# Pectolytic and Cellulolytic Enzymes of Two Populations of *Ditylenchus dipsaci* on 'Wando' Pea (*Pisum sativum* L.)[1]

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*Abstract:* 'Wando' pea is susceptible to *Ditylenchus dipsaci* from Raleigh, N. C. (RNC) but resistant to the same species from Waynesville, N. C. (WNC). Homogenates of RNC and WNC were analyzed for pectolytic and cellulolytic enzyme activity; both had high $C_x$ activity with WNC two to three times more active than RNC. Polymethylglacturonase activity was three to five times higher in RNC, but polygalacturonase was up to 100 times higher in WNC. Polygalacturonate-*trans*-eliminase was not detected although a $Ca^{++}$-stimulated pectin methyl-*trans*-eliminase was present. Enzyme analyses of healthy and infected pea tissue showed only slight enzyme activity unrelated to that in nematode homogenates. No correlation between enzyme activity and the differing pathogenicities could be detected. *Key Words: Ditylenchus dipsaci*, Enzymes, Pathogenicity, 'Wando' pea, Polygalacturonase, Polymethylgalacturonase, Polygalacturonate-*trans*-eliminase, Pectin methyl-*trans*-eliminase.

Barker and Sasser (1) reported garden pea (*Pisum sativum* L., 'Wando') reacted differently to the Raleigh, N. C. (RNC) and Waynesville, N. C. (WNC) populations of *Ditylenchus dipsaci* (Kühn) Filipjev. The RNC population caused galling and cell separation and reproduced in the plant tissues. The WNC population, however, caused a hypersensitive reaction and reproduction was inhibited. These observations led to the present study of the enzymatic aspects of the differing pathogenicities.

Krusberg (9) suggested nematodes may utilize polysaccharide-degrading enzymes to aid stylet penetration. Although no work has been conducted on the pectolytic and cellulolytic enzyme activity of the WNC population of *D. dipsaci*, Krusberg (6) reported pectin methylesterase activity in homogenates of the RNC population by other techniques, but failed to detect decreased viscosity of sodium polypectate solution at pH 6.3. In a later study, Krusberg (9) reported homogenates were most active in decreasing the viscosity of solutions of pectin N.F., less active toward pectin L.M. (low methoxyl), and least active toward sodium polypectate. With pectin N.F., reducing groups and end-products of pectin-*trans*-eliminase activity were detected. Tracey (17), Dropkin (4), and Krusberg (6) reported the presence of cellulase activity in homogenates of *D. dipsaci* based upon decreased viscosity of carboxymethylcellulose solution and the presence of reducing groups.

Several investigators have suggested pectolytic enzymes secreted inside plant tissues by *D. dipsaci* may be primarily responsible for cell separation. Quanjer (15) observed that the cells beneath the stomatal aperture of young potato shoots enlarged prior to penetration of larvae and suggested that pectinases dissolved the middle lamellae. Goodey (5) found that slices of healthy potato tubers and narcissus bulbs stained more deeply with ruthenium red than did nematode-infected tissue and suggested that

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nematode enzymes dissolved the pectin. Krusberg (8), however, reported no significant differences in pectin content in healthy, mature alfalfa tissues and those galled by the RNC population of *D. dipsaci*. In addition, he reported absence of pectinase activity in extracts of healthy or nematode-galled alfalfa tissues and suggested that more meaningful results could be obtained from seedlings where infected tissues were highly uniform and in active gall development.

In the present study the pectolytic and cellulolytic enzyme activities of the RNC and WNC populations of *D. dipsaci* are compared and their possible role in pathogenesis is discussed.

## MATERIALS AND METHODS

PREPARATION OF NEMATODE HOMOGENATES: The RNC and WNC populations of *D. dipsaci* were Baermann-extracted from 6 to 8 week-old alfalfa callus tissue cultures (7) and surface sterilized by a modification of the Peacock method (14). Nematode suspensions were pelleted by centrifugation at 680 *g* for 3 min, transferred to a sterile graduated centrifuge tube, suspended 8 min in an equal volume of 0.1% chlorohexidine diacetate (Hibitane®; Ayerst Laboratories, N.Y.), and again concentrated by centrifugation. They were then resuspended and rinsed 5 times with sterile distilled water. Following the last rinse, the fresh nematode volume was determined after centrifugation at 680 *g* for 2 min. The nematodes were stored at –20 C until used.

Homogenates were prepared by a modification of the method reported by Myers (12). The frozen nematodes were homogenized 15 min at 0 C in 0.3 M NaCl (1:12 v/v) using a 15-ml Ten-Broeck ground glass tissue grinder. Homogenates were centrifuged at 10,000 *g* for 10 min at 4 C and the supernatant immediately tested for enzyme activity.

PREPARATION OF TISSUE EXTRACTS: 'Wando' pea seeds were germinated 36 hr in running tap water. Those with emergent epicotyls were planted in rows in flats of white quartz sand. About 35,000 RNC or WNC nematodes in an equal volume of 1% carboxymethylcellulose solution were placed over the seedlings in each flat, which was covered with a plastic sheet to maintain a high humidity. Flats containing both inoculated and uninoculated seedlings were incubated four days in a growth chamber at 15 C and 8 hr/day fluorescent illumination. Four days after inoculation, the pea plants were removed from the sand and washed in tap water. The diseased portions of RNC- and WNC-inoculated plants were removed with scissors, weighed, and stored at –20 C. Corresponding non-infected portions of uninoculated plants were processed in the same manner.

Crude enzyme extracts were obtained by grinding frozen plant tissue in 0.5 M NaCl (1:3 w/v) with a mortar and pestle. The extracts were filtered through cheesecloth, centrifuged at 10,000 *g* for 10 min, and then dialyzed against several hundred volumes of deionized water overnight at 5 C and immediately tested for activity.

TESTS FOR POLYMETHYLGALACTURONASE, POLYGALACTURONASE, AND CELLULASE $C_x$: Viscometric and reducing sugar tests were used to assay polymethylgalacturonase, polygalacturonase, and cellulase $C_x$ activity. Enzyme activities of nematode homogenates were compared at pH 5, 6, and 7 in 0.2 M potassium phosphate buffer. For tissue extracts, a 0.1 M sodium citrate buffer (pH 4, 5, 6) and a 0.2 M Tris buffer adjusted with 0.2 M HCl to pH 7, 8, and 9 were used.

Relative enzyme activity (RA) was measured as the reciprocal of the number of min

required for 25% viscosity loss, the quantity multiplied by $10^3$. Viscometer (Ostwald-Fenske, size 300) reaction mixtures contained 2 ml of enzyme preparation plus 5 ml of buffered substrate solution; the latter was either 1% pectin N.F. (Sunkist Growers, Ontario, California), 1% sodium polypectate (Sunkist), or 1% carboxymethylcellulose (CMC 7MP, Hercules Powder Co., Wilmington, Delaware). Reducing end-group activity (RG) of the digestion products was measured using the dinitrosalicylic acid (DNS) test (10). The absorbency was measured at 575 m$\mu$ using standard curves for galacturonic acid or glucose. The RA and RG of boiled enzyme-substrate mixtures were used to obtain the spectrophotometer null point.

The products from reaction mixtures with maximum RA were examined by single dimension ascending paper chromatography. For both healthy and diseased tissues, reaction products were obtained 24 hr after initiation of the viscometric tests. Because of the high dilution of nematode extracts in viscometric studies, separate tests with greater numbers of nematodes were used for the detection of end-products. For the nematode homogenate analyses 1.5 ml of boiled or non-boiled homogenate were placed in test tubes containing 2 ml of each of the following substrates: 1% pectin (pH 6), 1% sodium polypectate (pH 5), or 1% CMC (pH 7) and incubated at 30 C for 24 hr prior to analysis. For analysis of pectin and sodium polypectate reaction products, 50 $\mu$l of the reaction mixtures were applied to Whatman No. 1 chromatography paper. The solvent system contained 15 ml of 88% formic acid, 85 ml of 77% ethanol, and 60 mg of sodium formate (13). To analyze CMC reaction products, 50 $\mu$l of reaction mixture on Whatman No. 3 chromatography paper were exposed to an ascending solvent system composed of ethyl acetate:pyridine:water (8:2:1 v/v/v) (16). Galacturonic acid, glucose, and cellobiose standards were co-chromatogrammed with the reaction products. After irrigation, the papers were air dried and developed with aniline reagents. An aniline reagent, containing equal volumes of 1.3 ml aniline in 50 ml acetone and 0.6 ml phosphoric acid in 20 ml acetic acid plus 30 ml acetone, was used for the detection of the pectin and sodium polypectate hydrolysates. The reagent used to detect CMC hydrolysate products contained 1 ml aniline plus 1 gm diphenylamine made to 100 ml with acetone. Ten volumes of this were combined with one volume 85% phosphoric acid. After spraying, the papers were placed in an oven at 105 C for 10 min to enhance color development of spots.

TESTS FOR PECTIN METHYL-*TRANS*-ELIMINASE AND POLYGALACTURONATE-*TRANS*-ELIMINASE: Pectin methyl-*trans*-eliminase and polygalacturonate-*trans*-eliminase activity of the nematode homogenates were determined by a method similar to that of Moore and Couch (11). Two series of tests were conducted. In one test, 1 ml of Tris-buffered substrate (1% pectin or 1% sodium polypectate at pH 7.5, 8, 8.5, 9, or 9.5) was added to 1 ml of 0.003 M $CaCl_2$ and 1 ml of enzyme preparation. An equal volume of distilled water was substituted for the $CaCl_2$ in the second test. The reaction mixtures were added to 1.2 ml of 1 N HCl, 5 ml of 0.01 M thiobarbituric acid, immersed in boiling water for 30 min and cooled in an ice bath. The mixtures were then centrifuged at 10,000 *g* for 15 min and the absorbency read at 548 m$\mu$. Results were expressed in units of *trans*-eliminase activity, one unit being arbitrarily defined as that amount of enzyme in 3 ml of reaction mixture that caused the absorbance at 548 m$\mu$ to increase 0.005 units in 24 hr.

Two separate tests, each consisting of two

TABLE 1. Relative polymethylgalacturonase activity (RA) and increased reducing activity (RG) of pectin substrates incubated 24 hr with crude enzyme preparations from homogenates of RNC and WNC populations of *Ditylenchus dipsaci*.

| | RNC | | WNC | |
|---|---|---|---|---|
| pH | RA[a] | RG[b] | RA | RG |
| 5.0 | 150.0 | 1861.1 | 40.5 | 716.6 |
| 6.0 | 225.0 | 1972.2 | 47.0 | 783.3 |
| 7.0 | 96.5 | 1183.3 | 21.0 | 455.5 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of galacturonic acid per ml reaction mixture as determined by DNS test.

replicates, were conducted for all enzyme characterizations. An overall average was computed for each enzyme.

## RESULTS

POLYMETHYLGALACTURONASE AND POLYGALACTURONASE ACTIVITY: With pectin as the substrate, homogenates of the RNC and WNC populations of *D. dipsaci* exhibited polymethylgalacturonase activity (Table 1). Maximum reduction in viscosity and increases in reducing activity occurred at pH 6. Activity was three to five times greater in the RNC than in the WNC homogenates. Polymethylgalacturonase activity in extracts from healthy or RNC- and WNC-infected tissues, however, did not differ significantly; each contained less than 3 RA units of activity (Table 2) and there appeared to be no correlation between RA and RG.

TABLE 2. Relative polymethylgalacturonase activity (RA) and increased reducing activity (RG) of pectin substrates incubated 24 hr with crude enzyme preparations from healthy and RNC- and WNC-infected 'Wando' pea shoots.

| | Tissue | | | | | |
|---|---|---|---|---|---|---|
| | Healthy | | RNC-Infected | | WNC-Infected | |
| pH | RA[a] | RG[b] | RA | RG | RA | RG |
| 4.0 | 0.0 | 38.8 | 0.0 | 16.6 | 0.0 | 0.0 |
| 5.0 | 0.0 | 0.0 | 0.0 | 138.8 | 1.1 | 155.5 |
| 6.0 | 2.0 | 0.0 | 2.6 | 38.8 | 2.8 | 27.7 |
| 7.0 | 0.0 | 0.0 | 0.0 | 11.1 | 0.0 | 177.7 |
| 8.0 | 0.0 | 50.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 9.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of galacturonic acid per ml reaction mixture as determined by DNS test.

TABLE 3. Relative polygalacturonase activity (RA) and increased reducing activity (RG) of sodium polypectate substrates incubated 24 hr with crude enzyme preparations from homogenates of RNC and WNC populations of *Ditylenchus dipsaci*.

| | RNC | | WNC | |
|---|---|---|---|---|
| pH | RA[a] | RG[b] | RA | RG |
| 5.0 | 1.5 | 83.3 | 105.5 | 311.1 |
| 6.0 | 0.6 | 77.7 | 6.5 | 33.3 |
| 7.0 | 0.6 | 5.5 | 0.6 | 16.6 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of galacturonic acid per ml reaction mixture as determined by DNS test.

Homogenates of both nematode populations degraded sodium polypectate substrates, exhibiting polygalacturonase activity (Table 3). Greatest reduction in viscosity and increases in reducing activity occurred at pH 5. At this pH, activity of the WNC homogenate was about 100 times that in the RNC homogenate. Extracts from healthy or WNC-infected tissue contained less than 2 RA units of polygalacturonase activity and exhibited no well-defined peaks of activity (Table 4). Preparations from RNC-infected tissue did not reduce the viscosity of sodium polypectate 25% after an incubation period of 24 hr. Increases in reducing activity were recorded but did not correspond with decreased substrate viscosity.

Chromatograms of WNC homogenate reaction mixtures with pectin or sodium polypectate substrates revealed the presence of galacturonic acid. For the RNC population, however, galacturonic acid was detected only when pectin was used as the substrate. In chromatographic analyses of reaction mix-

TABLE 4. Relative polygalacturonase activity (RA) and increased reducing activity (RG) of sodium polypectate substrates incubated 24 hr with crude enzyme preparations from healthy and RNC- and WNC-infected 'Wando' pea shoots.

| | Tissue | | | | | |
|---|---|---|---|---|---|---|
| | Healthy | | RNC-Infected | | WNC-Infected | |
| pH | RA[a] | RG[b] | RA | RG | RA | RG |
| 4.0 | 0.0 | 0.0 | 0.0 | 116.6 | 0.0 | 100.0 |
| 5.0 | 0.0 | 38.8 | 0.0 | 105.5 | 1.1 | 94.4 |
| 6.0 | 0.0 | 27.7 | 0.0 | 0.0 | 0.8 | 50.0 |
| 7.0 | 1.1 | 22.2 | 0.0 | 27.7 | 1.1 | 111.1 |
| 8.0 | 0.7 | 27.2 | 0.0 | 11.1 | 0.9 | 20.0 |
| 9.0 | 0.7 | 22.2 | 0.0 | 0.0 | 0.9 | 15.0 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of galacturonic acid per ml reaction mixture as determined by DNS test.

tures containing RNC- and WNC-infected tissue extracts with pectin or sodium polypectate substrates, traces of galacturonic acid were present. No galacturonic acid was detected in reaction mixtures containing healthy or boiled tissue extracts.

PECTIN METHYL-*TRANS*-ELIMINASE AND POLYGALACTURONATE-*TRANS*-ELIMINASE ACTIVITY: Without added calcium, homogenates of both nematode populations had similar *trans*-eliminase activity (Table 5). Maximum activity with pectin substrates occurred at pH 7.5–8 for RNC homogenates and at pH 7.5–8.5 for WNC homogenates. When $CaCl_2$ was incorporated into the reaction mixtures at pH 9.5, pectin methyl-*trans*-eliminase activity of the RNC homogenates was 101 *trans*-eliminase units compared to no activity for the WNC population. With sodium polypectate substrate, WNC and RNC homogenates exhibited little or no polygalacturonate-*trans*-eliminase activity throughout the pH range tested and was not significantly enhanced by the addition of calcium. In the presence or absence of $CaCl_2$, pectin was the preferred substrate for *trans*-eliminase activity in homogenates from the RNC and WNC populations.

TABLE 5. Pectin methyl-*trans*-eliminase and polygalacturonate-*trans*-eliminase activity of reaction mixtures incubated with crude enzyme preparations from homogenates of RNC and WNC populations of *Ditylenchus dipsaci*.

| Substrate | pH | RNC | WNC |
|---|---|---|---|
| Pectin without $CaCl_2$ | 7.5 | 9[a] | 9 |
| | 8.0 | 10 | 8 |
| | 8.5 | 6 | 8 |
| | 9.0 | 3 | 1 |
| | 9.5 | 3 | 0 |
| Pectin plus $CaCl_2$ | 7.5 | 10 | 5 |
| | 8.0 | 21 | 7 |
| | 8.5 | 52 | 25 |
| | 9.0 | 77 | 8 |
| | 9.5 | 101 | 0 |
| Sodium polypectate without $CaCl_2$ | 7.5 | 0 | 0 |
| | 8.0 | 0 | 0 |
| | 8.5 | 0 | 0 |
| | 9.0 | 0 | 2 |
| | 9.5 | 0 | 0 |
| Sodium polypectate plus $CaCl_2$ | 7.5 | 0 | 2 |
| | 8.0 | 2 | 0 |
| | 8.5 | 6 | 1 |
| | 9.0 | 5 | 2 |
| | 9.5 | 5 | 2 |

[a] One unit of *trans*-eliminase activity was arbitrarily defined as the amount of enzyme in 3 ml of reaction mixture which caused the absorbance at 548 mμ to increase 0.005 in 24 hr.

CELLULASE $C_x$ ACTIVITY: RNC and WNC homogenates exhibited high levels of $C_x$ activity (Table 6). Activity was maximum at pH 6 for RNC and at pH 7 for WNC homogenates. At either of these pH values

TABLE 6. Relative cellulase activity (RA) and increased reducing activity (RG) of CMC 7MP substrates incubated 24 hr with crude enzyme preparations from homogenates of RNC and WNC populations of *Ditylenchus dipsaci*.

| | RNC | | WNC | |
|---|---|---|---|---|
| pH | RA[a] | RG[b] | RA | RG |
| 5.0 | 1150.0 | 563.3 | 416.5 | 713.3 |
| 6.0 | 1600.0 | 500.0 | 2000.0 | 480.0 |
| 7.0 | 1300.0 | 550.0 | 4000.0 | 420.0 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of glucose per ml reaction mixture as determined by DNS test.

TABLE 7. Relative cellulase activity (RA) and increased reducing activity (RG) of CMC 7MP substrates incubated 24 hr with crude enzyme preparations from healthy and RNC- and WNC-infected 'Wando' pea shoots.

| | Tissue | | | | | |
|---|---|---|---|---|---|---|
| | Healthy | | RNC-Infected | | WNC-Infected | |
| pH | RA[a] | RG[b] | RA | RG | RA | RG |
| 4.0 | 0.0 | 0.0 | 0.0 | 36.6 | 0.0 | 13.3 |
| 5.0 | 1.7 | 20.0 | 3.5 | 43.3 | 4.4 | 106.6 |
| 6.0 | 3.6 | 0.0 | 5.5 | 0.0 | 5.4 | 66.6 |
| 7.0 | 1.8 | 3.3 | 2.7 | 0.0 | 3.8 | 60.0 |
| 8.0 | 1.1 | 6.6 | 1.7 | 10.0 | 0.0 | 16.6 |
| 9.0 | 0.6 | 0.0 | 1.0 | 6.6 | 0.0 | 10.0 |

[a] RA units represent the reciprocal of the time (min) for 25% loss of reaction mixture viscosity multiplied by $10^3$.
[b] RG expressed in μg of glucose per ml reaction mixture as determined by DNS test.

the WNC activity was greater than the RNC. The liberation of reducing groups was similar for both populations but did not correspond with reduction in substrate viscosity.

Similar (slight) $C_x$ activity was detected in healthy and both RNC- and WNC-infected tissue extracts (Table 7). Maximum reduction in substrate viscosity for all three tissue extracts occurred at pH 6, while maximum increase in reducing activity occurred at pH 5. RA and RG were, however, greater in infected than in healthy tissue extracts.

Chromatograms of reaction mixtures containing homogenates of nematodes and extracts of healthy and infected tissue with CMC substrate revealed the presence of cellobiose. Cellobiose was not detected in boiled extracts. Glucose was not detected in any of the reaction mixtures.

## DISCUSSION

Nematodes of the two populations differ in their abilities to produce certain pectolytic and cellulolytic enzymes. The activity of both polymethylgalacturonase and polygalacturonase appears to be of the endo- type since high RA was accompanied by a relatively low RG as determined chromatographically and by the DNS test. Since the chromatographic analyses revealed that CMC was degraded to cellobiose rather than glucose, both populations apparently lack β-glucosidase.

Although the pectolytic and cellulolytic enzymes were quantitatively different in the two nematode populations, these differences were not reflected in the host-nematode interactions; there was no correlation between the differential responses of the host and the levels of pectolytic and cellulolytic enzyme activity in host tissue or nematode homogenates.

Since the numbers of nematodes in nematode homogenates were much higher than those in galled tissue, one would not expect as high a relative activity in crude enzyme preparations of tissue extracts as in nematode homogenates. However, if the pectolytic and cellulolytic enzymes assayed played a role in pathogenesis, one would expect a difference in the relative activities between healthy and infected tissue extracts. No significant differences were detected.

It is possible, however, that enzymes functional in the early stages of pathogenesis were subsequently inactivated. There is a need, therefore, to assay these enzymes at various stages, particularly during the early stages of infection, to define the role of pectolytic and cellulolytic enzymes in disease development. Additional studies should also be conducted to determine the limitations of the preparation and analytical techniques since such methods (i.e. dilution and dialysis of enzyme extracts and freezing of nematodes and plant material) may affect the results (2, 3).

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