Pectolytic Activity of Aphanomyces Cochlioides in Culture and in Diseased Sugarbeets[1]

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Introduction

Black root of sugarbeet, caused primarily by *Aphanomyces cochlioides* Drechs., is a disease of economic importance in Ohio. Although varieties currently used in the Great Lakes Region possess a degree of resistance to *A. cochlioides,* additional resistance is needed. Conceivably, a better understanding of the mechanisms of pathogenesis of *A. cochlioides* might contribute to this goal.

Hydrolysis of cell wall constituents, particularly pectins, is one feature of pathogenesis common to a wide variety of plant pathogens (6, 21)[3]. Pectolytic enzymes are involved in tissue maceration and inter-cellular invasion (5, 6, 21), and possibly in killing of host cells (21).

Except for a histochemical study (13), information on pectolytic enzymes of *A. cochlioides* and their role in pathogenesis of sugarbeet is lacking. In contrast, the pectolytic activity of the related species *A. euteiches* Drechs., pathogenic on peas, has been intensively investigated (1, 2, 3). The hypothesis has been advanced that the endopolygalacturonase produced by *A. euteiches* facilitates parasitism by partially macerating or softening pea root tissues (1).

The purpose of the present study was to ascertain the pectolytic capabilities of *A. cochlioides* in culture and in diseased sugarbeets.

Materials and Methods

Culture Methods

A virulent, single-zoospore isolate of *A. cochlioides,* selected from a mass culture from diseased sugarbeet, was used. Stock cultures were maintained by weekly transfers to slants of Difco corn-

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meal agar (CMA). Inoculum used for enzyme production cultures consisted of either: a) agar disks cut from CMA plate cultures (single disk/50 ml of medium), b) zoospores (10^6/400 ml medium) produced by a previously described method (10 or c) diced (3-6 mm pieces) CMA cultures, one-half a petri plate culture or one-half a prescription bottle (180 ml capacity) slab culture per 400 ml medium. Petri dishes and prescription bottles contained 20 ml CMA and were used for inoculum after *A. cochlioides* mycelium covered the agar surface. In most instances the culture medium used for enzyme production was the glucose-peptone-asparagine (GPA) medium developed by Wood and Gupta (20) for pectolytic studies of *Pythium debaryanum* Hesse. During early phases of this study, cultures of *A. cochlioides* were produced in 250 ml flasks containing 50 ml medium. In subsequent tests reagent jugs (2.5 liter capacity) containing 400 ml GPA were used. Incubation was at 25°C, mostly as stationary cultures, with one week the usual period. In limited tests a modification of the BM-1 synthetic medium (11) of Papavizas and Ayers (19) was also used.

Filtrates of liquid cultures were obtained by first filtering through a Buchner funnel (Whatman No. 1 filter paper) to remove the mycelial mats, followed by two passages through sterile 0.65 μm membrane filters. Culture filtrates were then either dialyzed (20-24 hr against ca. 100 volumes glass distilled water at 5°C) or used undialyzed. In either case, filtrates were layered with toluene and stored at 5°C until used.

In later studies, pectolytic enzymes in filtrates were concentrated by $(NH_4)_2SO_4$ precipitation. Precipitates were centrifuged at 20,000 x g for 15 min., dissolved in distilled water, re-precipitated with 95% ethanol, centrifuged (20,000 $\times$ g) and dissolved in water or buffer for use. Lesser use was made of two other methods of concentrating culture filtrates: a) vacuum evaporation at 35°C, and b) lyophilization. Relative protein content of concentrated filtrates was ascertained by measuring absorbance spectrophotometrically at 280 mμ (3).

Plant Extraction Methods

Sugarbeet material used for extraction of pectolytic enzymes included healthy and diseased hypocotyls and healthy petioles. Several varieties were used: 633, a highly susceptible breeding line[4]; US 401, a moderately resistant variety; and GW H-1, a moderately resistant commercial variety. Beet seedlings were produced by either the growth rack technique (12) or in greenhouse pots. Seed-

[4]Seed were generously supplied by Dr. G. E. Coe, Research Geneticist, USDA, ARS, Beltsville, Maryland.

lings were inoculated with zoospores of *A. cochlioides,* as previously described (12), 2 weeks after planting and harvested 10 days later. Healthy seedlings, used as controls, were harvested at 24 days. Healthy field-grown GW H-1 petiole tissue (10 weeks old) was also used as a control.

Harvested plant materials were weighed in polyethylene bags, quick-frozen in liquid nitrogen and stored at −20°C. Extractions were made using 0.25 M NaCl plus 0.01 M cysteine HCl in 0.01 M Tris-HCl buffer at pH 8.5 (9) by homogenizing in a Virtis homogenizer for 6 min. The homogenate was squeezed through several layers of cheesecloth, centrifuged at 12,000 × g for 10 min, dialyzed 20 hr in two changes of glass distilled water, layered with toluene and used immediately or stored for short intervals at 5°C. Protein content of plant extracts was determined by the Folin-Ciocalteu method of Lowry *et al.* (16) with egg albumin as the standard.

Enzyme Methods

Polygalacturonase (PG) activity was determined in size 300 Cannon-Fenske viscometers at 30°C, using the method of Bell *et al.* (7). Reaction mixtures consisted of 5 ml enzyme preparation and 5 ml 1.2% sodium polypectate (NaPP) or 1.2% pectin N.F. (source Sunkist Growers, Inc., Ontario, Calif.) in 0.05 M citrate buffer at pH 6.0 plus a drop of toluene, except where otherwise noted. Enzyme activity was expressed as viscosity-reducing units per ml of filtrate or enzyme preparation (VRu/ml) for culture filtrates and as VRu/mg protein for plant extracts. One unit was defined as the amount of enzyme resulting in a 50% loss in viscosity of the respective substrates in 100 min at 30°C (3).

The liberation of reducing groups by action of polygalacturonase was determined by the hypoiodate method (14). Pectin methylesterase (PME) activity was determined by Bateman's method (4). Activity was expressed as milliequivalents (μ eq) of methoxyl groups released (2). Possible occurrence of *trans*-eliminase activity was assayed by the optical density method of Nagel and Vaughn (17).

Chromatographic analysis of the end products of pectolytic hydrolysis were made using either descending 1-dimensional paper chromatography (Whatman 3MM) and the isobutryic acid-water solvent system and the ammonical $AgNO_3$ detector of Jermyn and Tompkins (15) or the ethanol-formic acid ascending chromatography method of Page (18).

Macerating activity was determined by Bateman's technique (5), using potato tuber cores cut with a No. 8 cork borer and sliced

into sections ca. 700 μm thick with a freezing microtome. Degree of maceration was rated at intervals using a 0-5 scale (0 = no maceration, 5 = complete loss of coherence of tissue). Assays were made at 30°C. In limited testing, 5 mm length pieces of sugarbeet hypocotyl were used to estimate maceration of beet tissue by culture filtrates.

Results

Pectolytic Activity of Culture Filtrates

a) *Factors affecting viscosity-reducing activity.* Initially with the small volume (50 ml) cultures viscosity-reducing activity of culture filtrates was consistently low (Table 1). Although VRu/ml at pH 5.0 and 6.0 were one-half that at pH 7.0 and 8.0 in phosphate buffer in actuality the magnitude of difference was small. Citrate buffer at pH 6.0 appeared to give greater activity than did phosphate buffer at pH 6.0 and comparable activity to that of phosphate buffer at pH 7.0 and 8.0.

Table 1.—Effect of substrate pH and buffer on viscosity-reducing activity of dialyzed culture filtrates. Cultures (50 ml medium/250 ml flask) inoculated by single 6. mm dia. agar disk. Substrate NaPP.

Buffer	pH	VRu/ml[a]
0.02 M phosphate	5.0	0.03
	6.0	0.03
	7.0	0.06
	8.0	0.06
0.05 M citrate	6.0	0.07

[a] Averages of duplicate assays.

A dramatic increase (ca. 6-fold) in the viscosity-reducing activity of culture filtrates resulted (Table 2) from use of dense zoospore inoculum compared with the single disk, small flask method (Table 1). Furthermore, at these higher levels of viscosity-reducing activity, culture filtrates buffered with phosphate at pH 8.0 were

Table 2.—Effect of different buffers and substrate (NaPP) pH on viscosity-reducing activity of undialyzed culture filtrates. Cultures (400 ml/reagent jug) inoculated with 10^6 zoospores/jug.

Buffer	pH	VRu/ml[a]
0.05 M citrate	6.0	0.43
0.02 M phosphate	7.0	0.38
0.02 M BES	7.2	0.48[b]
0.02 M phosphate	8.0	0.44

[a] Averages of duplicate assays.
[b] BES buffered controls increased in viscosity with time.

15% more active than at pH 7.0, whereas citrate buffer at pH 6.0 was approximately equal in activity to phosphate buffer at pH 8.0 (Table 2). The BES (N, N-bis(2-hydroxethyl)-2-aminoethane sulfonic acid) buffer caused an increase in viscosity in the buffer (no filtrate) control with time and thus could not be used.

Because the time involved in producing zoospores was somewhat inconvenient and contamination problems occurred occasionally, use of agar cultures as inoculum was reinvestigated. By dicing the agar cultures, activity levels similar to those of dense zoospore suspensions were obtained. In one test (four replicates/treatment), CMA inoculum gave high yields of viscosity-reducing activity (0.38 VRu/ml), comparable to zoospore inoculum (0.32 VRu/ml). No advantage for use of GPA agar inoculum was noted (0.30 VRu/ml).

Effects of aeration on viscosity-reducing activity of filtrates was investigated by rotating reagent jug containers (400 ml GPA/jug) on a mechanical roller (18 rpm) and comparing activity to that obtained with stationary cultures. In week-old cultures, 86.2 and 540.4 mg dry weight of mycelium (averages of duplicate cultures) were produced in stationary and rotated culture respectively. But VRu/ml of undialyzed culture filtrates was 10-fold greater in stationary culture than in rotated culture (0.40 vs 0.04; averages of duplicate assays of duplicate culture batches). A lack of correlation between growth (dry weight of mycelium) and viscosity-reducing activity was evident.

Another dimension was added to the investigation of aeration effects by ascertaining the effect of culture age on viscosity-reducing activity of rotated and stationary cultures (Table 3). A striking difference in activity after 1-day incubation was evident, with high activity in rotated and low in stationary culture. After 1 week, rotated cultures had very little whereas stationary cultures exhibited very high activity. At 3 weeks incubation stationary cultures had only slight activity.

Table 3.—Effect of culture age and aeration (stationary vs rotated culture) on viscosity-reducing activity of undialyzed filtrates. Zoospore inoculum, enzyme substrate NaPP.

Treatment	Age (Days)	VRu/ml[a]
Rotated culture	1	0.19
	7	0.02
Stationary culture	1	0.08
	7	0.45
	21	0.03

[a] Averages of duplicate assays of duplicate batches of culture filtrate.

Incubation temperature affected the viscosity-reducing activity of filtrates. Three-fold greater activity was obtained at 25°C (0.13 VRu/ml) compared with that at 30°C (0.04 VRu/ml; averages of duplicate assays). Growth (mycelial dry weights) gave opposite results with 137 mg at 25°C vs 223 mg at 30°C (averages of three replicates).

Concentrations of the organic constituents of GPA were varied as follows: a) reduced to one half of standard amounts, b) standard amounts and c) one and a half times standard amounts (Table 4). Highest activity was obtained using the standard concentration (3.0%) of glucose. With L-asparagine little difference in activity existed between one-half and standard concentrations, while activity was reduced at the highest concentration. Activity in the case of peptone increased with increasing concentrations.

Table 4.—Effect of various concentrations of the organic constituents of GPA on viscosity-reducing activity of undialyzed culture filtrates. Substrate NaPP.

Medium	VRu/ml[a]
GPA	
with 1.5% glucose	0.22
with 3.0% glucose	0.30
with 4.5% glucose	0.22
GPA	
with 0.23% asparagine	0.21
with 0.45% asparagine	0.18
with 0.68% asparagine	0.11
GPA	
with 0.23% peptone	0.14
with 0.45% peptone	0.25
with 0.68% peptone	0.33

[a] Averages of duplicate assays of pooled (triplicate) culture filtrates.

Substitution of DL- for L-asparagine resulted in a reduction in activity, 0.17 VRu/ml for the DL- form as compared to 0.28 VRu/ml for the L- form (averages of duplicate assays). Non-utilization of D-asparagine by *A. cochlioides* (in a synthetic medium) has been reported (11).

A. cochlioides culture filtrates obtained from the modified BM-1 medium did not yield any measurable viscosity-reducing activity. As a comparison control, BM-1 medium (19) filtrates of an *A. euteiches* isolate were assayed and only low levels of endopolygalacturonase activity were obtained in limited tests. However, *A. euteiches* cultured on GPA gave moderate endopolygalacturonase activity (0.15 VRu/ml; averages of duplicate assays) after 3 weeks incubation as shake cultures when the pH was adjusted to 6.0 twice during incubation.

GPA supplemented with 0.1% NaPP or 0.1% pectin was compared with standard GPA to ascertain the effects of these substrate supplements on viscosity-reducing activity of *A. cochlioides* filtrates. Medium supplementation with NaPP reduced activity, whereas supplementation with pectin increased activity. The following VRu/ml were obtained: a) GPA = 19.7, b) GPA + 0.1% NaPP = 10.0, and c) GPA + 0.1% pectin = 26.7 (averages of duplicate assays).

Substrate specificity of the viscosity-reducing enzyme was investigated and NaPP was found to be more susceptible to degradation than pectin (Table 5). As reported in the preceding paragraph, addition of 0.1% pectin to GPA increased enzyme activity. Dialysis caused a reduction in enzyme activity. Other results indicated considerable test-to-test variation in loss of activity following dialysis (trace to 50% reduction) that could not be accounted for on the basis of filtrate dilution alone.

Table 5.—Viscosity-reducing activity of undialyzed and dialyzed culture filtrates, as affected by culture medium, on two substrates; sodium polypectate (NaPP) and pectin.

		VRu/ml[a]	
Medium	Substrate	Undialyzed	Dialyzed
GPA	NaPP	0.24	0.21
	pectin	0.10	none
GPA plus 0.1% pectin	NaPP	0.37	0.23
	pectin	0.13	0.02

[a] Averages of triplicate assays.

b) *Macerating activity.* Culture filtrates of *A. cochlioides* caused considerable maceration of potato tuber tissue, but in limited tests, were inactive or only slightly active in macerating sugarbeet hypocotyl tissues.

A procedure for ascertaining the relative ability of filtrates to macerate potato tissue involved use of dilutions (100, 75, 50 and 25% filtrate) and comparison with similar dilutions of a commercial pectinase standard (Table 6). Results indicated consistent dilution effects (good reproductibility) and the existence of differences in activity between batches of culture filtrates were demonstrable.

c) *Nature of pectolytic activity.* No PME activity could be demonstrated in culture filtrates of *A. cochlioides* in tests at pH 4.0, 5.0, 6.0 or 7.0. Apparently no PME was produced in culture. Reducing values following incubation of dialyzed filtrate and NaPP substrate were negligible, indicating lack of reducing substances in enzyme reaction products. No evidence of *trans*-eliminase activity

Table 6.—Comparison of potato tuber disk maceration by undialyzed *Aphanomyces cochlioides* culture filtrates with maceration by commercial pectinase. Four concentrations of each enzyme preparation were used. Maceration rated on 0-5 scale; 0 = no maceration, 5 = complete maceration.

Enzyme source	Concentration percentage	Ratings[a] Time in hours 3	4	5	6	10
Culture Filtrate # 1	100	0	1	1	1	1
	75	0	1	1	1	1
	50	0	0	1	1	1
	25	0	0	0	0	1
Culture Filtrate # 2	100	1	2	2	3	4
	75	0	1	1	3	4
	50	0	0	0	2	3
	25	0	0	0	1	1
Pectinase Std; 0.1 mg/ml	100	3	4	5	5	5
	75	1	3	3	5	5
	50	0	3	3	5	5
	25	0	1	1	3	5
Boiled-Enzyme Blank	—	0	0	0	0	0
Buffer Blank	—	0	0	0	0	0

[a] Ratings based on five potato disks per enzyme concentration.

was detected in *A. cochlioides* culture filtrates. An *Erwinia* soft-rot bacterial culture, used as a procedural control, gave very high *trans*-eliminase activity, indicating that the procedure was satisfactory.

Chromatographic analysis of enzyme reaction mixtures did not reveal any hydrolytic products with Rf's similar to the galacturonic acid control spot. Thus, the mechanism of hydrolysis appeared to be random rather than terminal. An endopolygalacturonase (endo-PG) was indicated.

d) *Concentration of endo-PG activity of filtrates.* First efforts to concentrate endo-PG activity of culture filtrates were by rotary vacuum evaporation (3). In one test, 400 ml filtrate were reduced to 40 ml. The VRu/ml values were 0.25 in dilute filtrate and 1.18 in the concentrated filtrate (averages of duplicate assays). Based on volume reduction only 47% of the potential activity was retained after vacuum evaporation. Lyophilization of filtrate resulted in even less retention of activity, 27% based on the original activity per ml of the dilute filtrate and the reduction in volume.

Culture filtrate of *A. cochlioides* was precipitated successively with increasing concentrations (55 to 95% saturated at 16°C) of $(NH_4)_2SO_4$ (Table 7). Greatest viscosity-reducing activity was found in the fraction precipitated by 55% saturated $(NH_4)_2SO_4$, which also had the highest (280 mμ) absorbance value.

Table 7.—Concentration of endopolygalacturonase from culture filtrate by successive precipitation with increasing concentrations (% saturated at 16°C) of $(NH_4)_2SO_4$. Relative protein content estimated by absorbance at 280 mμ.

$(NH_4)_2SO_4$ % saturation	280 mμ absorbance	VRu/ml[a]
55	0.86	0.67
60	0.14	0.02
80	0.50	0.02
95	0.21	none

[a] Averages of duplicate assays.

The relative merits of single precipitation of culture filtrates versus successive precipitation of a given batch of filtrate were investigated (Table 8). The filtrate fraction precipitated at 40% saturation had very slight viscosity-reducing activity, whereas the fraction precipitated at 60% saturation had much higher enzymatic activity and greater absorbance at 280 mμ. The 40-60% saturated successive precipitation procedure caused a definite loss in enzyme activity and protein content. Consequently, the standard concentration procedure adopted was a single precipitation with 60% saturated $(NH_4)_2SO_4$.

Table 8.—Concentration of endopolygalacturonase from culture filtrate. Comparison of enzyme activity in single precipitated vs successively precipitated filtrate. Relative protein content estimated by absorbance at 280 mμ.

$(NH_4)_2SO_4$, % saturation	280 mμ absorbance	VRu/ml[a]
40 (single ppt)	0.53	0.01
60 (single ppt)	1.75	0.40
40-60 (successive ppt)	1.13	0.28

[a] Averages of triplicate assays.

Pectolytic Activity of Sugarbeet Extracts

a) *Macerating activity.* In two tests, extracts from diseased GW H-1 beet seedlings macerated potato tuber disks in 22 and 20 hr., respectively. In comparison a commercial pectinase standard (0.1 mg/ml) macerated tuber disks in 4 to 5 hr.

b) *Nature of pectolytic activity.* Preferred substrate for viscosity-reduction by plant extracts was NaPP (same as for culture filtrates). Chromatographic analysis of reaction mixtures did not reveal any hydrolytic products with Rf values similar to that of galacturonic acid. An endopolygalacturonase was thus indicated. *Trans*-eliminase activity was not detected. Pectin methyl esterase was detectable provided cystein HCl was included in the extraction fluid.

c) *Pectolytic activity.* Endo-PG and PME activities of Aphanomyces black root susceptible (633) and resistant (401) varieties were ascertained in both the healthy and diseased state (Table 9). Extracts of diseased 633 and 401 had higher endo-PG activity than did extracts from healthy plants (Table 9). In some tests higher endo-PG activity was obtained from "presumed healthy" than from diseased seedling extracts. Low endo-PG activity was obtained from healthy field-grown GW H-1 sugarbeets (Table 9).

Measurable amounts of PME were detected, except in the case of healthy 401 seedlings. Healthy and diseased 633 seedling extracts had similar PME activities, as did petiole extracts from healthy field-grown GW H-1 plants.

Discussion

The viscosity-reducing enzyme produced in culture by *A. cochlioides* was classified as an endopolygalacturonase by the system of Demain and Phaff (8), as presented by Bateman and Millar (6). The preferred substrate was NaPP and failure to detect galacturonic acid as a hydrolytic product indicated a random mechanism of hydrolysis. The enzyme was constitutive rather than adaptive, because presence of the substrates was not essential for its production. However, as was reported for *A. euteiches* (2), culture medium supplemented with 0.1% pectin enhanced enzyme production.

The low yields of endo-PG produced by *A. cochlioides* in the singly-inoculated, small flask cultures were unanticipated. Similar small volume cultures were successfully used by Ayers and Papavizas (1) for endo-PG production by *A. euteiches.* A further difference in endo-PG production between *A. cochlioides* and *A. euteiches* was in aeration effects. Ayers and Papavizas (1) reported increased endo-PG activity in aerated (shake) cultures of *A. euteiches,* whereas aeration of *A. cochlioides* cultures greatly reduced endo-PG in my tests. Probably, the small flask culture method also basically involved an aeration effect. When the larger volume cultures were inoculated with dense zoospore or multiple agar-inoculum pieces, aeration in the medium must have been considerably reduced relative to that in the small volume flasks, and considerably more endo-PG was produced in the large volume cultures.

In contrast to the effects of aeration on endo-PG production, growth was greatly increased in aerated *A. cochlioides* cultures compared to stationary cultures. An increase in incubation tem-

Table 9.—Endopolygalacturonase (endo-PG) and pectin methyl esterase (PME) activity of extracts from diseased and healthy beets. Endo-PG substrate NaPP.

Variety	State	Growth Conditions	Age	Pectolytic Activity/mg Protein	
				endo-PG[b] VRu	PME[c] μ eq.
633	healthy	pots	24 days	0.040	0.0005
633	diseased	pots	24 days[a]	0.125	0.0003
401	healthy	racks	24 days	0.071	nil
401	diseased	racks	24 days[a]	0.126	0.0004
GW H-1	healthy	field	10 weeks	0.018	0.0003

[a] 10 days after inoculation.
[b,c] Averages based on triplicate or duplicate determinations.

peratures also increased growth, but reduced endo-PG production. There was, thus, no correlation between growth and production of endo-PG. Lack of correlation between growth and production of polygalacturonases has been reported for other plant pathogens (21).

The endopolygalacturonases of *A. cochlioides* and *A. euteiches* exhibited similarities, as well as the differences previously noted. Activity of endo-PG obtained from *A. cochlioides* was affected by the buffer (citrate vs phosphate) as well as pH and, similarly, the pH optimum for endo-PG from *A. euteiches* differed depending upon whether phosphate or Tris buffer was used (1). Also, Ayers *et al.* (2) reported a variable loss in endo-PG activity in dialyzed culture filtrates of *A. euteiches,* ranging from 4 to 52%. This variable effect of dialysis was also found with *A. cochlioides.*

Culture filtrates of *A. cochlioides* readily macerated potato tuber tissue, but exhibited little maceration of sugarbeet hypocotyl tissues. Presence of a cuticular layer on beet hypocotyl pieces and absence of cuticle on tuber disks probably accounts for much of the observed difference in maceration.

Judging from the values of μ eq of methoxyl groups liberated in diseased and healthy plant extracts, PME does not appear to be important in pathogenesis of sugarbeet by *A. cochlioides.* In this context, it should be noted that sugarbeet pectin differs from most plant pectins in that it contains a relatively large percentage of acetyl groups (21).

The viscosity-reducing activity of diseased and healthy plant extracts was apparently due to endo-PG. Generally higher endo-PG activity was found in diseased compared to healthy plant extracts, and presumably this increase can be ascribed to the endo-PG activity of *A. cochlioides.* However, in some cases extracts from supposedly healthy plants had higher endo-PG activity than did diseased extracts. In those cases microbial contamination of the plant materials probably was involved. The low endo-PG activity obtained from healthy field-grown petioles indicates low activities are associated with healthy plants.

Bateman (4) expressed the opinion that the intercellular growth of *Rhizoctonia solani* Kühn in bean cortical tissue suggests involvement of pectolytic enzymes in dissolution of the middle lamella. In this context, Herr (13) reported *A. cochlioides* hyphae were both intercellular and intracellular in cortical tissue of beet hypocotyls, but no detectable changes in pectin content of diseased tissues were demonstrated by several histochemical tests. The production of endo-PG and maceration of potato tuber tissue by

culture filtrates of *A. cochlioides* clearly demonstrate the pectolytic capabilities of this pathogen *in vitro*. Evidence of pectolytic activity by *A. cochlioides* in diseased sugarbeets was found, but has not been unequivocally demonstrated. A role in dissolution of the middle lamella during intercellular growth of hyphae seems probable, but no general, extensive maceration of beet tissues implicating high enzyme activity has been observed in diseased plants (13).

Summary

In culture, *A. cochlioides* produced a constitutive endopolygalacturonase but no demonstrable pectin methyl esterase or *trans*-eliminase. Supplementation of the culture medium with pectin increased endo-PG activity, although its presence was not required for enzyme production. Maximum endo-PG yields were obtained with dense (concentrated) inocula; either zoospores or diced agar cultures. Aeration of cultures greatly increased growth, but decreased endo-PG production. Age of cultures, buffer, pH, nutrition, and incubation temperature also affected production of endo-PG in culture. The enzyme was concentrated by precipitation with 60% saturated $(NH_4)_2SO_4$. Culture filtrates readily macerated potato tuber tissue. Extracts from diseased sugarbeet tissue exhibited both endo-PG and PME activity, whereas extracts from healthy sugarbeet tissue exhibited (generally) lower endo-PG activity and PME activity essentially similar to that of diseased tissue extracts. Diseased plant extracts also slowly macerated potato tuber tissue.

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Literature Cited

(1) AYERS, W. A. and G. C. PAPAVIZAS. 1965. An exocellular pectolytic enzyme of *Aphanomyces euteiches*. Phytopathology 55:249-253.

(2) AYERS, W. A., G. C. PAPAVIZAS, and R. D. LUMSDEN. 1969. Factors affecting the pectolytic activity of *Aphanomyces euteiches* in vitro and in infected tissue. Phytopathology 59:786-791.

(3) AYERS, W. A., G. C. PAPAVIZAS, and R. D. LUMSDEN. 1969. Purification and properties of the endopolygalacturonase of *Aphanomyces euteiches*. Phytopathology 59:925-930.

(4) BATEMAN, D. F. 1963. Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extracts of Rhizoctonia-infected tissues of bean. Phytopathology 53:197-204.

(5) BATEMAN, D. F. 1963. The "macerating enzyme" of *Rhizoctonia solani*. Phytopathology 53:1178-1186.
(6) BATEMAN, D. F. and R. L. MILLAR. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119-146.
(7) BELL, T. A., J. L. ETCHELLS, and I. D. JONES. 1955. A method for testing cucumber salt-stock for softening activity. U.S. Dept. Agr. ARS 72-5. 15 p.
(8) DEMAIN, A. L. and H. J. PHAFF. 1957. Recent advances in the enzymatic hydrolysis of pectic substances. Wallerstein Lab. Commun. 20:119-140.
(9) HANCOCK, J. G. 1966. Degradation of pectic substances associated with pathogensis by *Sclerotinia sclerotiorum* in sunflower and tomato stems. Phytopathology 56:975-979.
(10) HERR, L. J. 1971. In vitro zoospore production, motility, and germination of *Aphanomyces cochlioides*. J. Am. Soc. Sugar Beet Technol. 16:508-515.
(11) HERR, L. J. 1973. Growth of *Aphanomyces cochlioides* in synthetic media as affected by carbon, nitrogen, methionine, and trace elements. Can. J. Bot. 51:2495-2503.
(12) HERR, L. J. 1974. Production of healthy and diseased sugar beet seedlings for host-pathogen investigations of Aphanomyces black root. Plant. Dis. Reptr. 58:157-160.
(13) HERR, L. J. 1975. Histopathology and histochemistry of sugar beet seedlings resistant and susceptible to *Aphanomyces cochlioides*. Can. J. Bot. 53:284-294.
(14) JANSEN, E. F. and L. R. MACDONNELL. 1945. Influence of methoxyl content of pectic substances on the action of polygalacturonase. Arch. Biochem. 8:97-112.
(15) JERMYN, M. A. and R. G. TOMPKINS. 1950. The chromatographic examination of the products of the action of pectinase on pectin. Biochem. J.47:437-442.
(16) LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
(17) NAGEL, C. W. and R. H. VAUGHN. 1962. Comparisons of growth and pectolytic enzyme production by *Bacillus polymyxa*. J. Bacteriol. 83:1-5.
(18) PAGE, O. T. 1961. Quantitative paper chromatographic techniques for the assay of products of polygalacturonase activity of fungus cultures. Phytopathology 51:337-338.
(19) PAPAVIZAS, G. C. and W. A. AYERS. 1964. Effect of various carbon sources on growth and sexual reproduction of *Aphanomyces euteiches*. Mycologia 43:816-830.
(20) WOOD, R. K. S. and S. C. GUPTA. 1958. Studies in the physiology of parasitism. XXV. Some properties of the pectic enzymes secreted by *Pythium debaryanum*. Ann. Bot., Lond., N.S. 22:309-319.
(21) WOOD, R. K. S. 1960. Pectic and cellulolytic enzymes in plant disease. Annu. Rev. Plant. Physiol. 11:299-322.