., 1995; Ehlers *et al*., 2002). The *Rk* locus in cowpea has been used extensively to breed root-knot nematode resistant varieties in the USA and other countries. This gene locus was first designated as *Rk* by Fery and Dukes (1980) and it confers resistance to many populations of *M. incognita, M. arenaria, M. hapla*, and *M. javanica*. Genetic studies have indicated that this locus may have several alleles including *rk, Rk*, and *Rk2*. *Rk2* confers broad-based resistance to different races of *M. incognita* and *M. javanica* (Roberts *et al*., 1996). It is to be confirmed whether *Rk2* is an allele at the *Rk* locus or is a tightly linked separate locus. A single recessive gene unlinked to *Rk*, which confers broad-based additive resistance when combined with *Rk*, was identified and named *rk3* (Ehlers *et al*., 2000). These resistance loci provide a good resource for future studies and cultivar development.

Compatible and incompatible reactions lead to differential plant responses to nematode infection. A complex cascade of plant genes is activated upon nematode invasion and there are some visible reactions observed in the plant cells (Williamson, 1999). Based on the limited number of reported studies, a common response to root-knot nematode attack in host plants carrying a resistance gene is an early hypersensitive reaction (HR)-mediated cell death around the nematode feeding site, which prevents the nematode from further feeding resulting in nematode death. For example, strong early HR responses have been observed in *Mi-1*-mediated resistance in tomato (Williamson, 1999), *Mex-1*-mediated resistance in coffee (Anthony *et al*., 2005), Me_3-mediated resistance in pepper (Pegard *et al*., 2005), and incompatible interactions in soybean (Kaplan *et al*., 1979). Accumulation of phenolic compounds, especially chlorogenic acid, at the site of infection was also reported in resistant pepper roots by Pegard *et al*. (2005).

Non-hypersensitive reactions have been observed in $Hsp1^{pro-1}$-mediated resistance in sugarbeet against the cyst nematode *Heterodera schachtii*, where the J2 died due to degradation of the feeding structure (Holtmann *et al*., 2000). A delayed hypersensitive cell death was observed in the case of *Hero*-mediated gene responses in tomato against the cyst nematodes *Globodera pallida* and *G. rostochiensis* (Sobczak *et al*., 2005), in which the nematodes became sedentary and died at a late juvenile stage due to HR-mediated cell death in the developed syncytium. Gene H_1 confers resistance to *G. rostochiensis* pathotype Ro1 in potato. Studies of root ultrastructure in resistant potato plants harbouring gene H_1 showed an early HR around the J2 (Rice *et al*., 1985; Williamson, 1999). Here, syncytial development was restricted by HR leading to the restriction in the development of the nematode and increased numbers of males and reduced numbers of females.

In cowpea there is no detailed histological documentation of RKN-induced changes during compatible and incompatible reactions. This study was done to provide a detailed histological characterization of *Rk*-mediated resistance in cowpea. It is known that an oxidative burst is typically associated with HR in incompatible host–pathogen interactions including nematode–plant interactions (De Gara *et al*., 2003). A significant oxidative burst has been recorded in incompatible tomato (*Mi-1*)–RKN interactions (Melillo *et al*., 2006). Therefore, the accumulation of reactive oxygen species in the *Rk*-mediated incompatible cowpea–RKN interaction was also investigated.

Materials and methods

Plant material

Two near-isogenic lines (NIL) differing in the presence or absence of the gene *Rk* were used. The two parents used to develop the NIL were *M. incognita* race 3 resistant cowpea genotype 'CB46' (homozygous resistant, *RkRk*) and a highly susceptible genotype 'Chinese Red' (homozygous susceptible, *rkrk*). The F_1 was backcrossed to recurrent parent CB46 (BC_1) and homozygous *Rk* plants

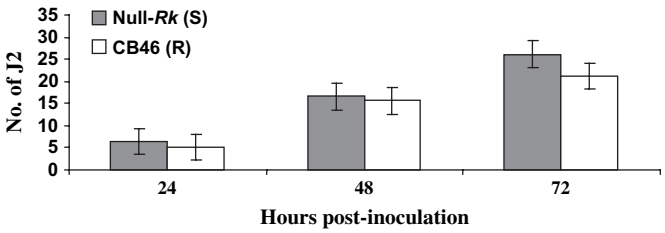


Fig. 1. Number of J2 penetrating into roots of CB46 (resistant) and null-*Rk* (susceptible) assayed at 24, 48, and 72 hpi. Values are means ±SE of two separate experiments.

were discarded in BC_1F_2 and non-segregating *rkrk* plants were advanced to the next back-cross (BC_2). Repeated backcrossing and selection was used to recover the *rkrk* line in the CB46 background. BC_4F_4 progenies were used for all the experiments described here. The *rkrk* line is referred to as the null-*Rk* line from here on.

Nematode inoculum

Eggs of *M. incognita* race 3 (isolate Beltran) cultured on susceptible tomato host plants were extracted from roots using 10% bleach solution (Hussey and Barker, 1973). This isolate is avirulent to gene *Rk* in CB46. Eggs were hatched in an incubator at 28 °C and J2 were collected in fresh deionized water. The J2 inoculum was prepared according to the experimental requirements.

Histological experiments

Seeds of CB46 and null-*Rk* cowpea genotypes were grown in growth pouches under controlled environmental conditions of 26.7±0.5 °C constant temperature and daily light/dark cycles of 16/8 h. This temperature was used because it lies within the optimum temperature range of 26–28 °C for development and reproduction of *M. incognita* on cowpea in growth pouches (Ehlers *et al*., 2000). Each pouch was inoculated with 3000 J2 in 5 ml of deionized water, 12 d after planting (dap). Three pouches from each genotype were mock inoculated with 5 ml of deionized water as negative controls. The presence of nematodes in the roots was confirmed by acid fuchsin staining (Byrd *et al*., 1983) 24 h post-inoculation (hpi).

Three root tips, each ~50 mm in length, were harvested randomly from two plants of each genotype at 3, 4, 5, 9, 14, 15, 16, 17, 18, 19, 20, and 21 d post-inoculation (dpi) and immersed in half-strength Karnovsky's fixative (2.5% glutaraldehyde and 4% formaldehyde in 50 mM phospahte buffer, pH 7.2). The roots were left overnight in the fixative at 4 °C. The roots were dehydrated by passing through a graded ethanol series (10–100%). Infiltration and embedding was done with a JB-4 methacrylate embedding kit (Polysciences Inc., Pennsylvania, USA). Semi-thin sections 4 μm thick were cut using a DuPont-Sorval JB-4 microtome using triangular glass knives. The sections were stained in 0.5% toluidine blue O in borate buffer (pH 4.4). Digital micrographs were taken using a Spot CCD camera (Spot RT colour system, model no. 2.2.1, Diagnostics Instruments Inc.) attached to a Leica DM LB2 compound bright-field microscope. Giant cell diameters were measured at 5, 9, 14, 19, and 21 dpi. Three well-developed giant cells were selected for each time point in both resistant and susceptible cowpea genotypes, being chosen from sections in a sequential series that optimized the giant cell size. The diameter was measured at three positions for each giant cell using a stage micrometer and the mean of the three measurements was used as the diameter for that giant cell. Giant cell measurements from root sections provide a relative measure of cellular changes in infected resistant and susceptible cowpea roots and do not represent an absolute measurement.

Root penetration studies

Penetration of avirulent nematodes in root tissue was studied on susceptible null-*Rk* and resistant CB46 cowpea genotypes. Plants were grown in growth pouches at 26.7 ± 0.5 °C constant temperature and daily dark/light cycles of 16/8 h. The inoculum level used was the same as for the histological experiments. Each pouch was inoculated with 3000 J2 in 5 ml of deionized water, 12 dap. The inoculated roots were harvested at 24, 48, and 72 hpi and immersed in 1.5% NaOCl solution for 15 min followed by rinsing with tap water to remove excess NaOCl. The roots were then stained with 1 ml of 3.5% acid fuchsin stain (Byrd *et al*., 1983), the solution was heated to boiling, followed by cooling to room temperature, and excess stain was removed by rinsing in running water. The root material was placed in acidified glycerin. The stained roots were pressed between glass slides and observed under the microscope. Three plants were selected for each sampling time point and three root tips from each plant were selected randomly and numbers of J2 inside the root tissue were counted.

An analysis of variance (ANOVA) was used to compare the penetration rate between resistant and susceptible roots. Data from all three root systems for a given genotype×time point were pooled together for analysis.

Egg mass production

Numbers of egg masses per root system were counted using an egg mass specific stain erioglaucine (Omwega *et al*., 1988; Ehlers *et al*., 2000) at 30 dpi to confirm the susceptibility of the null-*Rk* line used in the experiments. An inoculum of 3000 J2 per root system was used for the egg mass production assays and 20 plants each from CB46 and null-*Rk* were screened.

Quantitative detection of ROS release

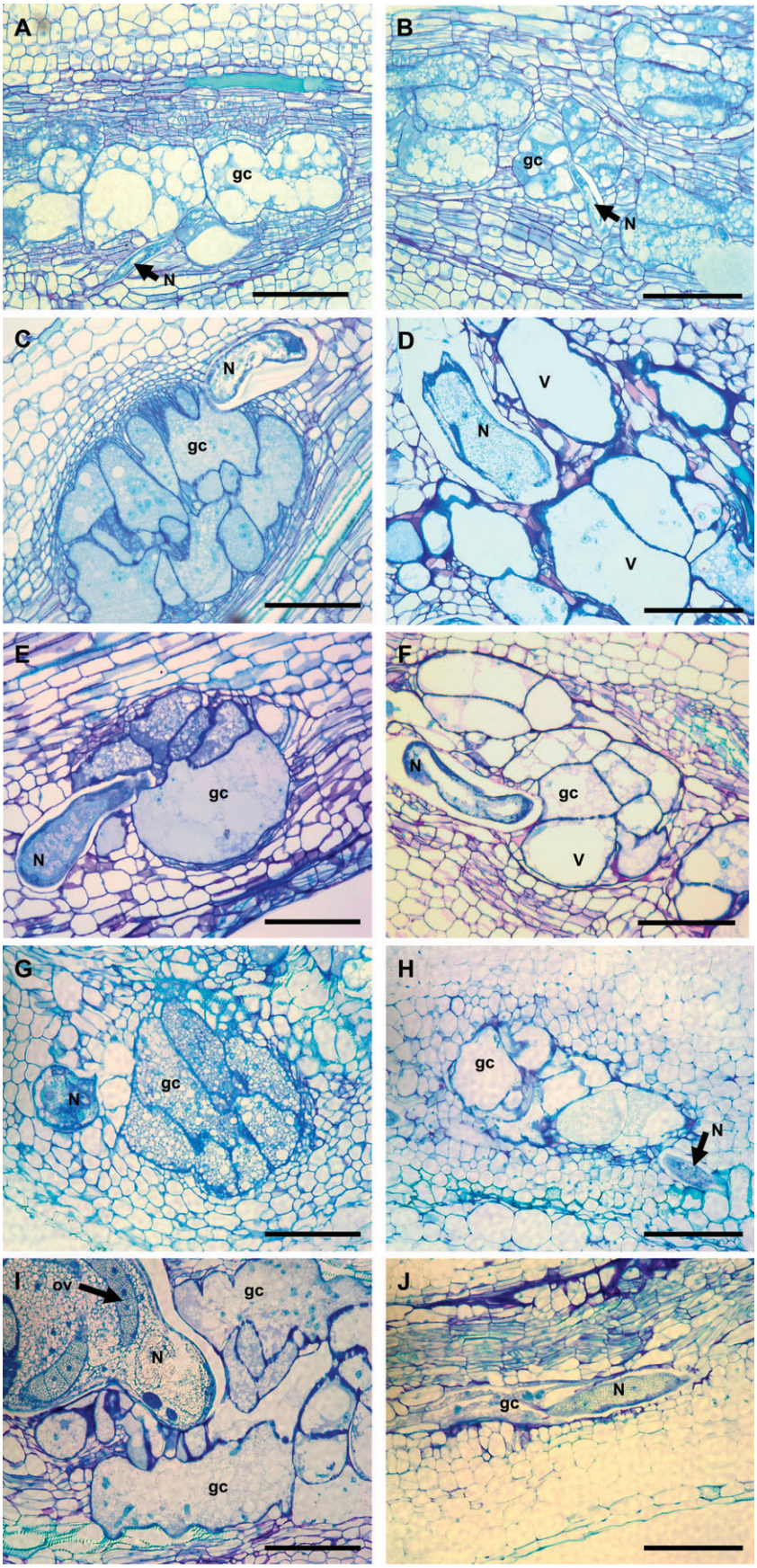
A fluorometric assay was designed to detect ROS accumulation using a membrane permeable probe, dichlorofluoroscein (DCFH). DCFH alone does not have fluorescence but when it reacts with ROS it oxidizes to DCF which is fluorescent. Root pieces were collected from infected root systems of the susceptible and resistant genotypes at 24, 48, and 72 hpi. Also, to detect the presence of a late oxidative burst, root samples were collected at 5, 9, and 14 dpi. The root samples (250 mg) were processed as described by Melillo *et al*. (2006). Phosphate-buffered saline (20 mM, pH 7.2) was used in place of potassium phosphate buffer. A 1 ml aliquot of the processed sample was collected and used to detect the increase in fluorescence (excitation 488 nm, emission 521 nm) caused by oxidation of DCFH using a fluorescence spectrophotometer (SPEX FluoroLog-3, Horiba Jovin Yvon). Mock inoculated roots (5 ml of deionized water per root system) were used as negative controls and mechanically injured roots were used as a positive control. Mechanical wounding was achieved by puncturing plant roots with a hypodermic needle. Five biological replicates were taken and the entire experiment was repeated once. The duplicate experiments did not differ according to ANOVA tests, therefore data from the independent duplicate experiments were combined for analysis. Blank samples without plant material were processed in parallel to eliminate any spontaneous change in fluorescence.

In order to test the specificity of the reaction, an experiment was done using the ROS scavenging reagent diphenylene-iodonium (DPI, an NADPH oxidase inhibitor). Root pieces (250 mg) from non-infected and 24 h-infected CB46 plants were harvested and pre-incubated for 30 min with 100 μM DPI followed by 30 min in DCFH reaction medium. The decrease in fluorescence was measured as described earlier and reagent blanks were used as reference.

Results

Root penetration

The presence of the gene *Rk* did not affect juvenile penetration into cowpea roots. The avirulent J2 were able to penetrate the roots of both cowpea genotypes and there was no effect of genotype ($P = 0.05$) on the number of J2 in roots up to 72 hpi (Fig. 1). In both genotypes, up to 24



hpi the number of J2 that had penetrated into roots was low, but the penetration rate was higher at 48 hpi and a gradual increase in penetration was observed up to 72 hpi.

Histological response to infection

The resistant line CB46 did not show a HR response to nematode infection. HR, in which a programmed cell death around the area of infection occurs and the development of the pathogen is arrested, is a common plant reaction against different pathogens in resistant genotypes. For example, in root-knot nematode–host plant interactions such as the *Mi-1*-mediated resistance in tomato an obvious HR occurs within 24 h of infection (Dropkin, 1969; Williamson, 1999). In this study, when 12-d-old seedlings were inoculated with 3000 J2 and longitudinal sections of roots were examined, there was no visible evidence of a HR response in resistant roots up to 21 dpi. The nematodes were able to establish healthy feeding sites in resistant roots in which the giant cells looked similar to those in susceptible roots up to 5 dpi (Fig. 2A, B). Lack of HR response was also confirmed by staining the roots with acid fuchsin at various time points (not shown). The first evident differences between the two genotypes were observed at 9 dpi when the giant cells adjacent to the nematode in resistant roots had some larger vacuoles (Fig. 2C, D), whereas the giant cells in the susceptible roots had uniformly dense cytoplasm with less vacuolation. The giant cells farthest from the nematode appeared to be metabolically more active than giant cells closer to the nematode in resistant roots (Fig. 2D). The nematodes at this time point were developing normally in the genotypes based on observations of their size, shape, and condition of internal contents. This trend in the giant cell conditions continued up to 14 dpi (Fig. 2E, F) when there was still no sign of visible feeding site deterioration in resistant roots. However, the nematodes associated with resistant roots at 14 dpi were arrested in development as they were slightly shrivelled and narrower than the nematodes in susceptible roots. This confirmed that although giant cell deterioration was not visible under bright field microscopy at this stage, giant cells were not metabolically active enough to provide optimum nutrients for nematode development. At 19 dpi (Fig. 2G, H) the differences in feeding sites between the two genotypes were clearly visible. At this stage most of the giant cells in the resistant roots appeared to be on the verge of collapse as they were devoid of any cytoplasm and the common cell walls between the giant cells were also thin, whereas in susceptible roots, healthy giant cell complexes with dense cytoplasm and thick cell walls were present. At 21 dpi the giant cell complexes in resistant roots had collapsed completely and nematode development was severely disrupted; they had a shrivelled appearance and had not advanced to a mature female stage based on lack of gonad development (Fig. 2J). In susceptible roots at 21 dpi most of the nematodes had developed to mature females and their well-developed ovaries could be seen in the sections (Fig. 2I).

Giant cell dimensions

Giant cell diameter did not differ between infection sites in resistant CB46 and susceptible null-*Rk* roots until 19 dpi. Giant cell measurements revealed that, by 5 dpi, the giant cells were fully developed and overall there was no significant difference in giant cell diameter between the two genotypes up to 19 dpi (Fig. 3). Although at 14 dpi the giant cells in susceptible roots were found to be larger ($P = 0.05$) than in resistant roots, it was probably because the giant cells selected for the measurement were not fully representative for this time point. However, at 21 dpi, the mean diameter of the giant cells in resistant roots was much smaller than in susceptible roots due to the collapse of giant cells (Fig. 3).

Root galling and egg production

Although the *Rk*-mediated resistance reaction was delayed, the development of female nematodes was arrested in resistant roots such that they did not reach reproductive maturity. The cortical cells surrounding the giant cells in resistant roots started to shrink rapidly at 12–14 dpi and at 19–21 dpi the cortical cells were almost normal in size. Therefore, at 19 dpi, only residual galling was visible on resistant roots even though the giant cells were not collapsed.

External observations of the roots at 21 dpi revealed large well-developed galls in susceptible roots whereas the resistant roots supported only some small residual swelling around the feeding sites (Fig. 4). Acid fuchsin staining at 21 dpi revealed that, in susceptible roots, the females had reached reproductive maturity and started to lay eggs, whereas in resistant roots, the under-developed female nematodes (approximately 90% J4 and 10% immature adults) showed no sign of egg production. This confirmed our observations from the histological root sections. At 30 dpi the number of egg masses per root system ranged from 43 to 99 (mean $\pm$SD = 65.5$\pm$15.9) in susceptible roots, whereas nematodes failed to produce any egg masses in resistant roots.

Fig. 2. Longitudinal sections of *M. incognita* feeding sites in inoculated cowpea roots. Section are stained with toluidine blue O. A, C, E, G, and I are null-*Rk* (susceptible) root sections and B, D, F, H, and J are CB46 (resistant) root sections at 5, 9, 14, 19, and 21 dpi, respectively. gc, giant cell; N, nematode; ov, ovary; V, vacuole. Bar = 200 μm.

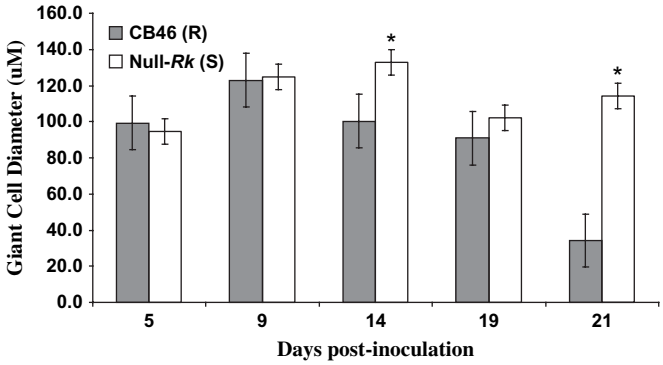


Fig. 3. Diameters (μm) of nematode-induced giant cells formed in CB46 (resistant) and null-*Rk* (susceptible) inoculated roots over a time period of 5, 9, 14, 19, and 21 dpi. Values are means from measurement of three giant cells. Each giant cell was measured at three different positions. Bars represent ±1 SE. * Significant at $P = 0.05$.

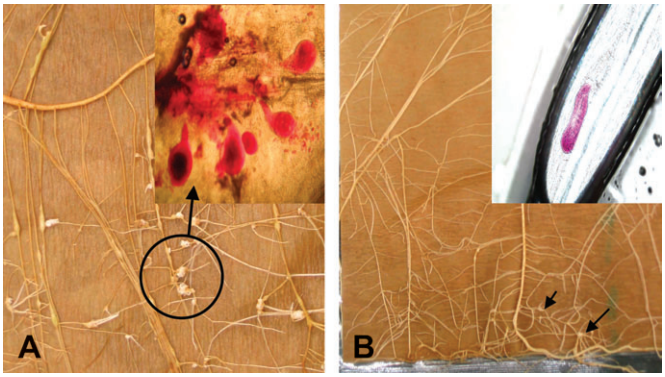


Fig. 4. Infection symptoms and *M. incognita* females in inoculated roots at 21 dpi. (A) Null-*Rk* (susceptible) roots showing nematode-induced galling (circled black) on the root surface, inset: a group of egg-laying females stained with acid fuchsin. (B) CB46 (resistant) roots almost free from galling except slight residual swelling indicated by black arrows, inset: a female nematode stained in acid fuchsin that has not developed to maturity and there is an absence of egg production.

Quantitative detection of ROS release

In a time-course experiment, ROS activity was studied, starting at the early stages of infection (24 hpi) until the later stages of infection at 14 dpi. Due to nematode infection, an early rise in ROS activity at 24 hpi was observed in root tissue of both resistant CB46 (157% compared with non-infected control) and susceptible null-*Rk* (153% compared with non-infected control) plants as shown in Fig. 5. This early oxidative burst continued up to 48 hpi in both CB46 (159%) and null-*Rk* (151%). ROS activity decreased after that with readings for ROS in infected roots compared to non-infected control at 72 hpi being 92% in CB46 and 85% in null-*Rk*. There was no differential ROS activity between the resistant and susceptible genotypes during these time points. During later time points (5, 9, and 14 dpi) very low levels of ROS activity were detected in both genotypes in infected and

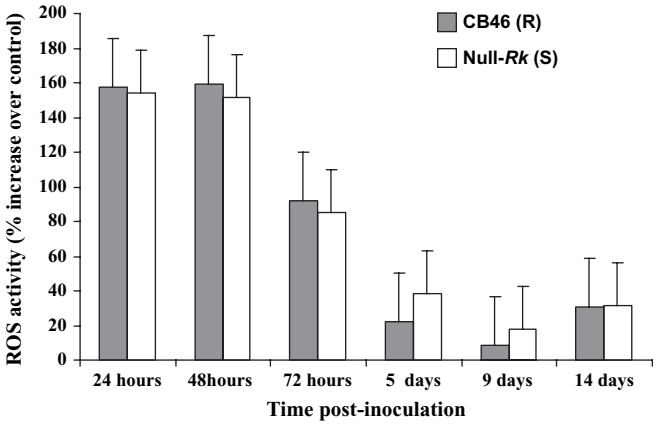


Fig. 5. Quantification of reactive oxygen species (ROS) in resistant CB46 and susceptible null-*Rk* cowpea root tissue assayed at 1, 2, 3, 5, 9, and 14 dpi. Increase in dichlorofluoroscein (DCF) is expressed as a percentage increase over the non-infected control. Fluorescence intensity was measured in counts per second (cps) with an excitation wavelength of 488 nm and emission wavelength of 521 nm. Values are means of combined data from two separate 5-fold replicated experiments. Bars represent 1 SE.

non-infected roots. Compared to non-wounded control plants, mechanically wounded roots (positive control) of both CB46 and null-*Rk* produced a significant early oxidative burst up to 48 hpi, which diminished at 72 hpi (Fig. 6), similar to the response in nematode-infected resistant and susceptible plants. Oxidation of DCFH in CB46 roots was inhibited by the superoxide O_2^- scavenger DPI (Table 1). DPI is an NADPH-oxidase inhibitor and was the most efficient inhibitor of ROS in *Mi-1*-mediated resistance in tomato (Melillo *et al.*, 2006). Upon DPI treatment ROS activity was reduced to 53% in RKN infected CB46 roots at 24 hpi. This confirmed that the enzymatic origin of superoxide contributed significantly to the early oxidative burst detected in infected cowpea roots.

Discussion

The gene *Rk* was identified almost three decades ago (Fery and Dukes, 1980) as a highly effective RKN resistance gene in cowpea. Although the *Rk*-based resistance has been studied genetically and has been used extensively in cowpea breeding, little was known about the mechanism of *Rk*-mediated resistance. Two types of mechanisms for RKN resistance in plants have been reported, including pre-infection resistance, where the nematodes cannot enter the plant roots due to the presence of toxic or antagonistic chemicals in root tissue (Haynes and Jones, 1976; Bendezu and Starr, 2003), and post-infection resistance in which nematodes are able to penetrate roots but fail to develop. Post-infection resistance is often associated with an early Hypersensitive

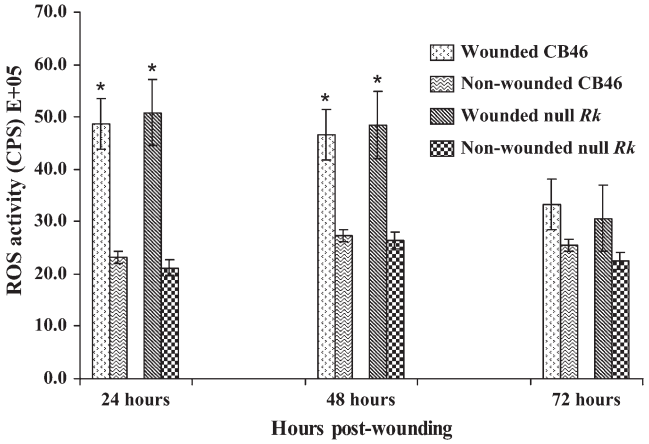


Fig. 6. Quantification of reactive oxygen species (ROS) in mechanically wounded (as a positive control), CB46 (resistant), and null-*Rk* (susceptible) roots over a time period of 24, 48, and 72 h post-wounding. Fluorescence intensity was measured in counts per second (cps) at excitation wavelength of 488 nm and emission wavelength of 521 nm. Values are means of combined data from two separate 5-fold replicated experiments. Bars represent ±1 SE. 1* Significant increase ($P = 0.05$) in ROS activity in wounded roots compared to non-wounded control within a genotype and time point.

Table 1. *Effect of DPI on ROS release in resistant CB46 roots 24 h after nematode infection compared to non-infected roots at the same stage*

Inhibitor	ROS release percentage[a]	
	Non-infected CB46	Infected CB46
None	100±1	100±2
DPI (100 μM)	80.7±2	53.2±2

[a] Values are means of combined data from two separate 5-fold replicated experiments ±SE.

Reaction (HR)-mediated cell death, in which rapid localized cell death in root tissue around the nematode prevents the formation of a developed feeding site, leading to resistance. Tomato (Dropkin, 1969; Williamson, 1999), pepper (Pegard *et al*., 2005), soybean (Kaplan *et al*., 1979), and coffee (Anthony *et al*., 2005) host plants that are resistant show typical HR upon avirulent RKN infection. In tomato, HR was observed as early as 24 hpi whereas in pepper, soybean, and coffee the HR was visible at 1–3 dpi, 2–3 dpi, and 4–6 dpi, respectively.

Interestingly in the current study, it was found that the presence of the gene *Rk* did not affect J2 penetration into cowpea roots and there was no evidence of early HR. In fact, the nematodes were able to initiate and maintain apparently healthy giant cells in resistant roots for about two weeks before visible signs of deterioration occurred, especially vacuolation and cell wall thinning, leading to giant cell collapse. This mechanism appears to be novel for RKN resistance. The only published report for a delayed resistance response against RKN was in tobacco (Powell, 1962) where a late HR was seen in developed giant cells. In cowpea there was no HR even during the later stages of infection. During this time the nematodes were able to feed and develop into late stage juveniles.

A common feature of pathogen-related HR is that it is preceded by loading of vacuoles with hydrolases and toxins and a calcium flux in the cytoplasm (Jones, 2001). A significant difference in vacuolation between the resistant and susceptible cowpea genotypes starting at 9 dpi was observed, and it is possible that the large vacuoles in resistant cowpea roots were filled with hydrolases and toxins that deprived the nematodes of nutrients and led to giant cell collapse, whereas in susceptible roots nematode feeding did not cause the formation of large vacuoles. In *Arabidopsis* a mutant called *dnd1-1* failed to produce an HR response against an avirulent strain of the bacterial pathogen *Pseudomonus syringae*, but an effective gene-for-gene resistance was still operative (Clough *et al*., 2000). *DND1* codes for a cyclic gated ion channel which facilitates passage of Ca^{2+}, K^{+} and various other cations. Thus host defence can be effective in the absence of HR and it might be dependent upon subtle changes in ion fluxes.

Reactive oxygen species (ROS) play an important role in plant defence, and during pathogen attack levels of ROS detoxifying enzymes like ascorbate peroxidase (APX) and catalase (CAT) are often suppressed in resistant plants (Klessig *et al*., 2000). As a result plants produce more ROS and accumulation of these components leads to HR in plant cells. For example, H_2O_2 plays a major role in triggering HR in incompatible interactions (Dangl and Jones, 2001). However, in the cowpea–RKN incompatible interaction mediated by the gene *Rk*, a classic HR, which is characteristic for most gene-for-gene resistance pathways, was not seen.

Our results of ROS quantification in RKN-infected cowpea roots showed that although there was an early oxidative burst in both the compatible and incompatible interactions in susceptible and resistant roots, respectively, there was no significant difference between the resistant and susceptible genotypes in level of ROS activity. Typically in incompatible interactions ROS is produced in a biphasic manner (Apel and Hirt, 2004), in which the initial rapid accumulation of ROS is followed by a more stable second burst. However, in cowpea, a biphasic pattern of ROS production was not seen in the incompatible reaction. These results indicated that the initial burst that was detected in cowpea roots upon RKN infection is a part of basal host defence reaction and is independent of gene *Rk*-mediated resistance. This response, which develops a few days after elicitation, seems to be related to an innate immunity in plants (Iriti and Faoro, 2007) and is triggered by changes in membrane potential, ion fluxes, and production of ROS. It is well known now that

perception of parasite and/or wounding and modulation of ROS contribute toward the activation of the plant defence response (Klessig *et al*., 2000; Gechev *et al*., 2006). This pattern of ROS production correlated well with the results of the histological profiles of the feeding site and the giant cell development that were found in the resistant and susceptible cowpea roots. The magnitude of H_2O_2 production apparently was not sufficient enough to trigger HR cell death in cowpea roots. It is also possible that the ROS scavenging mechanism was not suppressed to a level at which enough ROS could be diffused into the cells to trigger HR.

In conclusion, it is reported that *Rk*-mediated resistance in cowpea is a unique resistance mechanism involving the lack of a HR and based on a delayed defence response. The current study provides a strong platform for designing gene expression studies to identify candidate genes which play an active role in this defence pathway. An evaluation of the expression levels of genes coding for enzymes involved in ROS production and ROS scavenging will be useful for understanding the intricacies of redox changes occurring upon RKN infection in resistant cowpea.

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References

Anthony F, Topart P, Martinez A, Silva M, Nicole M. 2005. Hypersensitive-like reaction conferred by the *Mex-1* resistance gene against *Meloidogyne exigua* in coffee. *Plant Pathology* **54,** 476–482.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annual Review of Plant Biology* **55,** 373–399.

Bendezu IF, Starr J. 2003. Mechanism of resistance to *Meloidogyne arenaria* in the peanut cultivar COAN. *Journal of Nematology* **35,** 115–118.

Byrd DW, Kirkpatrick T, Barker KR. 1983. An improved technique for clearing and staining plant tissue for detection of nematodes. *Journal of Nematology* **15,** 142–143.

Clough SJ, Fengler KA, Yu I, Lippok B, Smith Jr RK, Bent AF. 2000. The Arabidopsis *dnd1* 'defence, no death' gene encodes a mutated cyclic nucleotide-gated ion channel. *Proceedings of the National Academy of Sciences, USA* **97,** 9323–9328.

Dangl JL, Jones JDG. 2001. Plant pathogens and integrated defence response to infection. *Nature* **418,** 203–206.

Davis EL, Hussey RS, Baum TJ, Bakker J, Schots A, Rosso MN, Abad P. 2000. Nematode parasitism genes. *Annual Review of Phytopathology* **38,** 365–396.

De Gara L, de Pinto MC, Tommasi F. 2003. The antioxidant systems vis-à-vis reactive oxygen species during plant–pathogen interactions. *Plant Physiology and Biochemistry* **41,** 863–870.

Dropkin VH. 1969. The necrotic reaction of tomatoes and other hosts resistant to *Meloidogyne*: reversal by temperature. *Phytopathology* **59,** 1632–1637.

Ehlers JD, Matthews WC, Hall AE, Roberts PA. 2000. Inheritance of a broad-based form of root-knot nematode resistance in cowpea. *Crop Science* **40,** 611–618.

Ehlers JD, Matthews WC, Hall AE, Roberts PA. 2002. Breeding and evaluation of cowpeas with high levels of broad-based resistance to root-knot nematodes. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M, eds. *Challenges and opportunities for enhancing sustainable cowpea production*. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture (IITA), Nigeria, Ibadan, 41–51.

Fery RL. 1985. The genetics of cowpeas: a review of the world literature. In: Singh SR, Rachie KO, eds. *Cowpea research, production and utilization*. New York: John Wiley and Sons, 25–62.

Fery RL. 1990. The cowpea: production, utilization, and research in the United States. *Horticultural Reviews* **12,** 197–222.

Fery RL, Dukes PD. 1980. Inheritance of root-knot nematode resistance in cowpea (*Vigna unguiculata* [L.] Walp.). *Journal of the American Society for Horticultural Science* **105,** 671–674.

Gechev TS, Van Breusegem F, Stone JM, Denv I, Laloi C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* **28,** 1091–1101.

Gheysen G, Fenoll C. 2002. Gene expression in nematode feeding sites. *Annual Review of Phytopathology* **40,** 191–219.

Haynes RL, Jones CM. 1976. Effects of the *Bi* locus in cucumber on reproduction, attraction, and response of the plant to infection by the southern root-knot nematode. *Journal of the American Society for Horticultural Science* **101,** 422–424.

Holtmann B, Kleine M, Grundler FMW. 2000. Ultrastructure and anatomy of nematode-induced syncytia in roots of susceptible and resistant sugar beet. *Protoplasma* **211,** 39–50.

Hussey RS, Barker KR. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* **57,** 1025–1028.

Iriti M, Faoro F. 2007. Review of innate and specific immunity in plants and animals. *Mycopathologia* **164,** 57–64.

Jones AM. 2001. Programmed cell death in development and defence. *Plant Physiology* **125,** 94–97.

Jones MGK, Northcote DH. 1972. Nematode induced syncytium: a multinucleate transfer cell. *Journal of Cell Science* **10,** 789–809.

Jung C, Wyss W. 1999. New approaches to control plant parasitic nematodes. *Applied Microbiology and Biotechnology* **51,** 439–446.

Kaplan DT, Thomason IJ, Van Gundy SD. 1979. Histological study of compatible and incompatible interaction of soybeans and *Meloidogyne incognita*. *Journal of Nematology* **11,** 338–343.

Klessig DF, Durner J, Noad R, *et al*. 2000. Nitric oxide and salicylic acid signaling in plant defence. *Proceedings of the National Academy of Sciences, USA* **97,** 8849–8855.

Mellilo MT, Leonetti P, Bongiovanni M, Castagnone-Sereno P, Bleve-Zacheo T. 2006. Modulation of reactive oxygen species activity and H_2O_2 accumulation during compatible and incompatible tomato-root-knot nematode interactions. *New Phytologist* **170,** 501–512.

Omwega CO, Thomason IJ, Roberts PA. 1988. A nondestructive technique for screening bean germ plasm for resistance to *Meloidogyne incognita*. *Plant Disease* **72,** 970–972.

Pegard A, Brizzard G, Fazari A, Soucaze O, Abad P, Djian-Caporalino C. 2005. Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. *Phytopathology* **95,** 158–165.

Powell NT. 1962. Histological basis of resistance to root-knot nematodes in flue-cured tobacco (abstract). *Phytopathology* **52,** 25.

Rice SL, Leadbeater BSC, Stone AR. 1985. Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes. I. Potatoes with resistance gene H_1 derived from *Solanum tuberosum* ssp. *andigena*. *Physiological Plant Pathology* **27,** 219–234.

Roberts PA, Frate CA, Matthews WC, Osterli PP. 1995. Interactions of virulent *Meloidogyne incognita* and *Fusarium* wilt on resistant cowpea genotypes. *Phytopathology* **85,** 1288–1295.

Roberts PA, Matthews WC, Ehlers JD. 1996. New resistance to virulent root-knot nematodes linked to the *Rk* locus of cowpea. *Crop Science* **36,** 889–894.

Singh BB, Asante SK, Florini D, Jackai LEN, Fatokun C, Wyrda K. 1997. Breeding for multiple disease and insect resistance. In: *IITA Annual Report, 1997*. International Institute of Tropical Agriculture, Ibadan, Nigeria, 22.

Sobczak M, Avrova A, Jupowicz J, Phillips M, Ernst K, Kumar A. 2005. Characterization of susceptibility and resistance responses to potato cyst nematode (*Globodera* spp.) infection to tomato lines in the absence and presence of the broad-spectrum nematode resistance *Hero* gene. *Molecular Plant–Microbe Interactions* **18,** 158–168.

Williamson VM. 1999. Plant nematode resistance genes. *Current Opinion in Plant Biology* **2,** 327–331.

Williamson VM, Kumar A. 2006. Nematode resistance in plants: the battle underground. *Trends in Genetics* **22,** 396–403.