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Pectolytic Enzymes in Three Populations of *Ditylenchus dipsaci*[1]

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Abstract: Extracts of nematodes of the Raleigh, North Carolina (RNC), Waynesville, N. C. (WNC), and onion populations of *Ditylenchus dipsaci* were examined for pectolytic activity. RNC nematodes contained a NaCl-stimulated endo-polymethylgalacturonase with optimal pH for activity of 6.0, whereas nematodes of the WNC and onion populations possessed a NaCl-stimulated endo-polygalacturonase with pH optimum of 4.0. Nematodes of each population also contained a $CaCl_2$-activated endo-pectin methyl-*trans*-eliminase with optimal pH of 9.0. Nematode extracts containing 0.5 M NaCl macerated potato discs. RNC and onion nematodes induced gall formation in Wando pea seedlings, but WNC nematodes induced a resistant, hypersensitive response. Thus pectolytic activity was not correlated with pathogenicity of *D. dipsaci* on Wando pea. *Key Words:* stem nematode, pea, pathogenicity, resistance.

Pectolytic enzymes have been investigated in several nematodes (4). Because of the widespread occurrence of cell separation in plants infected by *Ditylenchus dipsaci* (21), pectolytic activity in extracts or secretions of various races or populations of this nematode has frequently been described (5, 10, 12, 13, 16, 19, 20, 24). Krusberg (13) demonstrated both hydrolytic and *trans*-eliminative degradation of pectin with extracts of nematodes of the Raleigh, North Carolina (RNC) alfalfa population. Riedel and Mai (19) detected endo-polygalacturonase (endo-PG) and endo-pectin methyl-*trans*-eliminase (endo-PMTE) in aqueous extracts of an onion population of *D. dipsaci* from Orange County, New York (ONY). Endo-polymethylgalacturonase (endo-PMG) and PMTE in RNC *D. dipsaci* were confirmed by Muse et al. (16), but extracts of the Waynesville, North Carolina (WNC) alfalfa population contained endo-PG and PMTE. These two populations differ in that RNC nematodes induce galling and stunting of *Pisum sativum* 'Wando', whereas WNC nematodes induce a hypersensitive reaction (8).

Riedel and Mai (19) suggested that differences in pectolytic activities among nematodes of RNC, WNC, and ONY populations possibly resulted from experimental variations. The purpose of this investigation was to study pectolytic enzymes of the RNC, WNC and ONY populations of *D. dipsaci* under uniform conditions.

MATERIALS AND METHODS

RNC, WNC, and ONY populations of *D. dipsaci* were cultured monoxenically on alfalfa callus (11). Nematodes were removed from 6- to 8-week-old cultures with modified Baermann funnels, washed 5 times with distilled H_2O, and diluted to 10 times their fresh packed volume with 0.5 M NaCl or 0.005 M $CaCl_2$. The resulting suspension was passed twice through a French pressure cell and centrifuged 10 min at 9,000 *g*. The supernatant was dialyzed overnight at 4 C against one liter of 0.5 M NaCl or 0.005 M $CaCl_2$ and used as the source of enzyme. Control extracts were inactivated by boiling for 15 min. Protein in trichloroacetic acid precipitates of extracts was estimated by the Lowry method (14); casein solutions were standards.

Depolymerizing pectolytic activity, i.e., that consisting of any fragmentation (exo-

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or endo-) of a pectic substance at the bond connecting two galacturonic acid residues, was measured by determination of increase in reducing power of pectic substances following enzymic action. Substrate was 1.0% citrus pectin (Nutritional Biochemicals Corp., Cleveland, Ohio) or 1.0% sodium polypectate (Nutritional Biochemicals Corp.) buffered at pH 4.0, 5.0, 6.0, or 7.0 with citrate-phosphate buffer (0.1 M citric acid plus 0.2 M Na_2HPO_4) or at pH 8.0 or 9.0 with 0.2 M tris(hydroxymethyl) amino methane. One ml of active or boiled extract of *D. dipsaci* in 0.5 M NaCl, or one ml of 0.5 M NaCl was incubated with 4 ml of substrate for 3 h at 30.1 C. Two drops of toluene were added to inhibit bacterial growth (10). The reducing power of a 0.5-ml aliquot from each reaction mixture was measured by the dinitrosalicylic acid (DNSA) method (15), with galacturonic acid solutions as standards.

The type of depolymerization, i.e. exo- or endo-, was determined by viscometry. Substrate was 1.0% pectin or 1.25% Na polypectate, buffered as previously described from pH 4.0 through 9.0. In tests with extracts of *D. dipsaci* in 0.005 M $CaCl_2$, substrate contained 0.003 M $CaCl_2$. One ml of active or boiled nematode extract was added to 5 ml of substrate in No. 300 Ostwald-Fenske viscometers. Loss in viscosity (10) was determined after incubation for 30 min at 30.1 C.

For measurement of *trans*-eliminase activity, one ml of active or boiled extract of *D. dipsaci*, 0.5 M NaCl, or 0.005 M $CaCl_2$ was added to 4 ml of 1.0% pectin or 1.0% Na polypectate buffered from pH 4.0 through 9.0 and containing 2 drops of toluene. Solutions were incubated for 24 h and unsaturated degradation products were measured by the thiobarbituric acid (TBA) method (17). Attempts were also made to detect such breakdown products directly by measurement of increases in absorbance at 230 nm (1).

Pectin methylesterase (PME) activity was measured by a modification of the method of Smith (22). Substrate was 0.5% pectin and 0.2% phenol in 0.1 M NaCl. Three ml of active or boiled extract of nematodes in 0.5 M NaCl was added to 30 ml of substrate, and pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 with NaOH solution. Reaction mixtures were incubated at 30.1 C, and pH was measured after 3 and 24 h.

Liberation of galacturonic acid by hydrolysis of pectic substances was detected by ascending paper chromatography. One ml of active or boiled extract of *D. dipsaci* in 0.5 M NaCl was added to 4 ml of 1.25% pectin buffered at pH 6.0 (RNC population), or 1.25% Na polypectate buffered at pH 4.0 (WNC and ONY populations) and containing 2 drops of toluene. Solutions were incubated for 48 h at 30.1 C and centrifuged 20 min at 9,000 g. Ten-μl aliquots from the supernatants and galacturonic acid standards were spotted on Whatman No. 1 paper. Chromatograms were developed with 75% isopropanol: glacial acetic acid (9:1) (3), dried, sprayed with *P*-anisidine HCl reagent (18), and heated for 10 min at 130 C.

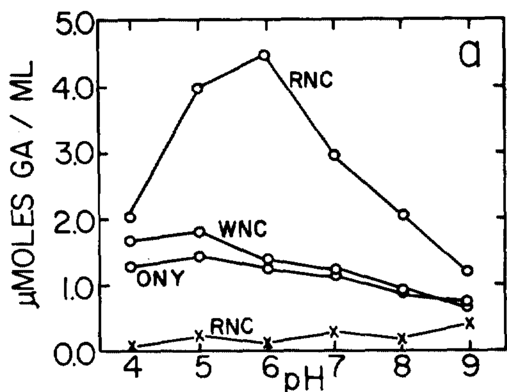
Enzymic maceration of potato discs was detected by immersing tuber sections 1.5 mm thick in a solution comprised of 2 ml of active or boiled extract of *D. dipsaci* in 0.5 M NaCl, 2 ml of buffer at pH 6.0 (RNC population) or pH 4.0 (WNC and ONY populations), and 2 drops of toluene. Discs were scraped with a knife after 5, 10, and 20 h to determine degree of maceration subjectively. This procedure was repeated with nematode extracts in 0.005 M $CaCl_2$, but solutions were buffered at pH 9.0 rather than pH 4.0 or 6.0.

Pathogenicity of nematodes of each population on 'Wando' pea was investigated by the procedure of Hussey and Krusberg (7). Infected seedlings were observed for symptoms, boiled in lactophenol-cotton blue, and cleared in lactophenol to determine location of nematodes (6).

All experiments were repeated once with different harvests of nematodes.

RESULTS

Depolymerizing pectolytic activity was detected in solutions of pectic substances incubated with extracts of *D. dipsaci* (Fig. 1). Pectin was the preferred substrate for RNC nematode extracts, whereas extracts of the WNC and ONY populations were more active towards Na polypectate. Optimal pH for enzyme activity was 6.0, 4.0, or 5.0 for the RNC, WNC, or ONY populations, respectively.



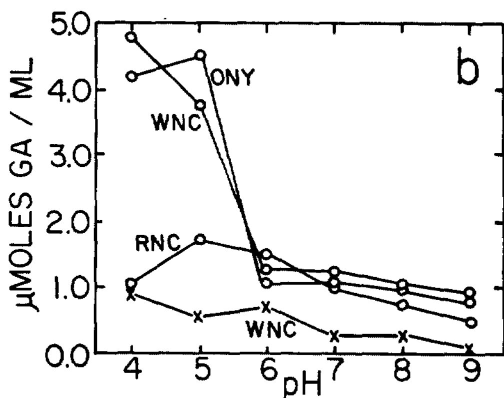
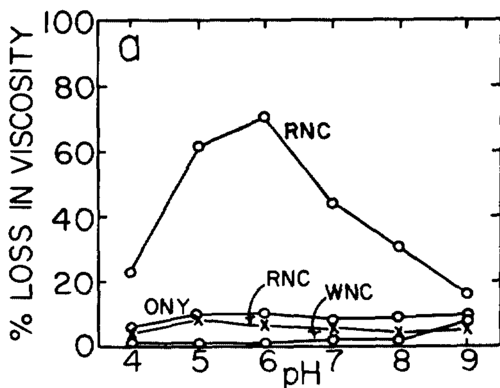
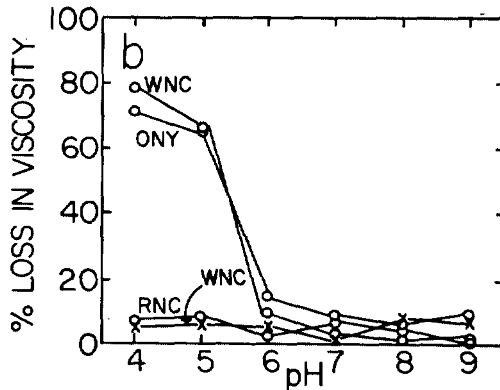


FIG. 1-(a-b). Production of reducing groups by pectin (a) and Na polypectate (b) solutions incubated for 3 h with active (o) extracts of the RNC, WNC, and ONY populations of *Ditylenchus dipsaci* in 0.5 M NaCl (x = boiled extract). Reducing groups are expressed in µmoles of galacturonic acid liberated/ml of reaction mixture.





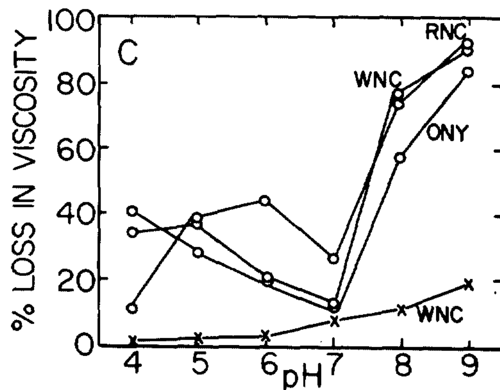


FIG. 2-(a-c). Reduction in viscosity of pectin (a,c) and Na polypectate (b) solutions incubated for 30 min with active (o) extracts of the RNC, WNC, and ONY populations of *Ditylenchus dipsaci* in 0.5 M NaCl (a,b) or 0.005 M $CaCl_2$ (c). x = boiled extract.

The endo-type of depolymerizing activity was great in pectin solutions degraded by RNC nematode extracts, but minimal in Na polypectate solutions (Fig. 2-a,b). Extracts of the WNC and ONY populations degraded both substrates with substantially greater activity occurring in Na polypectate solutions. Optimal pH was 6.0 for extracts of RNC and 4.0 for extracts of WNC and ONY populations. Viscosity of pectin solutions decreased markedly when they were incubated with extracts of *D. dipsaci* in 0.005 M $CaCl_2$ (Fig. 2-c). Extracts of all three populations caused extensive reduction in viscosity with a pH optimum of 9.0. Na polypectate solutions could not be tested because they instantly formed gels upon addition of $CaCl_2$.

Trans-eliminase activity was detected in nematode extracts (Fig. 3). Extracts of all three nematode populations in 0.005 M $CaCl_2$ were active at high pH when pectin was the substrate; optimal pH was 9.0. Extracts in 0.5 M NaCl were slightly active towards pectin solutions; optimal pH was 7.0. Activity was not observed when Na polypectate was the substrate or when

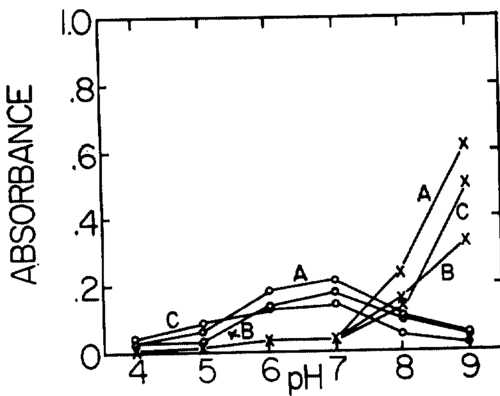


FIG. 3. Absorbance at 548 nm of products of TBA reaction with pectin solutions incubated for 24 h with active extracts of nematodes of the RNC (A), WNC (B), and ONY (C) populations of Ditylenchus dipsaci in 0.5 M NaCl (o) or 0.005 M $CaCl_2$ (x).

boiled extracts were used. High levels of pectin-degrading *trans*-eliminase activity were also detected in nematode extracts in the presence of $CaCl_2$ by measurement of absorbance of reaction mixtures at 230 nm. Again, activity was greatest at pH 9.0. Similar measurements with reaction mixtures containing NaCl were inconclusive because turbidity of these solutions interfered with measurement of absorbance.

No differences in pH developed between pectin solutions incubated with active extracts and those incubated with boiled extracts. Apparently, none of these nematodes possess pectin methylesterase.

Breakdown products of pectolytic action upon pectic substrates were detected by paper chromatography (Fig. 4). Galacturonic acid standards became red when they were sprayed with *P*-anisidine HCl. All spots formed by reaction mixtures were red except for a faint yellow degradation product (R_f 0.34) detected in reaction mixtures containing active RNC nematode extract. Pectin and Na polypectate retarded movement of galacturonic acid. Galacturonic acid was not detected in controls, but was found in reaction mixtures containing active extract of nematodes of any population. Digalacturonic acid was detected in one reaction mixture containing WNC nematode extract.

Potato discs incubated with active extracts of any population of *D. dipsaci* in 0.5 M NaCl were partly macerated after

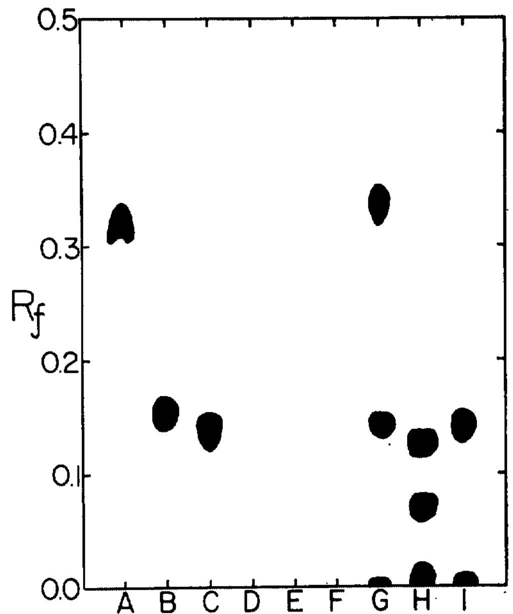


FIG. 4. Representative chromatogram of galacturonic acid standards and solutions of pectic substances incubated with extracts of nematodes of each population of *Ditylenchus dipsaci*. Papers were spotted with 10 μg galacturonic acid (A), 10 μg galacturonic acid plus 10 μl of 1.25% pectin (B), 10 μg galacturonic acid plus 10 μl of 1.25% Na polypectate (C), 10 μl of 1.25% pectin (D), 10 μl of 1.25% Na polypectate (E), 10 μl of any reaction mixture incubated with boiled extract of nematodes of any population (F), 10 μl of 1.25% pectin incubated with RNC nematode extract (G), 10 μl of 1.25% Na polypectate incubated with WNC nematode extract (H), and 10 μl of 1.25% Na polypectate incubated with ONY nematode extract (I).

5 h. Large pieces of tissue were easily scraped from discs after 20 h. No maceration occurred in discs incubated with boiled extracts. Maceration observed in tests with extracts containing $CaCl_2$ was slight to none.

WNC nematodes induced a hypersensitive reaction, death of the apical meristem, and proliferation of lateral buds of Wando pea (Fig. 5). Subsequently, a lateral bud became dominant and grew normally. Nematodes of the RNC and ONY populations caused galls and stunting (Fig 5). Nematodes were observed inside these galls.

DISCUSSION

RNC nematodes have endo-polymethylgalacturonase activity with optimal pH of 6.0, whereas endo-polygalacturonases with

FIG. 5. Nineteen-day-old Wando pea seedlings uninoculated (A) or inoculated with nematodes of the RNC (B), WNC (C), or ONY (D) populations of *Ditylenchus dipsaci*.

pH optima of 4.0 are present in the WNC and ONY populations. This difference is indicated by: reducing group data, which indicate substrate preference and pH optima of the NaCl-stimulated enzymes; failure of TBA to condense with degradation products in reaction mixtures containing nematode extracts in 0.5 M NaCl; and large losses of viscosity in solutions of pectic substances when extracts of nematodes in 0.5 M NaCl are added. Paper chromatography would suggest that galacturonic acid is the terminal degradation product.

Nematodes of all three populations have a $CaCl_2$-activated endo-pectin methyl-*trans*-eliminase with optimal pH of 9.0. This fact was indicated by detection of unsaturated degradation products by reaction of TBA with reaction mixtures containing $CaCl_2$ and by rapid reduction in viscosity in pectin solutions incubated with extracts of *D. dipsaci* in 0.005 M $CaCl_2$.

Our results generally support the findings of several researchers (13, 16, 19). Therefore, earlier reported variations in pectolytic activity among nematodes of each population were not due to variations in culturing or experimental technique, but to real differences among populations. The only discrepancy between our findings and those published previously consists of the detection of small amounts of PME in RNC nematodes by Krusberg (10).

The only known biochemical differences between RNC and WNC nematodes are variations in pectolytic enzymes and possession by RNC nematodes of a unique protein band visible after gel electrophoresis (9). Although pectinases may contribute to cellular death (2, 23), they have never been associated with the necrosis characteristic of the hypersensitive reaction in resistant plants. The fact that ONY nematodes have pectolytic activity similar to WNC nematodes but cause galls in Wando pea seedlings like RNC nematodes further suggests that presence of a certain pectolytic enzyme is not the limiting factor in pathogenesis of a given population of *D. dipsaci* towards Wando pea. Instead, resistance of Wando pea to WNC nematodes may largely depend upon *(i)* additional differences in substances secreted by nematodes of each population, *(ii)* possible presence or induction by nematode feeding of a toxin in Wando pea that inactivates WNC nematodes, or *(iii)* production by Wando pea of substances characteristic of an immune response to a WNC nematode protein.

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