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Research article

Differential expression of antioxidant enzymes and PR-proteins in compatible and incompatible interactions of cowpea (*Vigna unguiculata*) and the root-knot nematode *Meloidogyne incognita*

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ABSTRACT

This study aimed to evaluated the resistance and susceptibility of 10 cowpea cultivars to *Meloidogyne incognita* in field studies and to analyze the kinetics of the enzymes superoxide dismutase, catalase, peroxidase, chitinase, β-1,3-glucanases and cystein proteinase inhibitors in the root system of two contrasting cowpea cultivars after inoculation with *M. incognita*. The cultivars CE-31 and Frade Preto were highly resistant; CE-28, CE-01, CE-315, CE-237, were very resistant; CE-70 and CE-216 were moderately resistant, whereas Vita-3 and CE-109 were slightly resistant. In the roots of the highly resistant cultivar CE-31 the activity of the antioxidant enzyme superoxide dismutase increased and catalase decreased and those of the pathogenesis-related proteins chitinase, β-1,3-glucanase, peroxidase and cystein proteinase inhibitor increased in comparison with the root system of the slightly resistant CE-109, during the course of *M. incognita* infestation. Thus the changes in the activities of these enzymes might be related to the smaller final population of *M. incognita* in CE-31 and may contribute to the high resistance of this cowpea cultivar against infection and colonization by this nematode species.

1. Introduction

The root-knot nematodes (RKN) (*Meloidogyne* spp.) are obligate endoparasites that infect various plant species and are among the most damaging crop pathogens in the world [1,2]. Because they parasitize the root system, which takes up water and nutrients, the whole plant is affected. Consequently, crop yield decreases, leading to considerable economic losses of several millions of dollars worldwide [2]. Infection takes place when the motile second-stage juvenile (J2), the infective form, is attracted to the root system by root exudates and enters the elongation zone just behind the root tip [3]. The parasite then moves intra- and intercellularly to reach the primary phloem or the undifferentiated cells of the adjacent parenchyma, where it becomes sedentary and establishes a feeding site [4,5]. Feeding sites are characterized by the presence of hypertrophic multinucleated cells (giant cells) generated by successive mitotic divisions that occur without cytokinesis. During its development, the nematode feeds from these cells by means of a stylet, draining nutrients from the host plant and resulting in abnormal partitioning of photosynthates to the feeding site of the nematode. This impairs plant growth, causes wilting, increases the susceptibility of the plant to other pathogens and under some conditions may kill the plant. Some varieties of plant species are naturally resistant to specific nematode attack. Therefore, it is of great interest to determine which genes [6–8], molecules and biochemical mechanisms present in naturally resistant plant varieties allow them to resist infection by RKN.

A primary and immediate reaction of plants to pathogen attack is the overproduction of reactive oxygen species (ROS), such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), that lead to the hypersensitive response (HR) characterized by the cell death at the site of infection. Following HR, the challenged plant usually develops a systemic acquired resistance (SAR), which guarantees long-lasting systemic immunity toward not only the primary pathogen that induced the response, but also secondary infection

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by other pathogens [9]. A perfect synchrony of ROS generation and scavenging in plant tissues is of paramount importance because excess ROS can cause irreversible cellular injuries [10]. Several enzymes work together to tightly regulate the plant antioxidant network to maintain the steady-state level of ROS in plant cells [11]. For example, superoxide dismutase (SOD; EC 1.15.1.1) promotes dismutation of superoxide anions to H_2O_2 and O_2. Catalase (CAT; EC 1.11.1.6) hydrolyzes H_2O_2 into H_2O and O_2. Guaiacol peroxidase (POX; EC 1.11.1.7) is also capable of reducing the level of H_2O_2 by promoting the H_2O_2-dependent polymerization of hydroxycinnamyl alcohols during lignin biosynthesis and the H_2O_2-dependent cross-linking of cell wall proteins, such as hydroxyproline-rich glycoproteins and proline-rich proteins, which promotes reinforcement of the cell wall.

Other proteins involved in plant defense mechanisms are the pathogenesis-related proteins (PR-proteins) chitinases (CHI; EC 3.2.1.14), β-1,3-glucanases (GLU; EC 3.2.1.39), and proteinase inhibitors [12]. CHI and GLU catalyze the hydrolysis of chitin and glucans, respectively, which are structural carbohydrates present in the cell walls of phytopathogens and pests. Protein inhibitors affect the proteolytic enzymes excreted by viruses, bacteria, fungi and proteinases of the digestive tracts of insects and nematodes [13].

Cowpea is a nutritious plant that is widely cultivated in parts of Asia, Oceania, the Middle East, Africa, and Central and South America. This crop is affected by various nematode genera, but a disease known as meloidogynosis caused by root-knot nematodes (RKN), frequently *Meloidogyne incognita* and *Meloidogyne javanica,* is prevalent [1,14].

In this study, we evaluated the resistance and susceptibility of 10 cowpea cultivars to *M. incognita* in field studies and chose two contrasting cowpea cultivars, CE-31 (highly resistant) and CE-109 (slightly resistant), to analyze the kinetic of the enzymes SOD, CAT, POX, CHI, GLU and cystein proteinase inhibitors (CPI) in their root systems up to 10 days after inoculation with *M. incognita*, in order to contribute to the understanding of the role of these proteins in the defense of cowpea against this nematode species.

2. Results

2.1. Field screening experiment for identification of cowpea cultivars resistant and susceptible to M. incognita

The results of the resistance evaluation carried out in a field naturally infected with *M. incognita* showed that the egg mass numbers present in the root systems of the 10 studied cowpea cultivars varied from 0.7 ± 0.4 to 41.3 ± 16.5 (Table 1). In particular, the cultivar CE-109 had the most numerous and largest egg masses and was the most strongly affected. In addition to the severe symptoms in its root system, it showed stunted growth, wilting and a diseased appearance compared to the other cultivars. On the other hand, the cultivars CE-31, Frade Preto, CE-28, CE-01, and CE-315 had significantly ($p \leq 0.05$) fewer eggs than the remaining cultivars, but CE-31 presented the fewest egg masses (0.7 ± 0.4) and was therefore considered to be highly resistant (hR), but not immune to the parasite. Based on this field experiments the contrasting cultivars CE-31 (hR) and CE-109 (sR) [15,16] were selected for comparative studies on penetration and development of *M. incognita* and expression of antioxidant and PR-proteins in cowpea roots.

2.2. Penetration and development of M. incognita in the root system of CE-31 and CE-109 cowpea cultivars

Analysis of the course of *M. incognita* infection in the root systems of CE-31 (hR) and CE-109 (sR) showed that the initial penetration of infective second-stage juveniles (J2) was similar for both cultivars (Fig. 1). In contrast, subsequent development of J2 toward adult reproductive females was dependant on cowpea cultivar. Indeed, in our study carried out under controlled conditions (Section 4.4), it was verified that the roots of CE-109 and CE-31 had on average 82 and 78 fusiform individuals, respectively, at 4 DAI. However, at 8 DAI, the number of fusiform juveniles in CE-31 roots decreased abruptly whereas in CE-109 they increased. Remarkably, at 28 DAI, whereas in CE-109 roots 534 reproductive adult females (adults with eggs) on average were counted only around 12 were present in roots of CE-31.

Table 1
Egg mass number per plant,[a] egg mass index (EI), and degree of resistance (DR) of 10 Brazilian cowpea cultivars planted in a field infested with *M. incognita.*

Cultivar	Egg masses	EI[b]	DR[c]
CE-31	0.7 ± 0.4 e	0.0	hR
Frade Preto	1.7 ± 0.6 e	1.2	hR
CE-28	4.7 ± 2.1 d	1.9	vR
CE-01	7.3 ± 3.0 cd	2.1	vR
CE-315	7.0 ± 3.2 cd	2.1	vR
CE-237	13.7 ± 6.6 bc	2.7	vR
CE-70	22.7 ± 7.8 ab	3.3	mR
CE-216	27.8 ± 12.8 ab	3.4	mR
Vita-3	32.7 ± 11.5 a	3.6	sR
CE-109	41.3 ± 16.5 a	3.7	sR

[a] Plants were examined 60 days after planting. Each value is the average of 30 plants examined per cultivar. Values followed by different letters are significantly different (Tukey's test; $P \leq 0.05$).
[b] EI values: 0 = no egg masses; 1 = 1–2 egg masses; 2 = 3–10 egg masses; 3 = 11–30 egg masses; 4 = 31–100 egg masses and 5 ≥ 100 egg masses [15].
[c] DR designations: EI range of 0.0–1.0 = highly resistant (hR); 1.1–3.0 = very resistant (vR); 3.1–3.5 = moderately resistant (mR); 3.6–4.0 = slightly resistant (sR); and 4.1–5.0 = susceptible (S) [16].

2.3. Kinetics of SOD, CAT, POX, CHI, GLU and CPI in the root system of CE-31 and CE-109 cultivars

M. incognita infected roots of CE-31 showed greater increases in SOD activity compared to CE-109 (Fig. 2A). An abrupt increase in activity occurred at 2 DAI and continued to increase by about two-

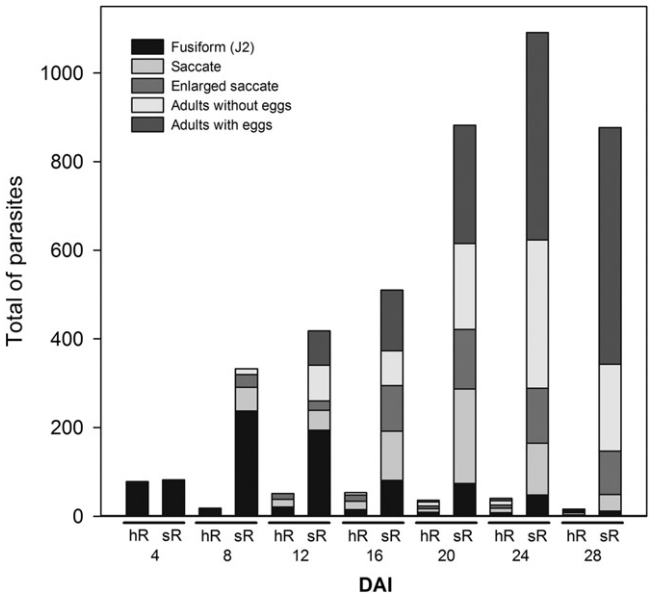

Fig. 1. Penetration and development of *M. incognita* in the root system of the highly resistant (CE-31) and slightly resistant (CE-109) cowpea cultivars after J2 inoculation (850 J2/root). Each value is an average of three replicates ($n = 6$ plants).

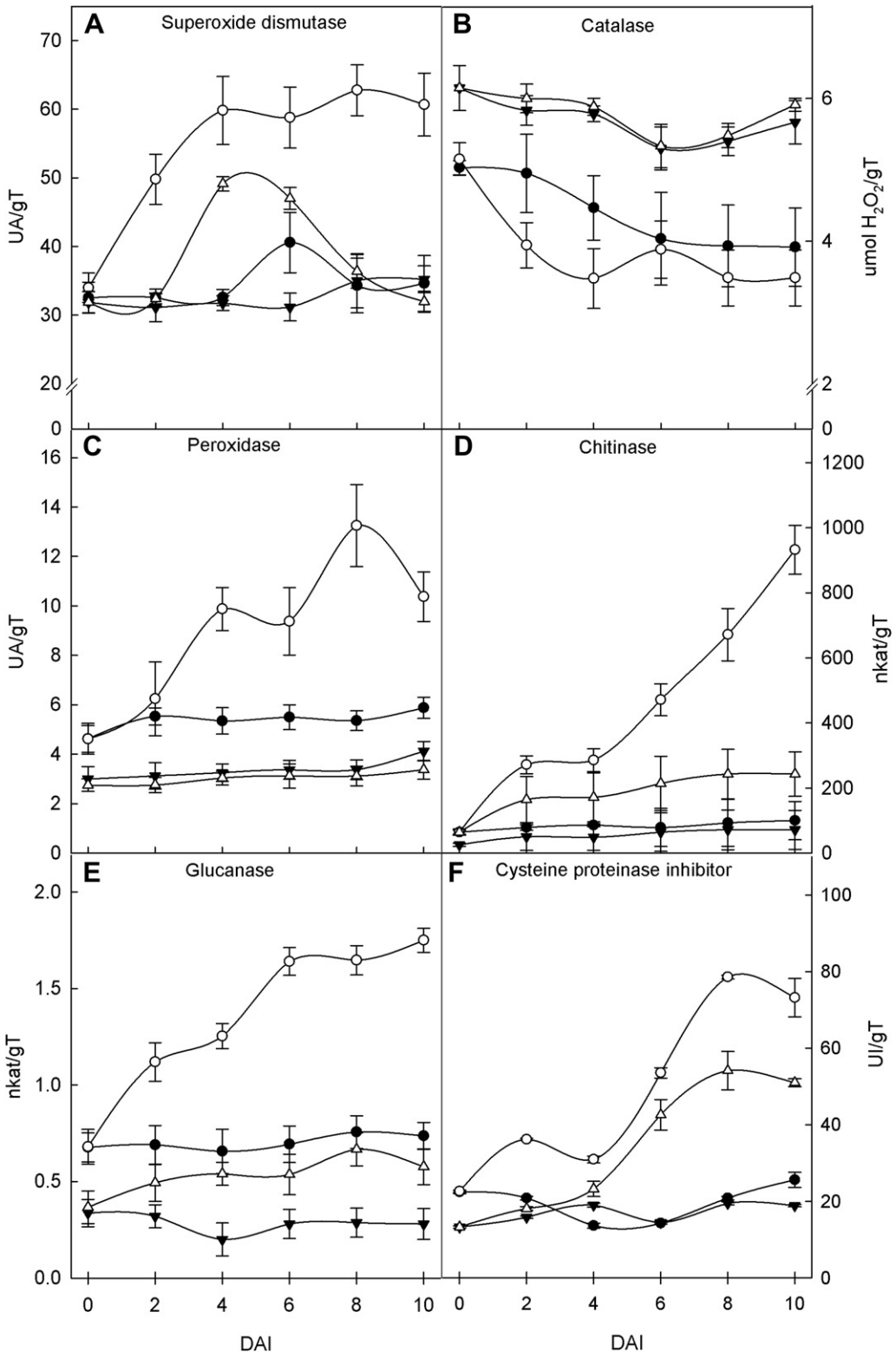

Fig. 2. Effect of *M. incognita* on antioxidant enzymes and PR-proteins in cowpea (*Vigna unguiculata*) roots. Activity of SOD (A), CAT (B), POX (C), CHI (D), GLU (E) and CPI (F) was measured in the root systems of the highly resistant (CE-31) and slightly-susceptible (CE-109) cowpea cultivars inoculated with *M. incognita* (850 J2/root). Infected CE-31 and CE-109 were compared with each other and with their respective non-infected controls. Each data point represents the mean of three independent experiments ± standard error (bar). Inoculated CE-31(—○—) and CE-109 (—△—); non-inoculated CE-31 (—●—) and CE-109 (—▼—).

fold at 4 DAI compared to controls. Then it leveled off until the end of the experimental period. Notably, SOD activity values for CE-31 were significantly greater ($p \leq 0.05$) than those for CE-109 during the entire experimental period of root-knot nematode (RKN) infection, even taking into account that SOD activity also increased in the root system of CE-109 compared to its corresponding control from 2 to 6 DAI. It was also verified a 2-day delay of SOD activity to increase in CE-109 compared with CE-31.

CAT activity measured in the root system of CE-31 inoculated with *M. incognita* (Fig. 2B) showed a rapid and significant decrease ($p \leq 0.05$) between 2 and 4 DAI as compared to its corresponding control. On the other hand, CE-109, which had greater basal CAT activity (control) compared to CE-31 (control), did not show any significant change upon *M. incognita* infection. However, CAT levels in CE-109 were consistently higher than that for CE-31 ($p \leq 0.05$) during the entire experimental period. The ratios between SOD and CAT activities (UA:μmol H_2O_2 reduced per gram of fresh roots) for inoculated CE-31 at 0, 2, 4, 6, 8 and 10 DAI were 6.60, 12.64, 17.19, 15.15, 17.98 and 17.38, respectively. For inoculated CE-109, at the same time points, SOD:CAT activity ratios were 5.18, 5.39, 8.35, 8.81, 6.64 and 5.40 UA:μmol H_2O_2 reduced. Because SOD generates H_2O_2 and CAT scavenges it, these data suggest greater H_2O_2 generation in RKN-inoculated CE-31 than in inoculated CE-109. However, H_2O_2 increase in the root of RKN-inoculated CE-31 cultivar (173.02 ηmol H_2O_2 g^{-1} fresh tissue) was significantly ($p \leq 0.05$) greater than its respective controls (150.50 ηmol H_2O_2 g^{-1} fresh tissue) only at 6 DAI (Fig. 3).

POX activity in the root system of CE-31 (Fig. 2C) was also significantly ($p \leq 0.05$) induced upon RKN infection. A trend of increasing POX activity was observed from 2 DAI and peaked at 8 DAI at a value two-fold higher than that of the corresponding non-inoculated control. POX activity in the root of CE-109 did not respond to RKN infection.

The PR-proteins CHI and GLU were also up-regulated in RKN-inoculated CE-31 compared to non-inoculated controls and RKN-inoculated CE-109 (Fig. 2D and E, respectively). Induction of CHI activity was rapid and remarkable upon RKN infection (Fig. 2D) in roots of CE-31 compared to its corresponding control and RKN-inoculated CE-109, during the entire experimental period. Notably, CHI activity level was approximately four-fold higher than that of RKN-inoculated CE-109 at the end of the experimental period.

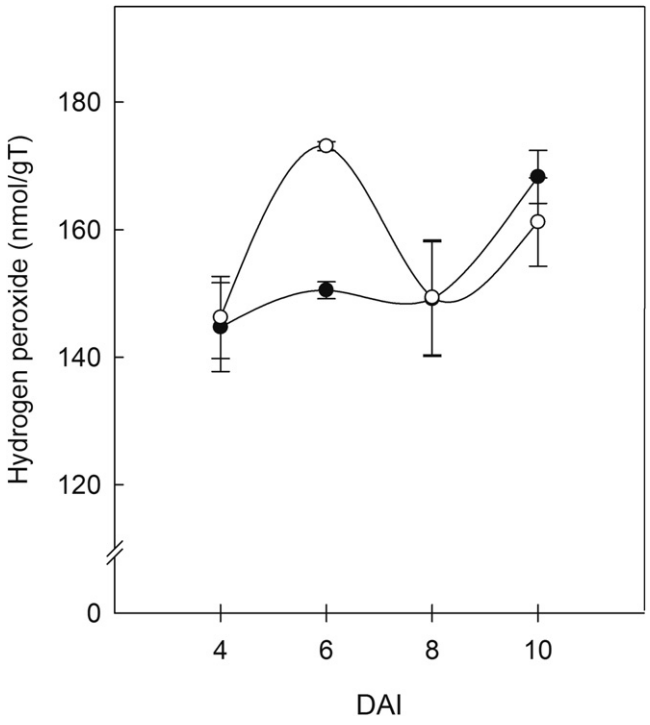

Fig. 3. Accumulation of hydrogen peroxide in the leaves of the highly resistant (CE-31) cowpea cultivar non-inoculated (—●—) and inoculated (—○—) with *M. incognita*. Each data point represents the mean of three independent experiments ± standard error (bar).

RKN-induction of GLU activity in root systems of CE-31 and CE-109 was also significant ($p \leq 0.05$) from 2 DAI onward (Fig. 2E). In CE-31, GLU activity doubled at 2 DAI and reached a maximum intensity of approximately eight and six-fold greater than those of non-inoculated controls and RKN-inoculated CE-109, respectively, at 10 DAI.

The activity of cysteine proteinase inhibitor (CPI) was markedly up-regulated in RKN-inoculated CE-31 and CE-109 compared to the respective controls (Fig. 2F). CPI began to accumulate in RKN-inoculated CE-31 from 2 DAI and was consistently present at higher levels than in CE-109. Moreover, CPI accumulation in CE-109 roots began after 4 DAI (Fig. 2F) which show a delay in CPI induction by RKN in the highly susceptible cultivar.

3. Discussion

In this present study, the resistance and susceptibility of 10 Brazilian cowpea cultivars were evaluated under field conditions using the egg mass numbers (Table 1) as criteria [15]. The cultivars were classed into four groups [16]. These groups comprised the highly resistant (hR), very resistant (vR), moderately resistant (mR), and slightly resistant (sR) cowpea cultivars (Table 1). The most significant differences in infection levels were found between the highly resistant CE-31 and the slightly resistant CE-109 cultivars. The reduced egg mass number observed in CE-31 under field conditions (Table 1) might be explained by emigration of J2 individuals that penetrated the root system, but may not have encountered favorable conditions to develop into reproductive adult females. In our study carried out under controlled conditions (Section 4.4), the number of nematodes in the root system of CE-31 decreased dramatically over the course of the experiment compared with CE-109 (Fig. 1), suggesting that the nematodes might have migrated out of the tissue. Thus, the reproduction of *M. incognita* was favored in the slightly resistant cultivar CE-109 and drastically reduced in the highly resistant cultivar CE-31. Das *et al.* [4] showed that the presence of the nematode-resistance gene *Rk* in a cowpea genotype (CB46) resistant to *M. incognita* does not affect J2 penetration into the roots. Resistance of cowpeas to *M. incognita* is due to a single gene or locus designated *Rk* with alleles *rk*, *rki, Rk*, *Rk2* and *Rk3* that effectively inhibit reproduction of *M. incognita* [6,17–21]. Unfortunately, the origin of the RKN-resistance of CE-31 is unknown, but it may carry the *Rk* gene whereas in CE-109 it lacks or it is masked in the presence of a dominant gene [22]. For instance, some cowpeas carrying the *Rk* gene are susceptible to some root-knot nematode populations [6]. Likewise there are populations of RKN that are virulent on tomato carrying the gene *Mi* which confers resistance to severa1 root-knot nematode species in tomato [5].

Similarly to cowpea the presence of a resistance gene in the cotton isoline 81-249 does not inhibit initial J2 penetration but arrests soon after infection further development into reproductive females, confirmed by the absence of egg masses on the roots at 40 DAI, in contrast to the susceptible isoline [23]. In *Lotus japonicus* the resistance genes do not alter root penetration by J2 nematodes, but ultimately inhibit their development into adult females [24]. Moreover, in several wild plant species, natural host resistance against *Meloidogyne* spp. reduces or arrests nematode development and reproduction [1].

Most pathogen/pest-resistant plants counterattack the invader by activating defense responses such as overproduction of reactive oxygen species (ROS), programmed cell death (PCD) with localized necrosis (hypersensitive response), at an early infection stage, and *de novo* synthesis of pathogenesis-related proteins (PR-proteins) at the site of infection [25]. Obligate parasites (biotrophic) require host cells to be alive during their development. Induction of PCD

resulting from hypersensitive response (HR) in the host would constitute an effective first line of defense against the obligate pathogen because dead tissue limits the amount of nutrients available to the pathogen, which may then opt to migrate out of the resistant cowpea root system. Interestingly, Das *et al.* [4] showed an early rise in ROS activity at 24 h post-inoculation (hpi) that continued up to 48 hpi in root tissue of both resistant CB46 and susceptible null-Rk cowpea genotypes compared with non-infected controls, but there was no typical HR in resistant cowpea roots and the nematodes were able to develop normal feeding sites and only from 14 to 21 DAI the female nematodes showed arrested development and deterioration [4].

Profound biochemical, anatomical and histological alterations occur in the root system of a plant challenged by root-knot nematodes [4,5,23,26]. In our study, a much greater increase of SOD and suppression of CAT activity were observed in CE-31 than in CE-109 (Fig. 1A and B, respectively). This observation suggests that much greater generation and accumulation of H_2O_2 should occur in the RKN-infected root system of the highly resistant cultivar CE-31 from 2 DAI. However, H_2O_2 accumulation in RKN-infected CE-31 was observed only at 6 DAI (Fig. 3). It is possible that the higher increase in POX activity in the root system of CE-31 from 6 to 8 DAI (Fig. 2C) may also have regulated the plant antioxidant network to maintain the steady-state level of H_2O_2 in plant cells. It is well known that H_2O_2 and other ROS can cause oxidative damage to proteins, DNA and lipids, leading to tissue necrosis [10]. Moreover, H_2O_2 is toxic to nematodes [27] and also acts as a signaling molecule to trigger various defense genes [28]. Often, H_2O_2 accumulation in resistant plants has been associated with HR and cell death (PCD), which in turn reduces the amount of nutrients available to the nematodes inside the dead tissue. For example, HR has been detected during penetration of *Meloidogyne arenaria* J2s in two resistant *Vitis* spp [29]. In tomato plants, the first 24 h seem to be critical in the plant–nematode interaction for determining the plant response to avirulent or virulent nematodes [30], as significant increases in ROS levels have been recorded in roots of the resistant tomato cultivar "Rossol" infected with an avirulent *M. incognita* pathotype at 12, 24 and 48 h post-inoculation.

The increased trend of POX activity observed as a response of resistant cowpea to *M. incognita* (Fig. 1C) might be associated with cell wall lignification. This increases the structural rigidity of plant tissues, which halts nematode penetration. It has been well-documented that POX activity is involved in the final steps of cell wall lignification. POX catalyzes the polymerization of the lignin precursors hydroxycinnamyl alcohols (ρ-cumaryl, coniferyl and sinapyl) [31] and the cross-linking of hydroxyproline-rich glycoproteins and proline-rich proteins to reinforce the cell wall.

The PR-proteins chitinase and β-1,3-glucanase promote degradation of chitin and β-1,3-glucanes which are essential constituents of pathogen cell walls. Overexpression of chitinase and β-1,3-glucanase has been reported in various plants in response to viruses, fungi, bacteria, and nematodes. Generally, plants contain low constitutive levels of both these enzymes which increase upon pathogen or insect attack [32]. This was verified in our study as both chitinase (Fig. 2D) and β-1,3-glucanase (Fig. 2E) activities were strongly induced in the incompatible relationship of *M. incognita* with CE-31 compared to inoculated CE-109. A class III chitinase was differentially expressed in the roots of coffee infected with *Meloidogyne paranaensis* [33] and β-1,3-glucanase activity was increased in roots and leaves of cucumber plants challenged with *M. incognita* [33]. Overexpression of the chitinase gene PjCHI-1, isolated from the fungus *Paecilomyces javanicus*, in transgenic tomato plants effectively reduced the production of egg masses and repressed the embryonic development of *M. incognita* [34]. In tomato roots infected with root-knot nematodes, defense genes that code for peroxidase, chitinase, lipoxygenase and proteinase inhibitors were induced within 12 h of inoculation [5].

Cysteine proteinase inhibitor (CPI) was markedly up-regulated in nematode-inoculated CE-31 compared to controls and inoculated CE-109 (Fig. 1F). CPI in CE-31 began to accumulate at an earlier stage of infection. Plant-parasitic nematodes have diverse types of active intestinal proteases, including cysteine proteases [35,36]. Many inhibitors of proteolytic enzymes from plants accumulate in plant tissues upon wounding or herbivory and are known to affect the efficiency of proteinases present in the digestive tracts of several pests [13]. Recently, we showed that a novel pathogenesis-related protein (PR-10) from *Crotalaria pallida* seeds with papain inhibitory activity acts against digestive proteinases from *M. incognita* and has nematostatic and nematicide effects on J2 *in vitro* [37]. Reinforcing this finding, transgenic tomato plants overexpressing *Colocasia esculenta* cysteine proteinase inhibitor (CeCPI) showed enhanced resistance to *M. incognita* based on diminished gall numbers, decreased proportion of reproductive female nematodes, and reduced egg masses [38]. Interestingly, as observed in our study, in the transgenic tomato plants the nematode invasion was not suppressed by overexpression of CeCPI, but the root gall number was dramatically reduced.

In a pioneering study using a soybean Affymetrix GeneChip expression array to assess the root responses of resistant cowpea genotype CB46 and a susceptible near-isogenic line (null-Rk) to *M. incognita* infection, Das et al. [26] showed that in the incompatible interaction 552 genes were significantly differentially expressed between the Rk-infected and non-infected treatments. Out of these 552 genes, 141 and 59 genes showed 1.5-fold or more up-regulation and down-regulation, respectively, in the Rk-infected compared with the Rk-non-infected treatment. Moreover, in the compatible interaction, out of 1060 genes differentially expressed 218 and 41 genes were 1.5-fold or more up-regulated and down-regulated, respectively, in the infected null-Rk compared to the non-infected null-Rk treatment [26]. In addition, comparison of gene expression between the infected Rk and infected null-Rk near-isogenic lines revealed that, for example, proteinase inhibitor 19 genes were up-regulated whereas iron superoxide dismutase, putative peroxidase and heme peroxidase genes were down-regulated in infected resistant (CB46) when compared to infected susceptible (null-Rk) cowpea plants at 9 dpi [26]. Our results that showed increased activities of SOD and POX in the incompatible reaction (RKN x CE-31) compared with the corresponding control and RKN-inoculated CE-109, are apparently contradictory with the above results by Das *et al.* [26]. However, there are various forms of SOD [copper- and zinc-containing SOD (Cu,ZnSOD), manganese-containing SOD (MnSOD) and iron-containing SOD (FeSOD)] in eukaryotes [39]. In agreement with this assumption, a recent proteomic study done by our research group showed that the expression of a Cu,ZnSOD was gradually up-regulated from 4 to 6 DAI in the roots of RKN-inoculated CE-31 compared with its uninoculated control, a finding that was confirmed by the analysis of the Cu,ZnSOD transcript (unpublished data). Peroxidases in plants also occur as isoenzymes. For example, 73 class III peroxidase isoenzymes were predicted to occur in *Arabidopsis thaliana* [40]. Thus we are tempted to suppose that the divergence of our results on SOD and POX, which were induced upon cowpea infection with RKN, with those of Das et al. [26] resides on the fact that the activities we measured do not make a distinction between the various isoenzyme classes and increases in activities may have occurred in specific SOD and POX isoenzymes. For instance, only the anionic POX, but not the cationic one, was induced on cowpea leaves upon salicylic acid treatment [41]. Other plausible explanation for the divergence discussed above is that the RKN-resistance associated gene of the CE-31 cultivar might be different from the Rk resistance gene previously described [6,17–21].

In conclusion, the greater activity of superoxide dismutase concomitant with the lower CAT activity and overexpression of peroxidase, chitinase, β-1,3-glucanase, and cysteine proteinase inhibitor in the resistant cowpea cultivar CE-31, compared to CE-109, may contribute to the resistance of CE-31 against infection and colonization by *M. incognita*. Thus transgenic overexpression of these enzymes and CPI in RKN-susceptible cowpeas and in the roots of other crops could be tested for the nematode control.

4. Materials and methods

4.1. Chemicals

Colloidal chitin was prepared from crab shell chitosan (Sigma Chemical Company). β-Glucuronidase was prepared as a Type H-2 crude solution from *Helix pomatia* (Sigma Chemical Company). Other chemicals used were reagent grade.

4.2. Plants and nematode inoculum

The plant material consisted of 10 cowpea cultivars, CE-31 (or Pitiúba), Frade Preto, CE-28, CE-01, CE-315, CE-237, CE-70, CE-216, Vita-3, and CE-109, obtained from the Agronomy School seed bank at the Federal University of Ceará, Brazil.

The second-stage juveniles (J2) of *M. incognita* (race 3) used in the kinetic studied of the antioxidant enzymes and PR-proteins were isolated from susceptible cowpea plants (Vita-3 cv.) growing in a greenhouse at the Federal University of Ceará Campus, Brazil. Egg masses from *M. incognita* were isolated from galled roots using a stylet under a stereoscopic microscope (ausJENA, Germany) and placed in Milli-Q grade water in a Petri dish. The infective, motile second-stage juveniles (J2) were allowed to hatch at around 26 °C in the dark. The J2 population was counted and used as inoculum within 1–3 days of collection.

4.3. Field screening experiment for identification of cowpea cultivars resistant and susceptible to M. incognita

Seeds of 10 cowpea cultivars (Table 1) were planted in a field naturally infested with *M. incognita* at the Agronomy School of the Federal University Campus in Ceará. Average temperature varied from 25 °C (night) to 35 °C (day). Relative humidity (RH) varied from 55% (day) to 80% (night). The plants were exposed to natural light [ca. 700 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at the plant canopy] and irrigated daily with tap water. The plants were arranged in a completely randomized block design with 10 plants per block with 3 replicates. The gall and egg mass numbers were obtained from 3 blocks (30 plants) of each cultivar and used to determine the resistance levels of the cowpea cultivars tested. Sixty days after planting, the whole plant was collected and the root system washed with tap water to remove soil. In all plants collected for examination, *M. incognita* was identified as the nematode species infecting the cowpea cultivars in the naturally infected field as previously described [42]. The presence of egg masses was assessed by staining the tissue with aqueous Phloxine B solution (0.15 g L^{-1}) for 15 min and then rinsing to remove excess stain [15]. The egg mass index (EI) was scored based on a scale of 0–5 according to the egg mass number per root system [15] as: 0 = no egg mass; 1 = 1–2 egg masses; 2 = 3–10 egg masses; 3 = 11–30 egg masses; 4 = 31–100 egg masses; 5 ≥ 100 egg masses. The host susceptibility of the studied cowpea cultivars were designated as degree of resistance (DR) according to Sasser *et al.* [16], employing the following relationship between EI values and DR: EI range of 0.0–1.0 = highly resistant (hR); 1.1–3.0 = very resistant (vR); 3.1–3.5 = moderately resistant (mR); 3.6–4.0 = slightly resistant (sR); and 4.1–5.0 = susceptible (S).

4.4. Development of M. incognita in the root system of the highly resistant and highly susceptible cowpea cultivars

CE-31 and CE-109, cowpea cultivars highly resistant (hR) and slightly resistant (sR) to *M. incognita*, respectively, were selected based on the field experiment (Table 1). Seeds were surface sterilized with 10 g L^{-1} sodium hypochlorite (0.5 g L^{-1} active chloride) for 5 min and rinsed thoroughly with Milli-Q grade water. After being soaked for 10 min in Milli-Q grade water to hasten germination, 5 seeds were planted per 0.5-L plastic jar containing river sand that had previously been thoroughly washed with tap water and autoclaved for 30 min at 120 °C and 1.5 Kgf cm^{-2}. Germination and plant growth occurred in a growth chamber kept at 25–30 °C and 70 ± 8% relative humidity with a 12-h photoperiod at ca. 280 μmol s^{-1} m^{-2} of PAR. Jars were irrigated daily with distilled water for up to four days after sowing, at which time seedlings were thinned to 2 per jar. Subsequently, plants were irrigated with five-time diluted nutrient solution [43]. Twelve days after planting (DAP), 850 J2 nematodes in 1.0 mL sterile water were deposited into a 2-cm-deep hole in the soil adjacent to the primary root. The hole was filled with river bottom sand. The pots containing 2 plants each were then arranged in a completely randomized block design with cowpea cultivars as treatments, with three replicates for each time point. Starting at four days after inoculation (DAI), six plants per cultivar were uprooted every four days until twenty-eight DAI. The collected root systems were stained with aqueous acid fuchsin [44] to check for infection and nematode development.

4.5. Kinetics of the antioxidant enzymes SOD, CAT, and the PR-proteins POX, CHI, GLU and CPI (cystein proteinase inhibitors)

To produce the time-course data for the enzyme kinetics three independent experiments were conducted in which the plants were arranged in a completely randomized block design with 8 plants (2 per jar) for each time point. Seeds of the cowpea cultivars CE-31 and CE-109 were planted and inoculated as described in 4.4. The roots uninfected and infected with *M. incognita* were collected at 0, 2, 4, 6, 8, and 10 days after inoculation (DAI) they were rinsed with distilled water to remove sand, frozen in liquid nitrogen, and kept at −20 °C until used.

For protein extract preparation, the frozen roots of 8 plants, from each experiment, and for each time point, were ground for 15 min in a mortar and pestle with 0.05 M sodium acetate buffer (pH 5.2) containing 0.50 M NaCl (1:5, w/v) over an ice bath. The suspension was filtered through one layer of cheesecloth and centrifuged at 10,000× *g* for 5 min at 4 °C. Next, the supernatant was recovered and dialyzed against the extracting buffer for 24 h (two changes with ten times the supernatant volume at a 12-h interval) at 4 °C. The extract thus obtained was utilized for enzymatic activity measurements which were made using an amount of protein in the linear range of the assay. Three enzymatic independent assays for each time point were carried out using three independent root extracts, each one obtained from 8 plants. The data presented is the mean of 3 independent results obtained for each time point. SOD activity [45] was measured in 96-well plates based on inhibition of the photo-reduction of nitroblue tetrazolium (NBT, Sigma Chemical Company). The reaction mixture consisted of 10, 15, or 20 μL of root extract, 0.05 M phosphate buffer (pH 7.8) to adjust the volume to 100 μL, 20 μL 0.13 M methionine, 20 μL 0.075 M NBT, 20 μL 0.001 M EDTA (GE Healthcare), 20 μL 2.5 g L^{-1} Triton X-100 and 20 μL 0.01 M riboflavin (Acros Organics Company). The reaction was developed by exposing the reaction

mixture to a 30 W fluorescent lamp for 5 min, at the end of which absorbance was measured at 630 nm in a microplate reader (ELX-800, Biotek Instruments). Control reaction mixtures were prepared the same way as experimental ones but were not irradiated with fluorescent light. Activity was calculated as the difference between the absorbance of the control and that of its experimental counterpart. One unit of enzyme activity (UA) was defined as the amount of enzyme that induces 50% inhibition of NBT reduction. Enzyme activity was expressed in units per gram fresh tissue (UA g^{-1}T).

CAT activity was determined as described by Sudhakar *et al.* [46]. The root extract (50 μL) was incubated at 30 °C for 10 min with 2950 μL of 0.02 M H_2O_2 prepared in 0.05 M potassium-phosphate buffer (pH 7.0). The decrease in absorbance at 240 nm was recorded and CAT activity was calculated using the molar coefficient of 36.0 M^{-1} cm^{-1} [47]. Activity was expressed as μmol H_2O_2 reduced per min per gram fresh tissue (μmol H_2O_2 g^{-1}T).

POX activity was determined as previously described [48]. Aliquots (10 μL) of the crude extract were added to 990 μL of 0.05 M sodium acetate buffer (pH 5.2), 500 μL of 0.06 M H_2O_2 and 500 μL of 0.02 M guaiacol (Sigma Chemical Company). The reaction mixture was incubated at 30 °C, and the increase in absorbance at 480 nm was recorded for 10 min at 1-min interval. POX activity was expressed as the change in absorbance per min per gram of plant fresh tissue (UA g^{-1}T).

GLU activity was measured by determining the amount of glucose liberated from laminarin (Sigma Chemical Company) used as substrate [49]. Laminarin (2.0 g) was dissolved in 1.0 mL of 0.050 M sodium acetate buffer (pH 5.2), boiled (98 °C) for 10 min and exhaustively dialyzed against acetate buffer to remove free glucose. For the assay, 100 uL of sample was incubated with 900 uL laminarin for 30 min at 50 °C. After addition of the appropriate reagents to the reaction mixture, according to Boller [49], absorbance readings were taken at 520 nm and the amount of reducing sugars liberated was calculated based on a standard curve created with known amounts (7.5–240 ug mL^{-1}) of commercial glucose (Sigma Chemical Company) dissolved in 0.050 M sodium acetate buffer (pH 5.2) containing 0.50 M NaCl. Activity was expressed as nanokatal per gram fresh tissue (nkat g^{-1}T). One nkat was defined as 1.0 nmol D-glucose produced per second at 37 °C.

CHI was assayed using a colorimetric procedure that detects N-acetyl-D-glucosamine (NAG) [50] produced by the combined hydrolytic action of chitinases and β-glucuronidase upon non-radioactive colloidal chitin used as substrate [51]. Colloidal chitin (250 uL; 10 g L^{-1}) was added to 100 uL of the root extract previously diluted with 150 uL of 0.050 M sodium acetate buffer (pH 5.2) containing 0.5 M NaCl and incubated at 37 °C. The reaction proceeded for 1 h and was stopped by boiling at 98 °C for 5 min in a water bath. After centrifugation (10,000× *g*, 25 °C, 5 min), 300.0 uL of the supernatant was transferred to a microcentrifuge tube to which 10 uL of sodium acetate buffer (pH 5.2) (endochitinolytic activity) or 10 uL β-glucuronidase (Sigma, type HP-2, 9.8 units ml) (exochitinolytic activity) were added. Both reaction mixtures were further incubated for 1 h at 37 °C. Next, the tubes were incubated at 98 °C for 5 min to stop the reactions and then cooled in a water bath. Then 190.0 uL of 0.050 M sodium acetate buffer (pH 5.2) containing 0.5 M NaCl was added, followed by 100.0 uL of 0.6 M potassium tetraborate. The tubes were heated again under the conditions described above and equilibrated to room temperature (25 °C). Finally, 1.0 mL ρ-dimethylaminobenzaldehyde (DMAB, Sigma) was added. Absorbance was read at 585 nm (UIltraSpect II, Pharmacia, Uppsala) and the amount of NAG produced was calculated on the basis of a standard curve produced with known concentrations (100–700 ng mL^{-1}) of commercial NAG dissolved in 0.050 M sodium acetate buffer (pH 5.2) containing 0.50 M NaCl. The assay results were compared with controls in which the enzyme extract and β-glucuronidase were omitted from the reaction mixtures. The assay was conducted five times, and chitinase activity was expressed as nanokatal per gram fresh tissue (nkat g^{-1}T). One nkat was defined as 1.0 nmol of NAG produced per second at 37 °C.

Cysteine proteinase inhibitor (CPI) activity was determined after the dialyzed root extract was heated at 98 °C for 30 min to abolish the endogenous proteolytic activity on azocasein (Sigma Chemical Company) used as substrate. The assay was carried out according to Michaud *et al.* [35] with modifications. Fifty μL of papain (5.0 mg mL^{-1} in 0.1 M sodium phosphate buffer, pH 7.8) plus 100.0 μL of the previously heated extract and 250 μL of 0.050 M Tris–HCl buffer (pH 7.5) were preincubated at 37 °C for 30 min to allow interaction of the inhibitor with papain (Sigma Chemical Company). Next, 100.0 μL of 20 g L^{-1} azocasein, prepared in the assay buffer, was added and the reaction was allowed to proceed at 37 °C for 30 min. The reaction was stopped by the addition of 500 μL of 100 g L^{-1} trichloroacetic acid (TCA). After 5 min at room temperature, the tubes were centrifuged at 12,000× *g* for 10 min, and 500 μL aliquots were withdrawn and neutralized with 500 μL of 1.0 M NaOH. Absorbance readings were taken at 440 nm. Blanks were prepared in which the TCA preceded the addition of azocasein. Activity was expressed as units of inhibitory activity per gram of plant fresh tissue (UA.g^{-1}T); under the experimental conditions used, 1 UA is equal to 50% inhibition of papain activity.

4.6. H_2O_2 determination

Fresh roots collected from inoculated and control plantlets were powdered with liquid N_2 and extracted separately for 30 min in a mortar and pestle with 0.05 M potassium tetraborate buffer (pH 8.4) over an ice bath. The suspension was filtered through one layer of cheesecloth and centrifuged at 20,000× *g* for 20 min at 4 °C. The supernatant obtained was immediately utilized for H_2O_2 measurements [52]. H_2O_2 contents were calculated based on a standard curve prepared by dilution of 300 g L^{-1} H_2O_2 and expressed as ηmol H_2O_2 per gram fresh tissue (ηmol H_2O_2 g^{-1}T).

4.7. Statistical analyses

Data from the field experiments (Table 1) and enzyme assays (Fig. 2, A–F) were subjected to analysis of variance followed by Tukey's test.

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