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Partial Characterization of Cytosolic Superoxide Dismutase Activity in the Interaction of *Meloidogyne incognita* with Two Cultivars of *Glycine max*[1]

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Abstract: The closely related soybean (*Glycine max*) cultivars Centennial and Pickett 71 were confirmed to be resistant and susceptible, respectively, to the root-knot nematode *Meloidogyne incognita*. Increases in superoxide dismutase (SOD) activity were detected in roots of both soybean cultivars 48 hours following inoculation. Superoxide dismutase activity increased in roots of the susceptible cultivar overall, but declined after 96 hours in roots of the resistant cultivar. The isoelectric points of SOD isolated from preparasitic and parasitic developmental stages of the nematode appeared to differ. The SOD activity increased dramatically as nematodes matured and enlarged. Plant and nematode SOD were present as ca. 40-kDa cuprozinc dimers. Initial increases in SOD activity in infected tissue appeared to involve nematode regulation of plant gene expression. However, as the nematode enlarged, SOD activity could be detected within the female body only.

Key words: enzyme, *Glycine max*, host–parasite interaction, *Meloidogyne incognita*, nematode, resistance, root, soybean, superoxide dismutase.

Successful development of sedentary plant-parasitic nematodes depends on the establishment and maintenance of feeding sites (15). Root tissues surrounding the nematode body must also remain in a condition conducive to nematode development. Plant responses that limit sedentary nematode development vary with nematode–plant (sometimes cultivar) interactions but generally are associated with hypersensitive reactions (16). Hypersensitive reactions to nematode infection generally occur after the nematode reaches the specific root tissues where feeding sites are normally established (4,20,22,26).

Root tissues parasitized by nematodes may produce or release compounds that limit nematode development following infection (16); these may adversely influence bioregulatory pathways essential to nematode development or to their feeding sites. In particular, superoxide ions (O_2^- free radicals), which are highly toxic to biological systems, have been detected in roots infected by nematodes and have been associated with host resistance (28,29). Superoxide dismutase (SOD) activity of plant origin has been hypothesized as essential to sedentary nematode and pathogen development (1,2,6,15,27,28,30). This enzyme converts superoxide ions to hydrogen peroxide, and presence of free radicals enhances SOD activity (10). We report experimental findings on SOD activity in relation to the susceptibility of soybean to the root-knot nematode *Meloidogyne incognita*.

MATERIALS AND METHODS

Nematode identification and extraction: A population of *Meloidogyne incognita* Chitwood race 1 was maintained in greenhouse culture on roots of tomato, *Lycopersicon esculentum* Mill. cv. Rutgers, and eggplant, *Solanum melongena* L. cv. Black Beauty. Nematode speciation was determined by comparing female perineal patterns, juvenile morphometrics, and differential host plant responses with known nematode phenotypes (8,9). Nematode eggs were ex-

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tracted from host roots with 0.53% NaOCl (w/v) for 30 seconds (14) and hatched at room temperature on a Baermann funnel. Preinfective second-stage juveniles (J2) that hatched within the first 24 hours were discarded. The experimental J2 were those hatched from 24–48 hours after extraction (14). Other life cycle stages used in experiments were teased from infected roots with dissecting needles under a dissecting microscope (40×).

Nematode reproduction on soybean cultivars: Greenhouse studies were conducted, as previously described (20), to determine the relative rate of reproduction of a single egg mass population of *M. incognita* race 1 in two closely related soybean (*Glycine max* (L.) Merr.) cultivars, Pickett 71 and Centennial. Pickett 71 is compatible with *M. incognita,* but Centennial is not (6,20).

Seeds of each cultivar were germinated and seedlings were inoculated with approximately 100 J2 as previously described (6,20). Trays containing inoculated soybean in sand were incubated in the dark at 27 C. There were 10 replicates of each treatment.

Approximately 24 hours after inoculation, the soybean seedlings were removed from the trays, and J2 that had not penetrated and extraneous sand were rinsed from the root surfaces with distilled water (dH_2O). The seedlings were placed on moist germination paper, and the position of the inoculated portion of each root was marked on the paper and covered with a second piece of moist germination paper. These were rolled into "ragdolls" and incubated at 27 C for either 24, 48, 96, or 168 hours. Control treatments were handled similarly, but nematodes were not added during the 24-hour inoculation period.

Processing of plant and nematode tissues: Segments of roots penetrated by an average of seven *M. incognita* J2 (as determined by acid fuchsin staining of randomly selected root pieces [17]) and comparable uninoculated root segments were isolated with a scalpel. The excised root samples containing the seven J2 (0.5–1.0 g fresh weight) were frozen in liquid N_2, ground in a cold mortar (−20 C), and suspended in 5.0 ml of cold extraction buffer (0.1 M K_2HPO_4; pH 7.8; 10 mg/ml insoluble polyvinylpolypyrrolidone [PVP]).

One to two million preinfective J2 were collected from Baermann funnels and suspended in 500 µl dH_2O. The nematode suspension was dropped into liquid nitrogen in a precooled mortar (−20 C), and the resultant nematode beads were collected with a cold spatula and ground in a precooled 25 ml Potter homogenizer at 4 C (C. H. Opperman, pers. comm.). The broken beads were resuspended in cold extraction buffer, and the process was repeated three times. The nematode suspension was repeatedly sonicated with a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) using a microtip with output control set at 5 and duty cycle at 90%, until inspection under a light microscope verified that the J2 were pulverized. The suspension was diluted with 500 µl of extraction buffer.

Both J2 and root preparations were centrifuged at 25,000*g* for 30 minutes at 4 C. The supernatants were removed, desalted (Econo-desalting columns, Bio-Rad, Melville, NY) at 4 C, and lyophilized in a Dura-Dry Freeze Drier (FTS, Stone Ridge, NY) and assayed immediately for SOD activity.

For parasitic nematode life cycle stages (80 sedentary late-stage J2, 20 females that had not produced egg masses, and 10 females with egg masses), nematodes were manually dissected from infected root tissues and ground in a 500-µl centrifuge tube containing 10 µl cold extraction buffer. The samples were centrifuged at 5,600*g* for 30 seconds, and the supernatant was electrophoresed. Only soluble cytosolic forms of SOD (i.e., nonmembrane bound) were analyzed.

Electrophoresis and isoelectric focusing: Two types of electrophoresis were used to separate SOD isozymes. Initially, native polyacrylamide gel electrophoresis (PAGE) was used to determine the number of isozymes in different nematode life-cycle stages (preparasitic J2 and female) as well as in

infected and uninfected root tissue of both cultivars. A Bio-Rad Mini Protean II electrophoresis system was used for PAGE. Isozymes were separated in 8.0% acrylamide gels run at 500 V at 4 C for 45 minutes. Later, isoelectric focusing (IEF) was used to characterize SOD isozymes based on their isoelectric point (pI). For IEF, a Bio-Rad Bio-Phoresis Horizontal Electrophoresis Cell was used in conjunction with Serva Precoats of pH range 3–6 (Crescent Chemical, Hauppauge, NY) that had been prefocused for 30 minutes at 4 W and run at a constant 4 W for 1.5 hours at 4 C with Serva anode and cathode buffer numbers 3 and 7 (Crescent Chemical), respectively.

Determination of superoxide dismutase activity: Following electrophoresis, SOD activity was detected (3,13,21,24) in native and IEF gels. Gels were rinsed with 50.0 mM K_2HPO_4 (pH 7.8) and soaked for 20 minutes in the dark in 2.0 mM nitroblue tetrazolium (NBT) in 50.0 mM K_2HPO_4, pH 7.8. The gels were rinsed in 50.0 mM K_2HPO_4 (pH 7.8) and incubated in the dark for 20 minutes in a solution containing 28.0 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) and 0.028 mM riboflavin in 50.0 mM K_2HPO_4; pH 7.8. Gels were developed in sunlight, air dried, and examined for SOD isozyme activity visually and with a densitometer (CS-9000 Gel Densitometer, Shimadzu, Kyoto, Japan). Absorbance at 560 nm was measured with a band width of 4 mm. A series of differing concentrations of bovine erythrocyte SOD standards (Sigma, St. Louis, MO) were used to construct a calibration curve for subsequent densitometer readings. For publication purposes, the gels were contact printed on Kodak Polyprint (Rochester, NY) such that the clear bands on the original gels where SOD activity inhibited NBT appear black in the figures (i.e., polarity is reversed). This experiment was repeated three times in its entirety.

Molecular weight determination: The molecular weight of bands with SOD activity was estimated by mobility in nondenaturing polyacrylamide gels (200 V, 25 C, 60 minutes) at acrylamide concentrations of 5.5, 7, 8, 9, and 10%. This was done three times for each gel concentration. A nondenatured protein molecular weight marker kit (Sigma) was used for calibration. Bovine serum albumin dimer (132 kDa), monomer (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and α-lactalbumin (14.2 kDa) were used as standards (25). After electrophoresis, proteins were stained with 0.1% Coomassie Blue R-250, and the molecular weight of the nematode protein was determined from a double-log plot of the negative retardation coefficients (slopes of plots of the logarithms of the relative protein mobilities against the percentage gel concentration) of all proteins against their known molecular weights, as outlined in the Sigma Technical Bulletin enclosed with the marker kit (25).

Sensitivity assays: Sensitivity of crude SOD activity to 1.5 mM KCN (7), 1.0% sodium dodecyl sulfate (SDS), and 5.0% β-mercaptoethenol (BME) was determined three times with published procedures (25).

Results

Effect of cultivar on nematode reproduction: The soybean cultivar Centennial suppressed nematode reproduction (65 eggs/g root fresh weight), in contrast to Pickett 71, which supported substantially greater nematode reproduction (2,698 eggs/g root fresh weight). That is, Centennial was resistant to the single egg mass population of *M. incognita;* Pickett 71 was not.

Isoelectric focusing of SOD: Superoxide dismutase activity was detected in root tissue of both nematode-infected and uninoculated Pickett 71 and Centennial soybean cultivars and in all nematode life cycle stages studied. The SOD activity per nematode appeared to increase as nematodes matured, so that as nematodes increased in size, fewer nematodes were required to detect SOD activity on IEF gels. Superoxide dismutase activity appeared comparable in homogenates of 1.5 million preparasitic J2, 80 J3 and J4 (sedentary

parasitic stages), 20 young females that had not yet produced egg masses, and 10 mature females separated from their egg masses (Fig. 1). The isoelectric points (pI) of bands detected with SOD activity were between pH 4.0 and 6.0. Although the precise pI's of the bands with SOD activity were not determined, the relative migration of bands with SOD activity differed and thereby indicated that the pI of SOD in preparasitic J2 differed from that detected in the parasitic stages of *M. incognita* (Figs. 1,2).

The overall pattern of bands with SOD activity detected in roots of the soybean cultivars differed from that of the nematodes (Fig. 2). The middle band detected in both control and infected soybean root tissue appeared to have the same pI as that of the parasitic stages of the nematode. The bands obtained from infected root tissue contained SOD activity from both nematodes and plant roots. The pI of the lower and upper bands in soybean root tissue were distinct from the pI of the bands from parasitic or preparasitic nematodes. Activity staining of the lowest band was more intense in nematode-infected root tissue of both soybean cultivars than in uninfected root tissue.

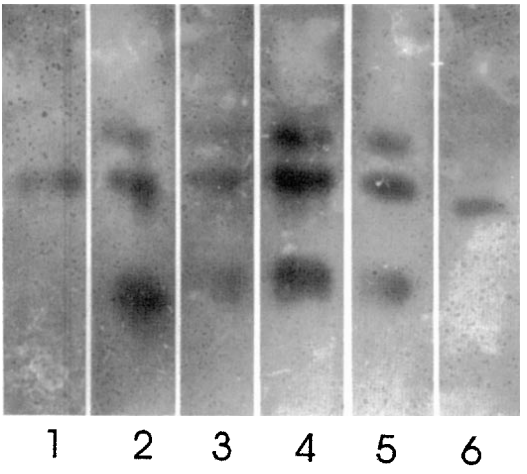

FIG. 2. Superoxide dismutase activity (nitroblue tetrazolium procedure) following isoelectric focusing of crude homogenates of healthy and *Meloidogyne incognita*-infected *Glycine max* cv. Pickett 71 and Centennial. Lane 1, homogenate of 10 mature females of *M. incognita;* lane 2, Pickett 71 (infected with *M. incognita;* 4 days postinoculation); lane 3, Pickett 71 uninfected (4 days postinoculation); lane 4, Centennial (infected with *M. incognita;* 4 days postinoculation); lane 5, Centennial (uninoculated; 4 days postinoculation); and lane 6, 1.5 million *M. incognita* preparasitic second-stages juveniles.

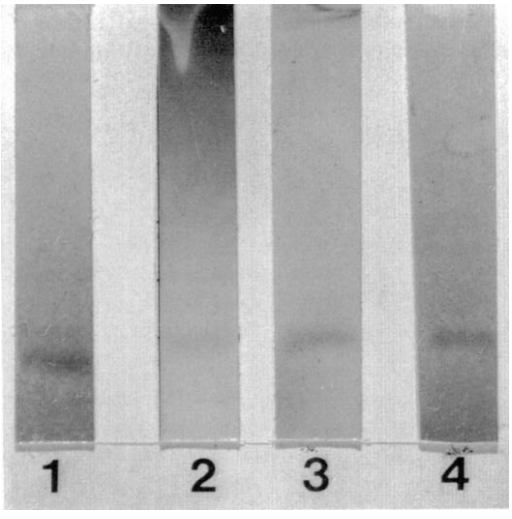

FIG. 1. Superoxide dismutase (SOD) activity (nitroblue tetrazolium procedure) following isoelectric focusing of crude homogenates of *Meloidogyne incognita* developmental stages. Lane 1, 1.5 million *M. incognita* preparasitic second-stage juveniles; lane 2, 80 third- and fourth-stage *M. incognita* juveniles; lane 3, 20 young *M. incognita* females; lane 4, 10 mature *M. incognita* females. Note that the isoelectric point for J2 SOD (lane 1 differs from that of parasitic stages (lanes 2–4).

The SOD activity (composite densitometer readings of all three bands) was determined for both uninfected and infected tissue. The SOD activity (units/μg protein) of uninfected root tissue was subtracted from that of infected root tissue for each cultivar over time. One unit of SOD activity will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25 C in a 3.0-ml reaction volume. The xanthine oxidase concentrations should produce an initial (uninhibited) ΔA_{550} of 0.025 ± 0.005 per minute. At 24 hours postinoculation, the level of SOD activity was identical for both cultivars. Increased differential SOD activity was detected in infected roots of both cultivars at 48 and 96 hours after inoculation but declined thereafter (Fig. 3). A decline in dif-

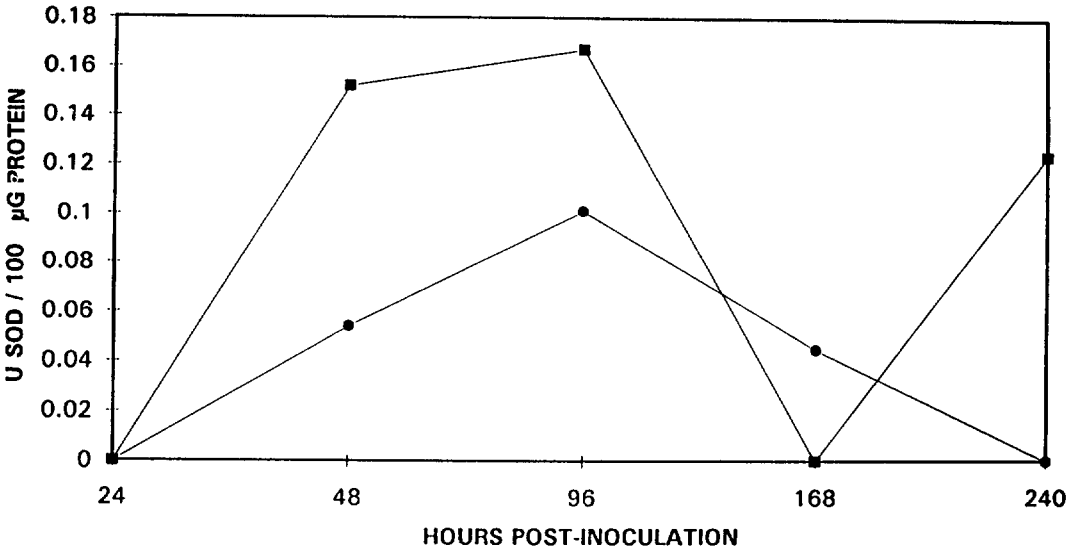

FIG. 3. Differential superoxide dismutase activity (infected minus healthy) in *Meloidogyne incognita*-infected root tissue of the soybean cultivars Pickett 71 (■) and Centennial (●). SOD activity is expressed in units per 100 µg protein.

ferential SOD activity in infected Pickett 71 root tissue at 168 hours and a subsequent increase in differential activity at 240 hours postinoculation was also detected. This decline in SOD activity at 168 hours and subsequent increase at 240 hours was detected in Pickett 71 each of the three times the experiment was conducted.

Detection of SOD activity via PAGE: The presence of SOD activity in nematodes and soybean roots was confirmed by PAGE. Four bands with comparable activity were detected in homogenates of 10 mature females and 1.5 million preparasitic J2. Seven bands with activity were detected in infected and uninoculated root tissue of both soybean cultivars. The relative intensity of the two lowest and the highest bands was enhanced in infected root tissue (Fig. 4). The position of bands with SOD activity relative to molecular weight standards suggested that both plant and nematode SOD dimers were approximately 40 kDa (Fig. 5).

Sensitivity assays: The SOD activity detected in both nematode and plant homogenates was completely inhibited by 1.5 mM KCN and 5.0% BME, but SOD activity was not inhibited by 1.0% SDS.

DISCUSSION

Increased SOD activity has been proposed as critical to sedentary nematode development; plant reactions that are incompatible with sedentary nematodes depend on the inability of the plant to enhance SOD activity to levels required to arrest

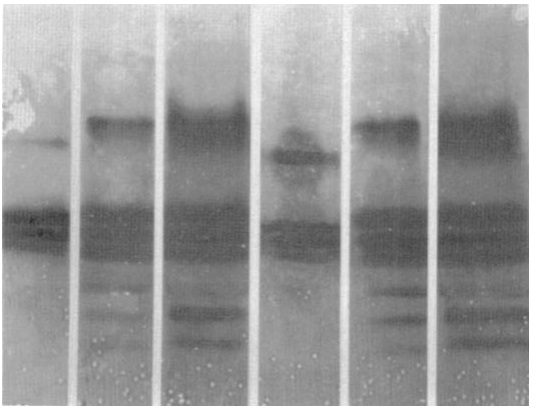

FIG. 4. Native acrylamide gels of superoxide dismutase activity. Lane 1, 1.5 million *Meloidogyne incognita* J2; lane 2, Centennial soybean uninoculated (168 hours); lane 3, Centennial inoculated (168 hours, postinoculation); lane 4, 10 mature *M. incognita* females; lane 5, Pickett 71 uninoculated (168 hours); lane 6: Pickett 71 inoculated (168 hours postinoculation).

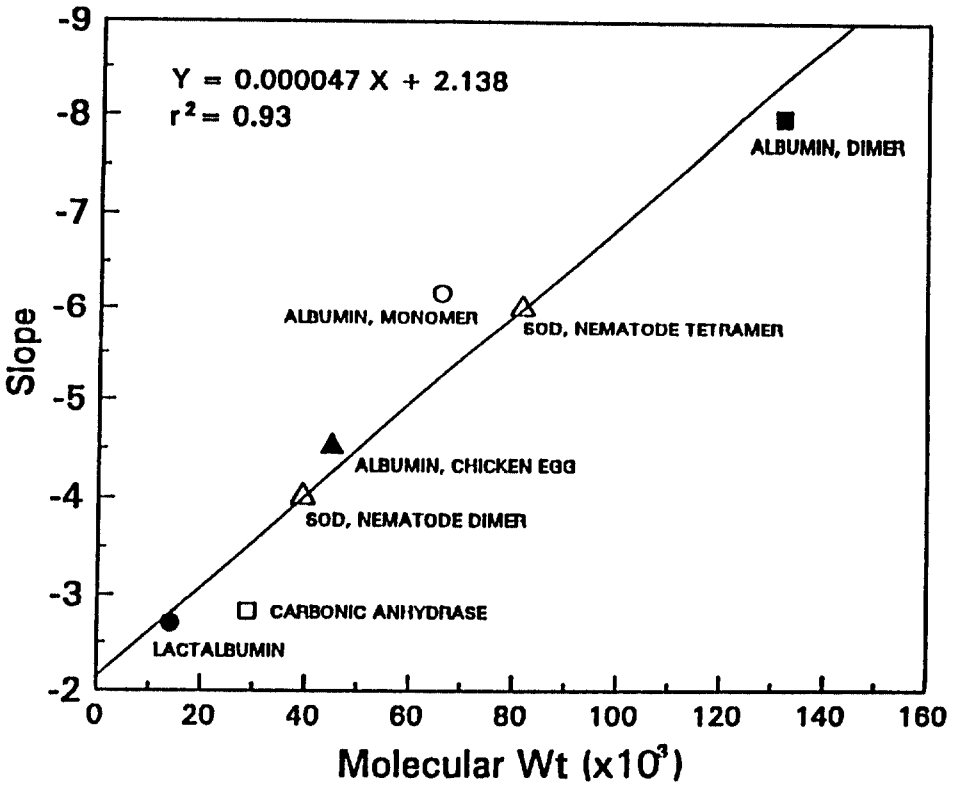

FIG. 5. Molecular weight determination of dimeric and tetrameric forms of superoxide dismutase in *Meloidogyne incognita* (1.5 million J2). Molecular weights of standards are lactalbumin (●) = 14.2 × 10^3, carbonic anhydrase (□) = 29.0 × 10^3, chicken egg ovalbumin (▲) = 45.0 × 10^3, bovine serum albumin monomer (○) = 66.0 × 10^3, bovine serum albumin dimer (■) = 132.0 × 10^3. Superoxide dismutase in *M. incognita* for dimer = 40.0 × 10^3 and for the tetramer = 80.0 × 10^3.

free radicals in infected tissues (1,2,12,27, 28). Previously, increased levels of SOD activity had not been detected in roots of susceptible plants until 4 or more days after inoculation. Conversely, SOD activity in infected roots of resistant plants was demonstrated to decline starting 4 or more days after inoculation (1,2,12,27,28).

Studies involving the interaction of root-knot nematodes with soybean (6,20) and tomato (4,22,26) suggest that recognition-related events critical to nematode development occur within the first 24 hours following infection and that subsequent expression of defense reactions are effectively completed within 72 to 96 hours (4,20,22,26). Because SOD activity in root tissues was not enhanced until 4 days following infection of susceptible roots (28), SOD might be involved with only secondary processes related to development and maintenance of feeding sites rather than the initial physiological processes that result in compatible or incompatible responses to root-knot nematode infection. Preliminary PAGE studies suggested that enhanced SOD activity might be of nematode, rather than of plant origin, because all of the SOD activity detected in galls was found within the mature female (Kaplan, unpubl.). The present IEF studies, however, clearly demonstrate that plant SOD activity increased within 48 hours of inoculation. This increase suggests that SOD activity does play a role in the nematode–plant interaction. The specific relationship of increased SOD activity to plant susceptibility is unclear, however, because increases in SOD activity were detected in both soybean cultivars.

This study reports the earliest increase in SOD activity following infection of roots

by a plant-parasitic nematode (27,28). Increased SOD activity occurred within the time frame when the isoflavanoid glyceollin accumulated in infected roots of Centennial (19). As a putative phytoalexin, glyceollin was related to the hypersensitive reaction that precluded nematode development in root-knot nematode infected soybean roots (19). Glyceollin impaired nematode activity in vitro, and inhibited respiration of isolated mitochondria (5,18). The role of increased SOD activity in hypersensitive tissues requires elucidation.

Our earlier detection of enhanced SOD activity and the identification of isozymes in the interaction of *M. incognita* with soybean may be attributed to differences in the sensitivity of assay methods (the IEF and densitometry used in the present study are considered to be 1,000-fold more sensitive than the spectrophotometry used previously) or to biological differences between the nematode–plant interactions studied (27,29). Initial increases in SOD activity appeared to be of plant origin and may reflect nematode regulation of plant gene expression. Enhanced SOD activity may also be stimulated as a result of the nonspecific release of free radicals as a consequence of damage to plant tissues incurred during nematode penetration. Superoxide dismutase converts the O_2^- free radical to less-toxic peroxides, which can then be detoxified by catalase; the presence of free radicals enhances SOD activity (23).

Early increases in SOD activity detected in Pickett 71 could not be of nematode origin because the number of preparasitic J2 required to detect SOD activity (1.5 million) far exceeded that present in roots. An average of 490 J2 that penetrated root tissues of the 70 roots used in each sample would be detectable only if their SOD activity was comparable to levels observed in the late J2 stages (168 hours). The increased intensity of the lowest band (Fig. 2), which is unique to the soybean roots, further suggests that early increases in SOD activity are of plant origin.

The SOD activity in *M. incognita* race 1 was similar to that characterized in *Caenorhabditis elegans*. The SOD activity in *C. elegans* and *M. incognita* was not inhibited by SDS but was inhibited by heat and BME (25). This suggested that the SOD activity present in cytosolic fractions of nematode and soybean crude homogenates was from a cuprozinc SOD (25). Based on size, the SOD in both species appears to be a dimer (10,23). Based on sensitivity assay data, the SOD likely contained Cu and Zn with subunits bound together by disulfide bonds (10,11,23,25). In addition, we report for the first time differences in the pI of SOD activity in preparasitic and parasitic stages of a plant-parasitic nematode; preparasitic J2 had a slightly lower SOD pI than the parasitic J2 and female.

The significance of enhanced SOD activity in nematode-infected root tissue relative to plant susceptibility remains uncertain. Increased SOD activity is associated with aging to biological organisms (25,31); nematodes have been used as a model system to study changes in SOD related to aging because they have relatively short life spans. The free radical theory of aging postulates that the deleterious effect of free radicals to the cells and tissues constitutes, or at least largely contributes to, the aging process. Enhanced SOD activity in aging organisms has been inferred as a natural process to resist oxidative stress to the organism (31). However, early detection of enhanced activity of SOD bands that appear to be plant specific suggests that SOD may be a nonspecific response during the early stages of the interaction, whereas later increases may reflect enhanced SOD activity within the developing nematode.

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