Relationship of Pectic Enzyme Activity and Presence of Sterols to Pathogenicity of Pythium ultimum on Roots of Antirrhinum majus

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ABSTRACT

Culture filtrates of *Pythium ultimum* exhibited marked pectic enzyme activity and rapidly macerated susceptible snapdragon (*Antirrhinum majus*) seedling root tissue, but not tolerant tissue. Tolerance to infection by the fungus was correlated with resistance of the root tissues to maceration by *P. ultimum* filtrates. Presence of β-sitosterol in the culture medium of *P. ultimum* depressed pectic enzyme activity of the culture filtrates. The time when pectic enzyme activity was reduced coincided with the first appearance of oospores in the cultures. β-sitosterol, campesterol, and stigmasterol were detected in snapdragon seedling roots. Other compounds, presumed to be oxidized sterols but not identified, were isolated from snapdragon and shown to induce oospore production by *Pythium*. It is proposed that seedling tolerance is based upon interacting physiologic factors. Substances such as β-sitosterol, present in tolerant host tissues, may switch fungal development from vegetative proliferation to reproductive activity. There may be a corresponding depression of enzymes which degrade cell wall material, and hence a lessening of virulence. Phytopathology 60:943-950.

A previous paper (24) showed that infection of snapdragon (*Antirrhinum majus*) seedlings by *Pythium ultimum* Trow. resulted in death if the seedlings were less than 15 days old, but not if the seedlings were older. Accordingly, the younger and older seedlings were, respectively, designated susceptible and tolerant. Tolerance was directly related to inherent factors in mature areas of primary and secondary root tissues, and was not associated with the presence of lignified tissues.

Casual observations of various plant species infected by *Pythium* spp. have shown that young tissues are rotted, whereas older tissues often are not. Frequently, oospores are found in the cortex of older roots while mycelium is rare, whereas, in younger roots mycelium is common but oospores are rare. Pectic enzymes, and possibly other polysaccharide-degrading enzymes, have been shown to be involved in tissue maceration in many plant diseases (3, 4, 6, 14, 23, 33). *Pythium ultimum, P. debaryanum,* and *P. aphanidermatum* have been found to produce several types of pectin-depolymerizing enzymes (1, 25, 32).

Several workers have shown that addition of sterols to chemically defined media may induce oospore formation in Pythiaceous fungi (9, 10, 15, 17). On the other hand, high concentrations of cholestanol may stimulate vegetative growth and inhibit sexual reproduction (8). Hendrix & Guttman (18) showed that estradiol inhibited sexual reproduction in *P. periplocum* but did not affect growth rate. Higher plants generally are good sources of β-sitosterol, stigmasterol, campesterol, and other sterols (12, 16). They have not been studied in snapdragon. It seems possible that sterol balances in host tissues might exert a regulatory effect not only upon the reproductive condition of the parasite, but also upon enzyme synthesis by the parasite. Therefore, we focused our attention upon the role of fungal enzymes and host sterols in the development of tolerance in snapdragon seedlings to infection by *P. ultimum*.

MATERIALS AND METHODS.—*Medium used for production of pectic enzymes by P. ultimum.*—Gupta's (14) medium was used for production of pectic enzymes. The medium was dispensed in Erlenmeyer flasks (25 ml/125-ml flask) and autoclaved at 121 C for 15 min. β-sitosterol (20 mg/liter), when used, was crystallized three times from chloroform-methanol, dissolved in methylene chloride, and added to the medium before sterilization. Ten mycelial plugs, 5 mm diam, taken from the edge of 24-hr-old cultures of *P. ultimum* growing on water agar, were placed in each flask and incubated at 22-27 C on a gyrotory shaker set at 128 rpm.

Pectic enzyme assay.—Pectic enzyme activity was measured viscometrically using sodium polypectate, pectin LM, or pectin NF citrus pectins as substrates. Pectin LM contained 4.2 to 5.3% methoxyl groups, and pectin NF contained 9.4 to 10.2% methoxyl groups by wt. Pectins were donated and analyzed by Sunkist Growers, Ontario, Calif. Solutions of 1.27% substrate (w/v) were prepared in 0.1 M Tris[tris(hydroxymethyl) amino methane]-maleate or phosphate buffer (13), and were used within 3 hr to minimize effects due to bacterial growth. Liquid cultures of *P. ultimum* grown in Gupta's medium were filtered through acid-washed Whatman No. 1 filter paper. The filtrate was used as the source of pectic enzymes after the pH was adjusted with 0.1 M NaOH or HCl. Reaction mixtures contained 12 ml substrate, 0.01 ml to 10 ml filtrate, and deionized water to make 22 ml. The reaction mixtures were incubated at 24 C (± 2 C) on the laboratory bench. Changes in viscosity were measured with pipette viscometers, and per cent loss in viscosity was calculated.

Macerating enzymes.—Filtrates of 6-day-old cultures

of *P. ultimum* grown in Gupta's medium were tested for their ability to macerate snapdragon root tissues. Two ml of a culture filtrate adjusted to pH 6.5 with 0.1 HCl and 1 ml of 0.1 M Tris-maleate buffer were placed in a 35 × 10 mm petri plate. Controls consisted of either 2 ml boiled culture filtrate plus 1 ml buffer, or 2 ml water plus 1 ml buffer. Cubes about 3 mm on a side from the cortex of mature portions of primary roots of 10-, 20-, and 30-day-old plants were used. Tissues were placed in each of the 3 solutions. After incubation at 25 C for 4, 11, or 22 hr, the amount of maceration was determined. The tissues were mounted in water on a microscope slide and covered with a coverslip; a 230-g wt was placed on the coverslip for 10 sec. The proportion of cells separated from one another was determined by microscopic examination, and the degree of maceration was estimated. The pH of each reaction mixture was determined after 12 and 24 hr.

The potential effect of bacterial contamination on maceration was determined. In one experiment, 50 ppm streptomycin sulfate was added to each plate to suppress growth. Bacteria did not affect the results, because the number of bacteria in boiled filtrates was approximately equal to that in unboiled filtrates. Furthermore, streptomycin suppressed numbers of bacteria but had no effect on maceration.

Production of plants for sterol analysis.—Seeds were germinated in flats in U.C. mix (2) in a growth chamber, transplanted to 4-inch clay pots, and placed in the greenhouse. They were watered daily and fertilized once a week with 1 M Hoagland's solution (19). The terminal flower buds were removed as they formed, and the plants were harvested when the flower buds on the lateral branches started to form. Young plants (up to 30 days old) were grown in flats in U.C. mix in the growth room and watered daily, but were not given supplemental application of fertilizer. When harvested, the roots and tops were separated, dried at 68 C, weighed, and ground.

Extraction and analysis of sterols.—A diagram of the extraction procedure is included in Fig. 1. Ground snapdragon tissue was extracted for 6 days with 95% ethanol in a Soxhlet extractor. The ethanol extract was saponified by shaking 3 hr at room temp with equal volumes of ethanol and 2 N H_2SO_4. Water was added, and nonsaponifiable lipids were extracted from the aqueous phase with ether. The ether phase was neutralized and dried over $MgSO_4$ and evaporated under reduced pressure.

For quantitative analysis, the ethanol-extracted tissue was re-extracted by refluxing for 8 hr in ethanolic KOH (1 g KOH/3 g tissue). It was filtered and washed with ethanol and ether. The ethanolic KOH, ethanol washings, and ether washings were pooled, water was added, and the nonsaponifiable lipids were extracted with ether. The ether solution was dried and added to the lipids obtained by Soxhlet extraction.

Sterols were precipitated as their digitonides by Windaus' method (31). The precipitate was filtered and washed carefully with cold ether rather than with ethanol, as 4,4-dimethyl sterol digitonides are less

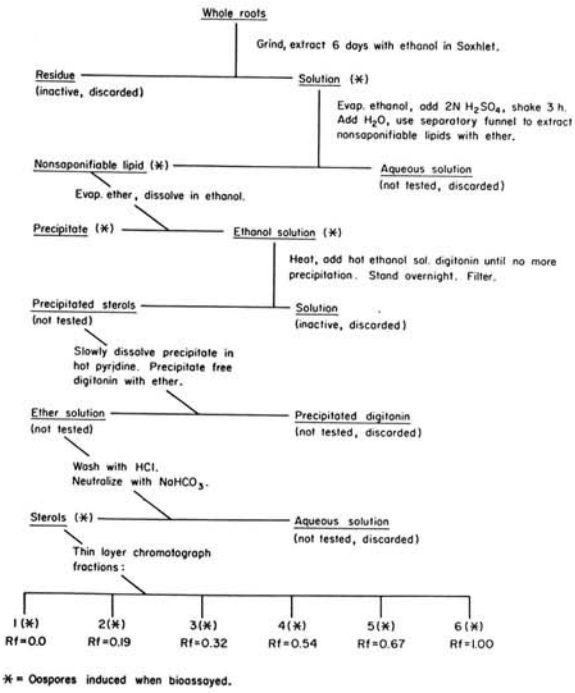

Fig. 1. Methods used in extraction of substances from roots of *Antirrhinum majus* capable of inducing oospore production by *Pythium ultimum*.

soluble in ether than in ethanol. The digitonin-sterol complex was cleaved and the free sterols were separated from the digitonin using Williams & Goodwin's modification (30) of Bergman's technique (5).

Total sterols were separated from other nonsaponifiable lipids by column chromatography over Brockman grade III alumina. The sterols were eluted with increasing amounts of ether in hexane after the nonpolar hydrocarbons were washed through with hexane. The presence of sterols in the fractions was detected by using thin-layer or gas chromatography.

Sterols also were separated by preparative thin-layer chromatography (TLC) over aluminum oxide. Aluminum oxide HF 254 (E. Merck A. G., Darmstadt, Germany) layers were activated by heating 4 hr at 110 C. The solvent systems were chloroform or chloroform-methanol (99:1). The edges of the plates were developed by spraying with 50% phosphoric acid and heating to 100 C for 15 min. Unsprayed areas at the same R_F as the standards were scraped off the plates. The sterols were eluted from the aluminum oxide with ether and methanol.

Thin layers of Silica Gel H were used for monitoring fractions collected from the alumina columns and for detecting sterols in fractions during isolation procedures.

A Perkin-Elmer Model 881 gas chromatograph fitted with a hydrogen flame ionization detector and glass injector was used to analyze trimethylsilyl ethers (TMS) of sterols prepared by the method of Klebe et al. (22). Quantitative analyses were made with a 2.4-m × 2.5-mm (inside diam) glass column packed by vacuum with 3% silicone gum rubber SE-30 on 60-80 mesh

regular chromasorb W. The TMS were injected on the column and the conditions used were: column temp 250 C; injector temp 295 C; detector temp 270 C; and N flow rate 45 ml/min. Rozanski's (28) peak area ratio technique was used to determine that stigmasterol did not interfere with the size of peaks due to β-sitosterol. For qualitative analysis, a copper column 1.8 m × 3.2 mm (outside diam) was packed by gravity with 1% Hi-EFF-8HP on 60-80 mesh acid-washed chromasorb W. The TMS were injected on the column under the following conditions: column temp 210 C; injector temp 250 C; detector temp 240 C; and N flow rate, 60 ml/min.

Oospore production.—Ability of various sterols and sterol fractions to induce oospore formation was tested by incorporating the fractions into the medium (EK) of Erwin & Katznelson (11). Ten-ml portions of media were autoclaved for 15 min at 121 C and stored at 4 C. The medium was diluted with 1.5% water agar (1:9), and 5 ml was poured into a 50- × 15-mm glass petri plate. The medium was diluted because luxuriant growth on the undiluted medium obscured oospores. Purified compounds and crude extracts were tested on this medium by dissolving them in diethyl ether and placing 1 ml on the solidified medium. After the ether evaporated, a plug of mycelium, 5 mm diam, from a culture of *P. ultimum* growing on EK was placed on the agar. After incubation in the dark at 22-27 C for 10 days, the mean number of oospores per microscope field (×250) in four randomly selected fields in each plate was determined. High light intensity (1,000-1,200 ft-c, 12 hr/day) inhibited oospore production. Significance was estimated using Duncan's multiple range test. Three control treatments were always used: (i) EK medium plus 20 mg β-sitosterol/liter; (ii) EK medium alone; and (iii) EK medium plus 1 ml diethyl ether.

RESULTS.—*Pectic enzyme production by P. ultimum.*—Of the three substances tested (sodium polypectate, pectin NF, and pectin LM), sodium polypectate gave the most rapid loss in viscosity. It was therefore used as the substrate for measuring enzyme activity of culture filtrates of *P. ultimum*. The optimum pH for the viscosity-reducing reaction in a range of values from pH 5.5-7.5 was pH 6.5 (Fig. 2). The pH of the reaction mixture remained constant, and the experiments were repeated with the same result.

The fungus was grown on Gupta's medium with and without β-sitosterol, and the filtrates from the pooled contents of 5 flasks were assayed for pectic enzyme activity. Addition of β-sitosterol to the culture medium significantly decreased pectic enzyme activity of the culture filtrates (Fig. 3-A). The pectic enzyme activity of the filtrate of the β-sitosterol-supplemented cultures reached a maximum in 4 days and then dropped sharply and steadily as age increased. In contrast, enzyme activity of filtrate from cultures produced on medium without β-sitosterol reached a maximum about 1 day later and remained level through the 10th day.

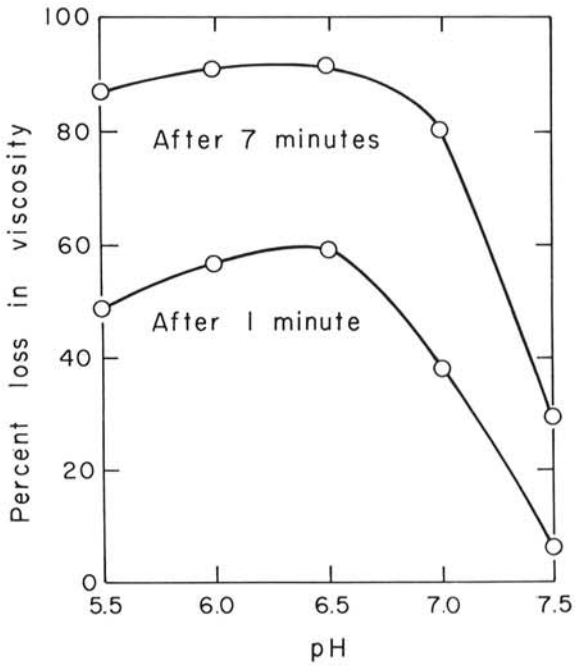

Fig. 2. Effect of pH and time on pectic enzyme activity of culture filtrates of *Pythium ultimum*.

The presence of β-sitosterol in the medium uniformly stimulated growth based on dry wt (Fig. 3-B). Thus, pectic enzyme activity was negatively correlated with increased growth stimulated by the addition of β-sitosterol in the culture medium. Oospores were not formed in medium without β-sitosterol, whereas they were observed in 5-day-old cultures that contained the sterol. The addition of β-sitosterol to the medium did not appear to affect pH (Fig. 3-C).

Relationship of age of root tissue to maceration by culture filtrates of P. ultimum.—Although no tissue was unaffected by the culture filtrate, increased resis-

TABLE 1. Maceration of roots of *Antirrhinum majus* of various ages by culture filtrates of *Pythium ultimum*

Tissue exposed	Disease reaction	Plant age (days)	Degree of maceration after hours of exposure[a]		
			4	11	22
Root tip	Susceptible	10	1	3	3[b]
Root tip	Susceptible	20	1	2	3
Root tip	Susceptible	30	1	3	3
Cortex of primary root	Intermediate	20	0	1	3
Cortex of primary root	Tolerant	30	0	0	1

[a] The degree of maceration was determined by estimating the ratio of intact to separated cells in a tissue.
[b] 0 = no maceration; 1 = less than 25% of the cells separated; 2 = 25-70% of the cells separated (Fig. 4-B); 3 = over 70% of the cells separated (Fig. 4-C).

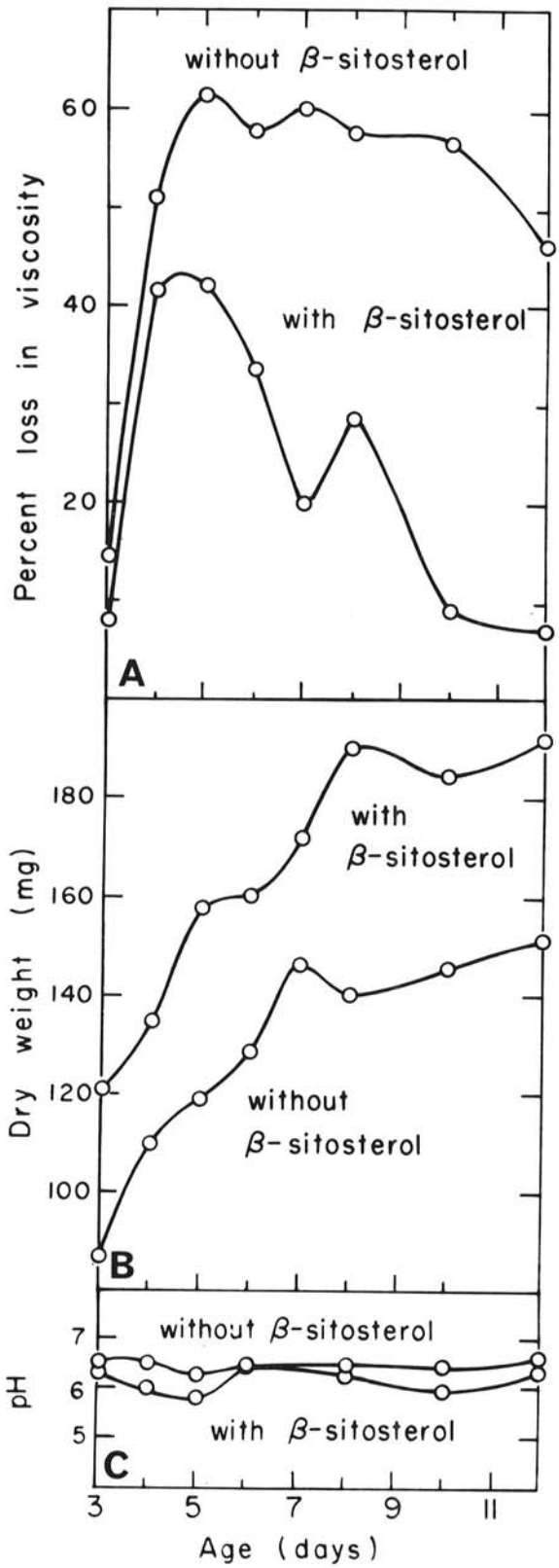

Fig. 3. Effect of addition of β-sitosterol (20 mg/l) to Gupta's medium on *Pythium ultimum:* **A)** Pectic enzyme activity of culture filtrate; **B)** dry wt of mycelium; **C)** pH of culture filtrate.

tance to maceration was related to increased age of the tissue (Table 1, Fig. 4). Root tips taken from all plants were macerated rapidly, sometimes completely, in 11 hr. Maceration of cortical tissue of the primary roots of 20-day-old plants was not detectable until 11 hr, and by 22 hr it was complete. Maceration of cortical tissue of the primary root of 30-day-old plants was not detected until 22 hr, indicating that this tissue was resistant to the culture filtrate. Tissue placed in water or boiled filtrates was not macerated.

In the root tips, tissues of the region of differentiation and the younger portion of the region of elongation were macerated first, whereas, tissues in the meristem and the region of maturation were not macerated so rapidly.

Extraction and identification of some oospore-inducing factors from roots of snapdragon.—Efforts were made to isolate and identify the oospore-inducing factors from roots to determine whether they were involved in the development of tolerance of snapdragon to *Pythium* (Fig. 1). At various stages plant extract fractions were bioassayed, and three compounds that induced oospores were identified.

The nonsaponifiable lipid fraction (2.65 g) isolated from whole root systems (94.8 g dry wt) of mature plants induced formation of oospores. Thin-layer chromatograms of this lipid showed spots with R_F values close to that of β-sitosterol. The total fraction was separated into two parts; one soluble in ethanol and one insoluble in ethanol; both parts induced oospore formation. The insoluble part was not investigated further.

Addition of digitonin to the ethanol solution gave a precipitate which contained all the oospore-inducing activity, indicating that β-hydroxyl sterols were probably responsible for the activity. The compounds precipitated by digitonin (100 mg) were separated into six fractions using preparative thin-layer chromatography, and each fraction was bioassayed (Table 2). Although all six fractions induced some oospore production in *P. ultimum,* only fractions 2 and 4, which had highest activity, were investigated further.

The components of fraction 2 were investigated but not identified. Gas chromatograms of fraction 2 were compared to those of β-sitosterol, lanosterol, stigmasterol, cholesterol, and ergosterol, but none of these was present. An infrared spectrum revealed the presence of hydroxyl and carbonyl absorption, but no definite

TABLE 2. Effect of six fractions of digitonin-precipitable sterols from *Antirrhinum majus* roots upon oospore formation by *Pythium ultimum*

Fraction[a]	R_F	Wt (mg)	No. oospores/ microscope field	
1	0.00	1.9	14	b, c[b]
2	0.19	1.2	28	a, b
3	0.32	1.6	14	b, c
4	0.54	74.1	40	a
5	0.67	2.7	8	c
6	1.00	18.0	2	c

[a] Fractions obtained by preparative thin-layer chromatography.

[b] Numbers followed by same letter were not significantly different at the 5% level.

conclusions were made because the sample was insufficient. Because the R_F values of these compounds were much lower than those of the common plant sterols, the compounds in group 2 were more polar than other sterols reported to have induced oospore formation in Pythiaceous fungi.

Fraction 4, which had similar R_F values to the desmethyl sterols, contained 74% of the total amount precipitated by digitonin. Gas chromatographic analysis of group 4 on a SE-30 column revealed peaks with the same retention time as β-sitosterol, stigmasterol, and campesterol. Analysis of chromatograms from a Hi-EFF-8EP column revealed two peaks having the same retention time as β-sitosterol and stigmasterol. About 34 mg of white crystals were isolated from this mixture by fractional crystallization from chloroform-methanol. Examination of the mass spectrum of this material revealed two compounds which contained parent ions and fragmentation patterns indicative of β-sitosterol and stigmasterol. The crystals induced oospores when assayed. The biological portions of this experiment were repeated with similar results.

Changes in concentration of β-sitosterol in roots of seedlings of different ages.—The amount of β-sitosterol in seeds and roots of 10-, 20-, and 30-day-old plants was determined using quantitative gas chromatography. The desmethyl sterols were isolated from the total nonsaponifiable lipid from each sample by column chromatography as described, and the TMS was prepared.

The TMS of each sample, plus one of a weighed amount of β-sitosterol (95% purity) which served as a standard, were analyzed. The average area of the peak due to β-sitosterol for two replicates of each sample was calculated.

Seeds contained 4.4 μg of β-sitosterol/mg of tissue, whereas all three root samples contained less than 2 μg/mg of tissue. The amount of β-sitosterol dropped from 1.77 μg/mg in 10-day-old roots to 0.77 μg/mg of tissue in 30-day-old roots. Stigmasterol and campesterol were present in all three root samples. Stigmasterol was also found in the seed samples, but campesterol was not detected.

Roots of 10-, 15-, 20-, 25-, and 30-day-old plants (180 plants/age group) were dried at 60 C, and the wt per root was calculated. The dry wt increased from 31 μg/root in 10-day-old plants to over 200 μg/root in 30-day-old plants (Fig. 5).

Thus, the concentration of β-sitosterol dropped in the roots on a wt basis as the plants aged, but the amount per total root systems increased.

DISCUSSION.—There was a striking correlation between susceptibility of tissues to macerating factors in the culture filtrate of *P. ultimum* and susceptibility to the fungus. It is reasonable to assume that resistance to maceration is due to changes in cell wall chemistry, since drastic changes take place in the chemical composition during maturation of cell walls (7, 20, 26, 27), and since pectic enzymes, which are involved in tissue maceration, show substrate preference (4, 33). We showed that sodium polypectate was a better substrate than pectin NF for pectic enzymes produced by this isolate of *P. ultimum,* indicating substrate preference here also. It is probable that one of the reasons for resistance of older roots to *P. ultimum* is that the enzymes produced by the pathogen cannot attack the substrates available in the cell walls of the resistant tissue.

Further work is necessary to determine the chemical nature of this barrier. Evidence presented by previous workers indicates that endopolygalacturonases are responsible for tissue maceration, although other enzymes, such as arabinase, may be involved. Since we used crude preparations, probably containing several enzymes, it would be necessary to determine which enzymes are involved in tissue maceration and also to determine whether they are present in infected tissue.

The fact that oospores were found in infected tissue of tolerant plants was the genesis of our hypothesis that sterols also may be involved in resistance to *P. ultimum*. Klebe (cited in 29) long ago postulated that the life cycle of the fungi was divided into an aggressive, rapidly growing, "summer" phase, and a resting period, the reproductive phase. Since sterols induce oospore formation in *Pythium,* it seemed possible that sterols in tolerant plant tissues might trigger the fungus to go into its reproductive stage and lose its pathogenic potential. In susceptible plant tissue lacking high enough concentrations of sterols, no such triggering might occur and the fungus would continue its vegetative growth. The effect could be due to qualitative or quantitative differences of sterols in the tissues.

Although the effect of steroids on resistance have received little attention in plant pathology, steroids are known to be involved in resistance to some diseases in animals (21). We concentrated on β-sitosterol, realizing that other compounds also might be involved.

The fact that β-sitosterol inhibited pectic enzyme activity in vitro was further evidence that oospore-inducers could be involved in resistance. Noteworthily, a sharp decline in pectic enzyme activity coincided with the appearance of oospores in the cultures.

The assumption that β-sitosterol is involved in resistance would have been strengthened if an increase in concentration of β-sitosterol in the roots (on a w/w basis) had been noted as the plants aged. Reconsideration of the data brought out important points. First we extracted whole roots, and the distribution of β-sitosterol within the various tissues was not determined. Second, in the older plants much of the dry wt is contributed by tissue inside the endodermis in mature portions of the primary and secondary roots, and this tissue is not colonized by the pathogen. Such tissue would contribute greatly to the dry wt values, but may not be significant so far as actual resistance mechanisms are concerned. Since there is an increase in total β-sitosterol per root system as the plants age, higher concentrations may exist in certain tissues than in others. For example, the concentration in the cortex of the primary root of a 30-day-old plant may be much higher than the concentration in the region of elongation of the root tip. Thus, to actually determine whether there is a correlation between increased resistance to *Pythium* and an increased concentration of β-sitosterol, one must examine the tissues of the roots actually involved.

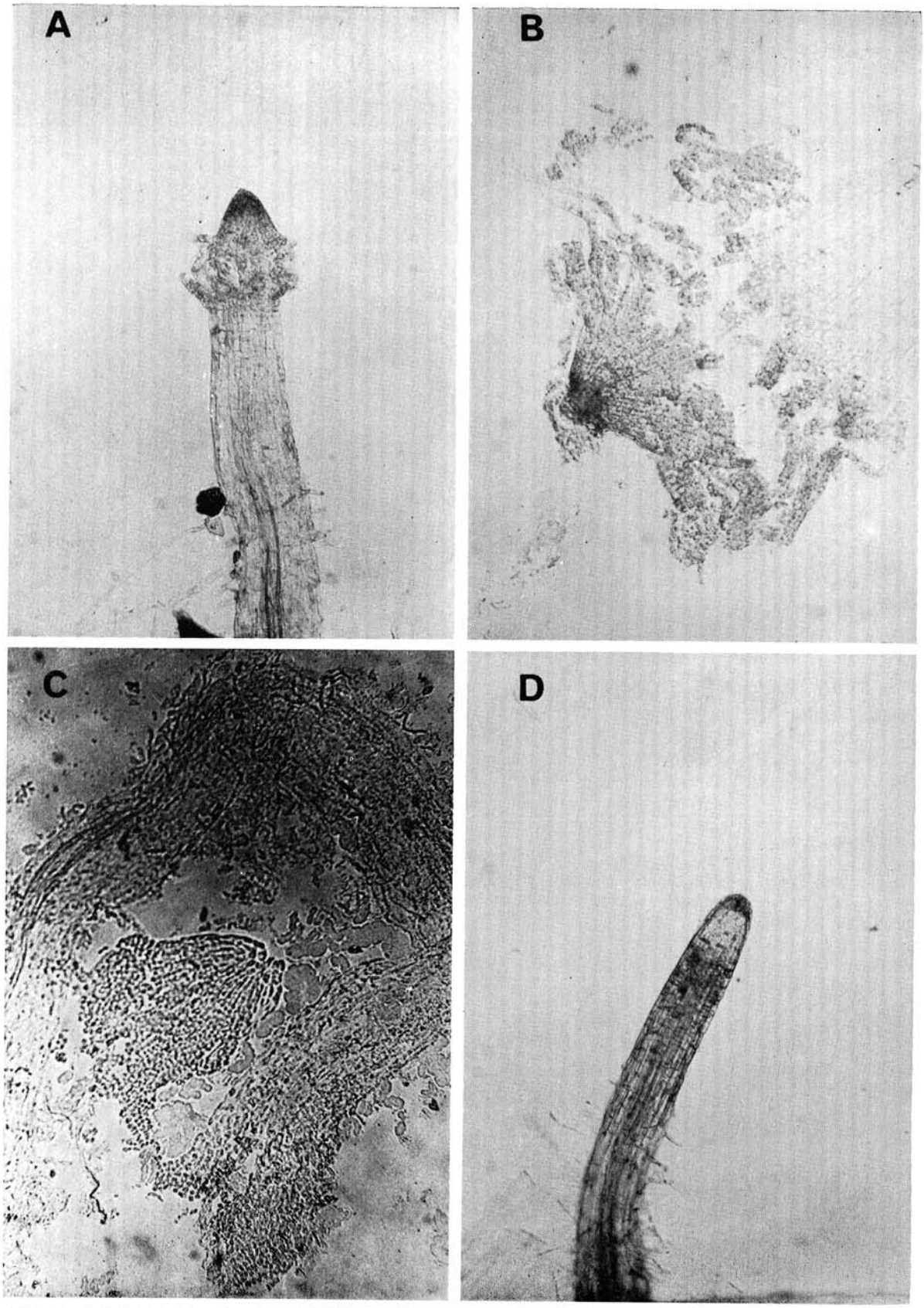

Fig. 4. Effect of culture filtrates of *Pythium ultimum* on root tips of 10 to 15-day-old snapdragon seedlings held at pH 7.0 after various times: **A)** 4 hr; **B)** 11 hr; **C)** 14 hr; **D)** 14 hr in boiled culture filtrate (control).

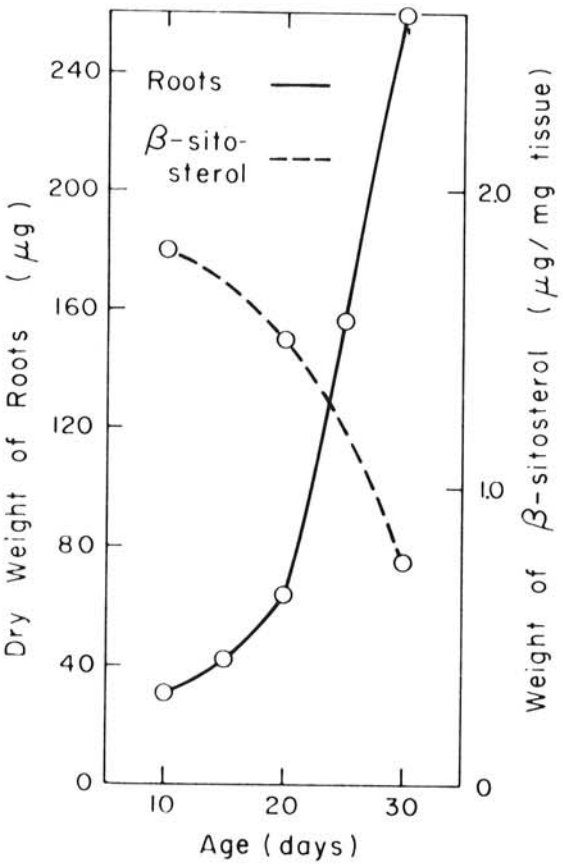

Fig. 5. Changes in concentration of β-sitosterol and root dry wt in relation to age of seedlings of *Antirrhinum majus*.

Of interest was the detection of two groups of compounds in addition to the identified β-hydroxyl sterols that induced oospore production in *P. ultimum*. This is important since only β-hydroxyl sterols have been reported to induce oospores in culture (15, 17). One group that was precipitated by digitonin was probably structurally related to the sterols because only β-hydroxyl sterols are precipitated in this reaction. However, the compounds were much more polar than simple β-hydroxyl sterols, and infrared spectra indicated the possible existence of other polar functional groups. The second group of compounds were those which were insoluble in ethanol after the total extract was treated with acid. It is possible, but not probable, that this group of compounds contained sterols that were not removed due to insufficient washing with ethanol.

In the analysis of snapdragon roots, β-sitosterol and stigmasterol were identified by mass spectroscopy in extracts of snapdragons, and campesterol was tentatively identified based on its retention time during gas chromatography. This represents the first report of these compounds in snapdragon.

While the roles of macerating enzymes of the fungus, sterols of the host, and age of tissues were not inexorably linked in these studies, sufficient correlations were found to lend credence to the conjecture that they have a profound influence on seedling resistance. The production of a substance like β-sitosterol by localized tissue in older regions of roots reasonably can be expected to affect the virulence of a pathogen, such as *P. ultimum*, by reducing its capacity to form macerating enzymes and to stimulate it to expend its energy into forming reproductive structures, thus converting it into a relatively nonvirulent parasitic stage.

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