Histochemical Root Pathology of *Brassica oleracea capitata* L. Infected by *Pratylenchus penetrans* (Cobb) Filipjev and Schuurmans Stekhoven (Nematoda:Tylenchidae)

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Abstract: Histochemical study of cabbage roots axenically parasitized by *Pratylenchus penetrans* revealed a five-fold increase in peroxidase activity (localized near lesions), pectic xylem plugs (resembling those caused by *Fusarium*), and accumulation of oxidase-mediated polyphenols in the region of mechanical injury. *Fusarium*-resistant cabbage was more susceptible to *Pratylenchus* than the *Fusarium*-susceptible varieties, particularly in the formation of oxidized phenolic compounds. Of 13 fluorescent compounds detected by paper chromatography, one major spot was found to be ferulic acid and a minor one, catechin. *Key Words: Pratylenchus penetrans*, Lesion nematode, Cabbage, Histochemistry, Pathogenesis, Pectin, Phenolics, Host-parasite relationships.

In recent years, cabbage plants and soil samples submitted from certain fields in Massachusetts have yielded high populations of the lesion nematode, *Pratylenchus penetrans* (Cobb) Filipjev and Schuurmans-Stekhoven. Roots with dark brown lesions characteristic of lesion nematode injury contained eggs, juveniles and adults of this species.

P. penetrans causes typical lesions although the extent of injury varies widely with host species and variety (7, 15, 16, 18, 25, 26). Lesion nematodes primarily cause necrosis and breakdown of root cortex cells. In general, symptoms are limited to the feeding site, and cavities are formed in the cortex in which eggs are laid and juveniles develop. In peach (15), large lesions form within a few hours, while in apples, lesions are much smaller even after several months (18). Many investigators have attributed the symptoms to the accumulation of phenolic compounds in the injured area and the activation of associated oxidative enzymes (14, 18, 20, 25, 26). The importance of these reactions and the role of phenolics in plant disease is not well understood.

The host-parasite relationships of *P. penetrans* and cabbage have not been described. Preliminary studies in our laboratory (Acedo, unpublished data) showed that cabbage reacts strongly to nematode feeding. This investigation was undertaken (i) to determine the histopathological development of the nematode in cabbage roots, (ii) to relate the changes induced in the cells to the general symptom expression of the plant, and (iii) to isolate and characterize the phenolic compounds from injured and healthy root tissues on three cabbage cultivars.

MATERIALS AND METHODS

Pratylenchus penetrans were axenically cultured on alfalfa ('Dupuits') callus grown upon nutrient agar by a modification of Krusberg's (10) methods. They were aseptically extracted from infected callus placed 24 hr on a 1.5 cm plug of nonabsorbant cotton in contact with 10 ml of distilled water in a previously autoclaved, foil-capped, 15-ml centrifuge tube. Unless otherwise stated, nematodes thus extracted were transferred directly to seedlings. Seeds of three cabbage varieties 'Early Jersey Wakefield'

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(EJW) which is *Fusarium* resistant, 'Golden Acre' (GA) and 'Danish Ballhead' (DB), both *Fusarium*-susceptible, were surface sterilized 15 min in 2.5% sodium hypochlorite, rinsed twice in sterile distilled water and germinated on 2% water agar in petri plates.

Healthy, uniform, 4-day-old seedlings of each variety were transferred (three seedlings per tube) into 150 × 25 mm tubes of nutrient agar prepared according to Chen *et al.* (5). When primary roots were about 3 cm long, 75–100 nematodes, mostly adults, were introduced in 0.05 ml water via a micropipette and the tubes incubated at 21 C and 12 hr photoperiod. Forty tubes of each variety were inoculated, and 40 seedling cultures served as uninoculated controls. Inoculated and uninoculated seedlings also served as sources of extracts of diseased and healthy tissues.

In the greenhouse, one-month-old seedlings of each cabbage variety were planted singly in 4-inch pots, and treated with inocula of 0, 100, 250, 500, 1000, and 10,000 aseptic nematodes obtained from axenic cultures on alfalfa callus. These were introduced close to the seedling root in a drop of water. Each inoculum level was represented by 10 single plant replicates and arranged in a single randomized block on a greenhouse bench where the temperature fluctuated between 23–30 C during the daytime and 20–23 C at night. A water soluble complete fertilizer with 20-20-20 analysis and containing micronutrients was applied (approx. 0.40 gm/pot) every two weeks. Depending on the maturity of each variety, the plants were harvested 60 (EJW), 76 (GA), or 90 (DB) days after inoculation. The nematodes in the soil in each pot were extracted by sugar flotation (8). The roots were incubated for 48 hr following the methods of Young (31) and were then chopped and placed over paper in a modified Baermann funnel. The numbers of nematodes recovered from the roots were added to the counts made from the soil. At the end of the experiment, shoots and roots were separately cut off at the soil, oven dried (100 C) for 18 hr, and weighed.

The infection process on seedlings was examined in several ways. Whole roots and frozen sections cut in a cryotome were analyzed for phenols directly on microslides with selective histochemical reagents such as diazotized sulfanilic acid (DSA), 2,6-dichloroquinone-4-chloroimine and tetrazotized O-dianisidine (Fast Blue) (1, 13, 23). Roots mechanically injured by puncturing with sterile microneedle were examined immediately after injury, and at 1, 3, 6, 12, and 24 hr thereafter. In addition, the influence of injury on the rate of browning by hydrolytic enzymes was studied by dipping injured whole roots in 1% aqueous solutions of Pectinol 59-L® (Rohm and Haas, Bristol, Pa. 19007), B-glucosidase (almond emulsin), horseradish peroxidase, cellulases (Cellulase 36, Rohm and Haas), or distilled water.

Paraffin sections of root lesions caused by *P. penetrans* and of punctured healthy roots were prepared following standard histological methods, using CRAF III fixation, normal butyl alcohol dehydration, and safranin-fast green (9).

Chromatographic and spectrophotometric analyses were made on extracts of healthy and diseased tissues. Peroxidase activity was determined by the method of Loebenstein and Linsey (12).

For chromatographic and subsequent spectrophotometric analysis, crude extracts were prepared from homogenates of necrotic areas, and from healthy roots. Root tissues were cut and weighed separately, dropped in boiling methanol, cooled, and homogenized in a glass grinder. Homogenates were centrifuged 5 min at 3100 *g* to remove cell fragments. Each extract was concentrated

to approximately 1 ml per gram of tissue in a rotary evaporator under reduced pressure at 40 C. Crude methanolic extracts were used without further purification.

Crude extracts (100 μl, equivalent to 0.03 gram fresh weight) were spotted on Whatman No. 1 (18½ × 22½ in) chromatography paper using the method described by Block, *et al.* (1). The most efficient descending solvents were 4:1:5 (v/v/v) n-butanol/acetic-acid/water (BAW), and 125:72:3 (v/v/v) benzene/acetic acid/water (BzAW). Other organic solvents were also used for further identification of specific compounds.

Chromatograms were observed under ultraviolet light (320–400 nm) before and after exposure to ammonia fumes for detection of aromatic compounds. Detecting agents used were DSA, DCQ, Fast Blue, tetrazotized benzidine, ferric chloride-ferric cyanide, ammoniacal silver nitrate and 0.3% alcoholic ninhydrin (1,13,23).

Unidentified fluorescent compounds from areas with the same R_f values were detected and outlined under UV light, cut out from unsprayed chromatograms and eluted 4 hr in methanol. The absorption spectra of the eluents were scanned and measured from 210–320 nm in a B & L 505 recording spectrophotometer.

Results and Discussion

Symptomatology: Root lesions are the most characteristic symptom in plants attacked by *P. penetrans*. Nematodes congregated readily around roots of seedlings growing in test tubes and began feeding within 3 hr following inoculation. Small, faint, yellow spots associated with nematode feeding eventually enlarged to elliptical brown lesions which appeared 24–36 hr later.

Disease development in greenhouse-grown plants resembled that in the laboratory except that all the roots showed lesions. Most of those showing necrosis were found near the soil surface, although many lateral roots were attacked. Browning was evident at the nodes and tips of rootlets. Many lateral roots had a beaded appearance due to large number of aborted root initials.

Numbers of nematodes increased in each of the three varieties tested. Populations were counted in 10 tubes of each variety 60 days after inoculation and were 18–43 times greater than the original inoculum (75–100 *P. penetrans*/tube). The mean differences of population increase between varieties were not statistically significant. In greenhouse experiments, we measured the effects of inoculum level (0, 100, 250, 500, 1000, 10,000 *P. penetrans*/pot) on growth of the host. Populations increased from 3–7 times, but total numbers of nematodes did not differ significantly between varieties when analyzed for fresh shoot weights (3 varieties, 10 replications/variety). However, within each variety, as the inoculum level rose there was a corresponding increase in population and a reduction in root weight. An inoculum of 250 nematodes resulted in a mean dry weight reduction of 38% in 8 weeks which was statistically significant at the 1% level. Tops of infected plants showed no symptoms other than reduced growth.

Varietal difference in the degree of browning and lesion formation were apparent. *Fusarium*-resistant EJW exhibited dark brown spots, which covered a large portion of the roots within a few days, whereas the *Fusarium*-susceptible GA produced discrete light brown lesions which coalesced only after several weeks. Root injury did not appear to affect nematode population adversely, in fact, the highest populations were recovered from roots with the most severe necrosis.

Histological Studies: *P. penetrans* destroyed parenchyma cells of the root cortex. Cell walls at feeding sites were thicker than

normal and stained darkly with safranin. Nematode movement within the cortex was both inter- and intracellular. All life cycle stages including eggs were present within the roots. A blue-staining gummy material associated with cortical injury was found in the xylem tissues of EJW 3 weeks after inoculation; GA and DB showed this reaction only after 6 weeks. Wilting of plants in the greenhouse was also observed at the same time after inoculation which possibly can be attributed to xylem plugging. Histochemical studies using ruthenium red (9) and hydroxylamine-ferric chloride (19), indicated that the plugs were of pectic material.

Cabbage plants responded similarly to *P. penetrans* and *Fusarium* infections in the formation of gel-like xylem plugs during pathogenesis. In *Fusarium* infection, Smith and Walker (24) concluded that the gummy deposit is probably a response of the host to injury rather than a form of resistance. Krusberg (11) reported that salivary secretions of plant parasitic nematodes containing high concentrations of pectic, cellulolytic, and proteolytic enzymes were capable of breaking down cells and releasing their contents. In addition, a β-glucosidase, similar to that of the wilt-causing Fusaria and capable of releasing phenols bound in phenolic glycosides is secreted by *P. penetrans* (14, 15).

P. penetrans is known primarily as a root cortex parasite; there are no published reports of this nematode in the stele. Our histological evidence indicates that the cortical cells at many feeding sites collapsed, leaving cavities surrounded by cells with a fast-blue staining, highly granular protoplasm. In cabbage, the nematodes were found probing the endodermal tissues 2 weeks after inoculation and had penetrated to the stele 4 weeks later. No trophic reaction was observed, only localized mechanical destruction. A mucilaginous substance, which stained dark blue, was found in the endodermis as early as 9 days before the nematode reached this tissue. The host parasite relations follow the general patterns of injury observed by Castillo (4) in alfalfa, by Pi (17) in tomatoes, and by Rohde (unpublished data) in carrots, in which the endodermis presents a barrier to invasion of the vascular elements. The resistance of the carrot and tomato endodermis to penetration, however, was only temporary since nematodes were found in the stele 1 month after inoculation. Likewise, in cabbage the endodermis only temporarily protected the stele for 3 weeks after inoculation. Results therefore indicate that *P. penetrans* can cause extensive endodermal and vascular destruction in cabbage.

A detailed discussion on the biochemistry, function, and protective significance of the endodermis was presented by Van Fleet (28). Uritani (27) stated that necrotic tissue may be a physical barrier to further penetration by the invading pathogen. Pathogenicity studies of *P. penetrans* by several workers have shown that the endodermis is often the region which becomes discolored and thus may serve as a barrier to nematode penetration into the vascular system (15, 18, 25, 26).

Phenols accumulated in the endodermis of infected cabbage roots far in advance of actual nematode feeding. Visible browning and polyphenol accumulation, as detected by the specific reagents DSA, DCQ, and Fast Blue, always occurred together. Paraffin sections showed a necrotic reaction mainly in the cortical and epidermal tissues (usually 2–3 cell layers) and endodermal tissue with deep affinity for stain in advance of the nematode penetration. In addition, the endodermal cells appeared to be filled with granular and densely-colored material.

MECHANICAL INJURY: Roots punctured with a sterile micro-needle developed lesions

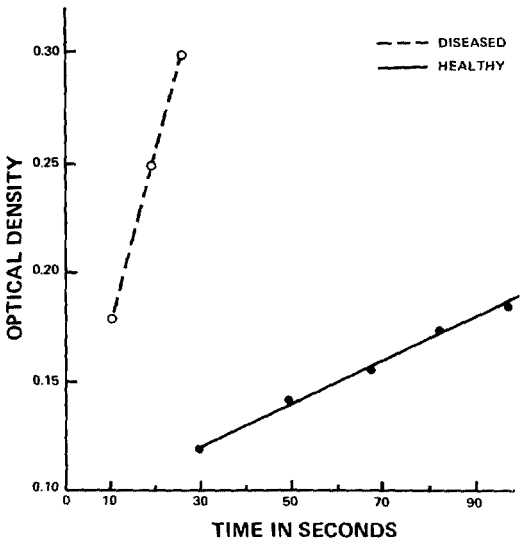

FIG. 1. Peroxidase activity of healthy, and nematode-infected, cabbage roots 72 hr after inoculation. Time-rate of optical density change in pyrogallol solution in the presence of H_2O_2 was measured at 420 nm. Each point represents the mean of 2 samples. Samples were divided into 5 subsamples and the determinations were averaged for the figure.

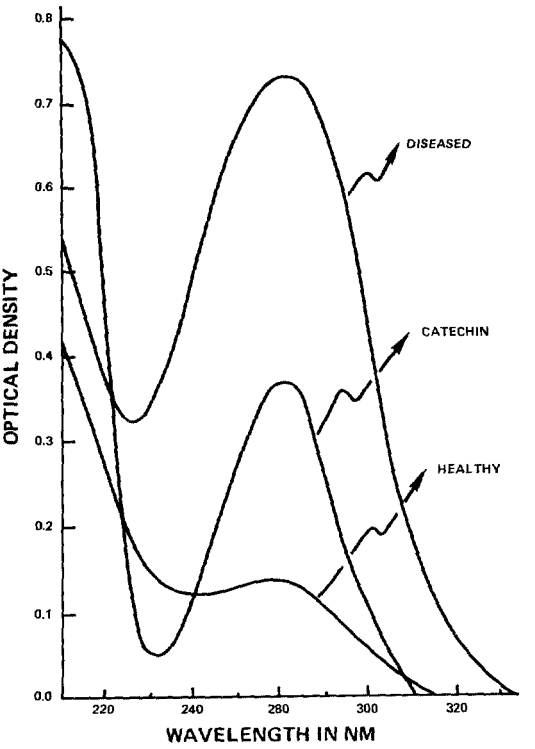

FIG. 2. Absorption spectra of a fluorescent compound (R_f 0.69 in BAW) from extracts of healthy, and nematode-infected, EJW cabbage. Peak absorption is at 280 nm. Note similarity of curves of the unknown spot at R_f 0.69, and authentic catechin.

within 36 hr resembling those caused by the nematode, but these remained localized near the site of injury. Addition of hydrolytic enzymes such as Pectinol 59-L®, emulsin, horseradish peroxidase and Cellulase 36 resulted in rapid discoloration of punctured areas with the development of a more intense brown color. Root lesions caused by nematodes did not differ in gross appearance from those resulting from mechanical injury.

ASSAY FOR PEROXIDASE ACTIVITY: In roots of nematode-infected plants, peroxidase activity was five times greater than in healthy roots. The differences are shown in Fig. 1.

Farkas and Kiraly (6) concluded that polyphenoloxidase and peroxidase activities were higher in infected than in healthy tissues. Preliminary work in our laboratory showed that tissue sections of cabbage roots infected by *P. penetrans* gave strongly positive reactions for peroxidase as measured by the method of Burstone (3) and that the reaction was more highly concentrated adjacent to injured tissues than in healthy areas.

The physiological role of peroxidase in metabolism is not clearly understood. Its activity is known to be high in wound meristem (28) and the enzyme is capable of oxidizing many mono- and diphenols and aromatic amines to quinones in the presence of hydrogen peroxide (2). Rubin and Chetverikova (21) suggested that the terminal oxidase in cabbage infected by *Botrytis cinerea* Pers. is a flavoprotein system which produces hydrogen peroxide which is removed by peroxidase.

CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC ANALYSES OF PHENOLICS IN CABBAGE: The phenolic compounds associated with lesion nematode injury to cabbage were detected by paper chromatography and ultraviolet absorption spectrophotometry. Thirteen fluorescent compounds were readily detected on chromatograms of methanolic extracts from tissue of healthy and infected cabbage roots. All varieties tested gave the same number of visible spots except that those from seedlings infected with *P. penetrans* were larger and brighter under UV light. Two of the fluorescent spots were presumed to be the main phenolics of cabbage. All varieties exhibited these two compounds with the same R_f values in four different solvents, viz., BAW, BzAW, benzene/methanol/acetic acid, 45:8:4 v/v/v and 8% acetic acid. The spots appeared sky-blue under UV light but reacted differently with chromogenic reagents. One of the major spots with an R_f value at 0.84 in BAW was identified as ferulic acid, the other major spot (R_f 0.93) was not identified. A weakly reacting spot (R_f 0.69, BAW and BMA) was identified as catechin by color reaction and UV absorption comparison with known standards. Fig. 2 shows a representative spectrum of the eluent from this spot. On the basis of O. D. at 280 nm, the amount of catechin in infected extract was 20 times more than in healthy extract. Rubin and Ivanova (22) reported a similar increase of this compound in cabbage infected by *Botrytis cinerea* Pers.

The synthesis of the above compounds suggests that penetration and feeding by nematodes stimulates injured cells to accumulate relatively large amounts of phenolic substances, apparently a result of the nematode-host interaction, since only a trace of these compounds and other unidentified spots was found in extracts of healthy tissues.

No definite conclusion was drawn regarding the mechanism involved in accumulation of these compounds in lesions or mechanical processes associated with this reaction. Phenolic compounds are either synthesized in the lesion by the injured cells or perhaps preformed phenols are released from compounds such as glycosides by increased activity of parasite enzymes. In either case, oxidized phenols are the principal factors responsible for browning and the production of necrotic tissues.

Discoloration due to subsequent polymerization of the oxidized phenols to brown substances has been shown to occur in a variety of plant diseases (6, 27). This type of browning reaction was observed by Wallace (29, 30) in areas of chrysanthemum leaves infested by foliar nematodes. In this case, chlorogenic acid and isochlorogenic acid were the principal polyphenols oxidized. Recently, Pi (17) compared the effects of lesion and root-knot nematodes on resistant and susceptible tomato varieties. Infection by either species led to the accumulation of phenolics, largely chlorogenic acid and its derivatives, in the endodermis.

In conclusion, injury produced by the feeding activity of *P. penetrans* on cabbage is similar to that on other known hosts of this species, that is, mechanical and enzymatic destruction of cells have caused the discoloration of nearby intact cells and subsequent accumulation of phenolic compounds. These compounds were presumed to influence the activity of nearby cells especially in the endodermis.

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