MONTANA STATE UNIVERSITY

Studies on chemotaxis of Aphanomyces cochlioides Drech. zoospores to sugar beet seedlings
by Palthad Vittal Rai

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Botany (Plant Pathology)
Montana State University
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STUDIES ON CHEMOTAXIS OF APHANOMYCES COCHLIOIDES DRECH. ZOOSPORES TO SUGAR BEET SEEDLINGS

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ABSTRACT

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Chemotaxis tests for zoospores of A. cochlioides with crude preparations of root exudates, individual fractions, the fractions in all possible combinations, and the individual compounds from neutral fraction and anion fractions showed that the crude preparations had the maximum attracting and growth influencing abilities. Gluconic acid had the maximum zoospore attracting ability among the individual compounds tested.

CHAPTER I

INTRODUCTION

Afanasiev (1948) reported the occurrence of black root, or damping off of sugar beet (Beta vulgaris L.) caused by A. cochlioides, in Montana. Since then this pathogen has been observed to cause noticeable damage to the beet crop in Montana, especially in heavily irrigated soils. The symptomatology of this seedling disease is "black root", discoloration of hypocotyls varying from dark brown to black and discoloration of petioles of lower leaves. The leaves remain green and turgid, but diseased plants are stunted in growth (1).

Zoospores of A. cochlioides are the primary means by which this fungus asexually propagates. MacWithey observed massing of zoospores of A. cochlioides concentrated on the hypocotyl of sugar beet seedlings. He also observed that germination of zoospores was better when they clumped on the host (unpublished). Using Aphanomyces euteiches Cunningham and Hagedorn (3) reported that zoospores massed on pea roots, especially in the region of elongation. Dukes and Apple (4) discovered abundant massing of zoospores of Phytophthora parasitica var. nicotianae at the cut ends of roots and on the wounded parts. Zentmyer (16) showed that zoospores of Phytophthora cinnamomi were attracted to the excised roots of susceptible avocado plants. He also observed that the response of zoospores was more pronounced in the region of elongation than at the tip or in more mature portion of roots. Furthermore, he reported that germ tubes of these germinating zoospores were uniformly directed towards the root from a distance of up to 2-3 mm. He also demonstrated that the zoospores

of Phytophthora citrophthora were attracted to roots of its citrus host but not to those of avocado, indicating specific attraction of zoospores. Many other studies with different organisms have also shown that attraction of zoospores is general to root exudates (3, 4, 8, 15). Zoospores of A. cochlioides have been observed accumulating on sugar beet, pea (Pisum sativum) and tomato (Lycopersicum esculentum) (unpublished). Moreover, no accumulation was observed on cucumber (Cucumis sp.) roots.

Chemotaxis of zoospores has been worked out in various saprophytic fungi by many workers; and compounds such as potassium salts, inorganic phosphates and many protein degradation products, e.g., alanine, leucine, aspartic acid, glutamic acid, α-aminobutyric acid, etc., caused attraction (9, 10). Dukes and Apple (4) reported that 1% sucrose solution acts as a strong attractant of zoospores of Phytophthora parasitica var. nicotianae. They also observed that glucose, fructose, rhamnose, maltose and combinations of several sugars and amino acids attracted zoospores, but not lactose, galactose, tap water and sodium chloride. Carlile and Machlis (2) observed that zygotes of Allomyces sp. responded to individual amino acids, such as cystine, proline and serine. Royle and Hickman (9) reported that glutamic acid was unique in causing both attraction and encystment of zoospores of Pythium aphanidermatum. They also observed that combination of sugars, (fructose, glucose and sucrose) and 18 amino acids in equal proportions by weight caused excellent attraction and clustering of cysts. Troutman and Wills (15) stated that zoospores of Phytophthora parasitica var. nicotianae always migrated towards the

negative electrode in the presence of an electric current and compared this principle to that of plant roots and rhizosphere.

Although previous investigators have demonstrated the chemotactic properties of various compounds found in root exudates, no study had included the quantitative aspect of such compounds as they naturally occur. Furthermore, few investigators have even considered the complete qualitative analysis of compounds in exudates which act as attractants. It is therefore the purpose of this report to show which compounds are present in sugar beet exudate, what concentration of such compounds are exuded, and which compounds are effective in zoospore attraction, germination and development.

CHAPTER. II

MATERIALS AND METHODS

Preparation of zoospore suspension: An A. cochlioides culture (courtesy Dr. M. M. Afanasiev, Montana State University, Bozeman) was maintained on corn meal agar and grown on a liquid medium (5). The organism was grown in 250 ml Erlenmeyer flasks containing 100 ml of autoclaved medium for four days at room temperature. After decanting the medium, the mycelial mat was rinsed thoroughly in sterilized distilled water six times and incubated in the last rinse for 24 hours at room temperature. Spot tests indicated that sugars or amino acids were not present in the final rinse water. Twenty-four hours after the rinse, the mycelial mat produced an abundance of actively moving zoospores.

Preparation of sterile sugar beet root exudate: Sugar beet seeds of the Great Western Sugar Company, variety number 359-602, pretreated with New Improved Cerasan (0.3 g Cerasan per 100 g seeds) were treated with 20% Chlorox for 20 minutes. After washing the seeds 8 to 10 times in sterilized distilled water, they were aseptically transferred to plates of potato dextrose agar and incubated at room temperature for 3 days. The clean germinated seeds were transferred aseptically to the sterilized growth vessel.

The growth vessel was a petri plate (9 cm diameter and 4½ cm depth) containing stainless steel wire mesh fitted inside, 1 cm above water. The wire mesh acted as a platform on which the germinating seeds rested. The developing roots were held in the water in the vessel and shoots grew upwards from the platform. Hence the water in the vessel served as

a reservoir of root exudates. The seedlings were grown for seven days in the vessel at room temperature. The plants and exudates were checked for contamination on nutrient agar (Difco). The plants were counted and the water in the vessel was reduced to 1.0 ml by a flash evaporation.

Analysis of root exudates: The concentrated root exudate was passed through Dowex 50 (H^+) and Dowex 1 (formate), respectively, in order to separate the sample into cation, anion and neutral fractions, respectively. (13). The fractions were evaporated to dryness by dry air and placed in P_2O_5, NaOH desiccator overnight.

The organic acid fraction was separated by one dimensional chromatography on Whatman No. 1 paper by using the following solvent systems: A) n-butanol - acetic acid - water (4:1:5 v/v), B) ethyl acetate - pyridine - water (8:2:1 v/v) and C) n-pentanol - 5 N formic acid (1:1 v/v). Organic acids were detected on the chromatograms according to the method of Trevelyan, et. al. (11), and by spraying of 5% brom-phenol blue in ethanol. Organic acids were quantitatively determined according to the method of Strobel and Hewitt (13).

Sugars were identified by one dimensional paper chromatography in solvent systems A and B. After elution from the chromatograms the reducing sugars were estimated quantitatively by the method of Nelson (6). Estimation of melibiose, raffinose and sucrose in the neutral fraction was made by Joyce-chromoscan densitometer, after treatment of the chromatogram with basic silver nitrate as prescribed by Trevelyan (14). Standard curves for these sugars were made by using 1, 2, 4, and 8 µg. Estimation

of sugars and organic acids were calculated on a per root basis.

A known amount of the amino acid fraction was separated by two-dimentional thin layer chromatography on silica gel H in the solvent system: Isopropanol-NH_4OH (67:33 v/v) followed by n-butanol-acetic acid-water (3:1:1 v/v). Known amino acids were also separated by two dimentional thin layer chromatography. Amino acids were detected by spraying 0.3% ethanol-ninhydrin on the developed chromatoplates. Amino acids present in the sample were identified according to their position corresponding to the position of the reference amino acids.

After separation of a given amount of sample the chromatoplates were air dried and sprayed twice with ethanolic ninhydrin and dried at 75 C for 10 minutes. The spots were scraped into a beaker with 7.65 ml distilled water, stirred well and filtered through Whatman No. 1 paper into a cuvette. Readings were taken in a Bausch and Lomb Spectronic 20 colorimeter at 570 mμ. Each reading was compared with the respective standard curve for that particular amino acid prepared in the same manner using known concentrations (0.5, 1.0, 1.5 and 2.0 μg) of the amino acid. Individual amino acids were also calculated per root basis.

Attraction tests: To test root materials under standardized conditions, a modified technique of Royle and Hickman was used (8, 9). The capillary root model was prepared with glass capillary tubes of 1 mm outer diameter and 8 cm in length. Two scratches were made at the 2 cm mark in each tube. The tubes were washed thoroughly in concentrated sulfuric acid and sterilized distilled water. Solutions for tests were mixed in equal

proportions with 0.5% purified agar (Difco) at about 50 C. Capillaries were filled by allowing the agar solutions to be drawn up by capillary action to the 4 cm mark (20 µl). After a few minutes when the substances inside the capillary tubes solidified, pieces of 2 cm length were made at the pre-cut marks. These root models were cleaned with cheesecloth and placed in plain Syracuse watch glass (diameter 2 5/8 inch) which was placed on the stage of a compound microscope. Two such root models were tested in each watch glass. There were 4 root models for each compound and the various fractions from beet root exudates. Agar, 0.25%, was used as a control in the root models.

Two ml of zoospore suspension were used in each watch glass. The tubes were arranged parallel to each other about 2 cm apart and the watch glass was covered with a lid. Readings were taken 6-8 hrs after the zoospore suspension was added. Concentrations of crude exudate and exudate fractions in the tubes were identical to the amounts produced by 70 plants. The concentration of other compounds in the tubes were identical to the amount produced by 5, 10, 15 and 20 plants. Readings were taken by counting the zoospores which lodged at the ends of root model in the microscope field. Furthermore, the germinating zoospores in each case were estimated. Readings of the randomly lodged zoospores were taken from randomly selected regions in the watch glass where there was no influence of the compounds which were in the root models. The proportion of zoospores at the root model ends to that of randomly lodging zoospores was calculated. The percentage of spore germination was also calculated in each case.

CHAPTER III

RESULTS

Gluconic acid was the predominant acid in the organic acid fraction (Table I, fig. 2). Two other compounds were present, lower in amount and detected in the solvent system, containing ethyl acetate-pyridine-water (8:2:1 v/v); the R_f's of which were 0.28 and 0.56. The neutral fraction yielded 8 sugars, fructose, glucose, melibiose, raffinose, ribose, sucrose, and 1 unidentified compound (fig. 3). Table I shows that glucose was present in quantities larger than any other sugar. Fourteen spots were found on the chromatoplates when the amino acid fraction was analysed. Eight of these were identified; these include alanine, arginine, aspartic acid, glutamic acid, glycine, lysine, phenylalanine and threonine (fig. 4). The quantitative estimation of each compound is presented in Table I.

Zoospores showed distinct differences in response towards different fractions tested in root models as shown in Table II. The crude preparation had an excellent ability to attract zoospores to support a high germination and to influence profuse mycelial growth. The mycelial growth was more prominant near the tip of the root model than at the farther regions (fig 1-B). The amino acid fraction was next best to crude preparation in supporting the development of the germ tubes, but it did not have a noticeable ability to attract zoospores. The zoospores lodged near the end of the root model which contained amino acid fraction germinated and developed much better than the others which lodged farther away from the tube ends (fig. 1-C). The neutral fraction showed a relatively good zoospore attracting ability, however, it somewhat retarded the germination of zoospores. The development of germ tubes in

the presence of the neutral fraction was poor, and the accumulation of zoospores seemed to be diffuse (fig. 1-D). Second to the crude preparation, the organic acid fraction showed the maximum zoospore attracting ability (fig. 1-E); however, the organic acids appeared to have no effect on the germination of zoospores and the development of hyphae. When the amino acid fraction was combined with neutral fraction there was no zoospore attraction above that of the neutral fraction alone. Likewise, the zoospores germinated and developed as they did in the amino acid fraction alone. The combination of the amino acid fraction and the organic acid fraction showed a poor zoospore attracting ability when compared to organic acid fraction alone. When the total effect of this combination was compared with the individual effects, the amino acid fraction seemed to suppress the attraction ability of the organic acid fraction. However, there was slight increase in the attraction ratio and germination percentage over the results observed in the amino acid fraction alone. When the organic acid fraction and the neutral fraction were combined the attraction ratio was less than the individual effect of each fraction but the germination percentage was only slightly less than the additive effect of both the compounds. The combination of all the three fractions (fig. 1-F) had relatively a better effect on attraction, germination and development of the fungus but in all cases was less than that of crude preparation. The check (fig. 1-A) had an attraction ratio of 1, which was considered as the base and 10% germination.

The ability of the different compounds and groups of compounds to attract zoospores were made according to the following formula:

$$\text{Ratio of zoospore attraction} = \frac{\text{No. of zoospores at the end of root model}}{\text{No. of zoospores randomly lodging}}$$

Among the individual compounds gluconic acid showed maximum ability to attract zoospores with fructose and glucose next in order (Table III). Maltose, sucrose, and xylose played a relatively small role in attracting zoospores. Melibiose had no effect whereas raffinose and ribose seemed to repel the zoospores. Gluconic acid and all the identified sugars from the root exudate were tested for attraction of zoospores in root models using concentrations of compounds as they were found to be exuded by 5, 10, 15 and 20 plants, respectively, (fig. 5). When all of the identified sugars were combined with gluconic acid the attraction ratio was more than that of the sugars alone and less than that of gluconic acid alone. Amino acids were not tested individually as the amino acid fraction did not show attraction for zoospores under conditions as they naturally occurred in sugar beet root exudate.

TABLE I

Qualitative and quantitative analyses of sugar beet root exudate

	Compounds exuded by sugar beet roots	Quantity in µg per root
I.	ORGANIC ACIDS	
	1. Gluconic acid	0.3560
II.	SUGARS	
	1. Fructose	0.4444
	2. Glucose	1.1389
	3. Maltose	0.2556
	4. Melibiose	0.0063
	5. Raffinose	0.0133
	6. Ribose	0.0778
	7. Sucrose	0.0002
	8. Xylose	0.1556
III.	AMINO ACIDS	
	1. Alanine	trace*
	2. Arginine	0.0024
	3. Aspartic acid	0.0022
	4. Glutamic acid	0.0021
	5. Glycine	trace
	6. Lysine	trace
	7. Phenylalanine	trace
	8. Threonine	trace

* Any compound which was less than 0.0001 µg was considered as trace.