# Predisposition Effect of Water Saturation of Soil on Phytophthora Root Rot of Alfalfa

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## ABSTRACT

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Root rot caused by *Phytophthora megasperma* f. sp. *medicagnis* was more severe (disease index 4.8, based on a 0–5 rating scale) on alfalfa seedlings grown in saturated soil 1 wk before inoculation than on those pregrown in unsaturated soil before inoculation (disease index 2.5). Yield of foliage was reduced from 60 to 30% of that of uninoculated controls by preinoculation saturation. The percent kill of seedlings axenically inoculated with motile zoospores ranged from 50% in unsaturated soil to 70% in saturated soil. The increased severity and incidence of root rot was associated with: increased chemotactic attraction of zoospores to roots grown in saturated soil; breaks on the surface of roots (visible by scanning electron microscopy) subjected to soil saturation; increased electrical conductivity of root exudate from 15 to 22 $\mu$mhos/cm; root exudation of amino acids increased by 30% and sugars by 10%. Results indicated that water saturation of soil predisposed alfalfa roots to Phytophthora root rot by increasing root damage and the exudation of nutrients which in turn increased the chemotactic attraction of zoospores to the roots.

Phytophthora root rot of alfalfa (*Medicago sativa* L.) was first described by Erwin in California (13–15). The name of the causal agent was recently designated as *Phytophthora megasperma* Drechs. f. sp. *medicaginis* by Kuan and Erwin (22) because of the specificity of isolates to alfalfa. This disease has been associated with excessive rainfall and poorly drained or heavily irrigated soils. Pulli and Tesar (26) reported that two supplemental irrigations in July and August was a stress factor that caused increased Phytophthora root rot severity in a field experiment. Erwin reported that increasing the interval between irrigations arrested the disease (14) in the field.

Generally, most root diseases are favored by wet soils (8). The effect of soil water on root diseases can be a function of an effect on the pathogen, on soil microorganisms, and/or on the host plant. Most research reports emphasize the effects of soil water on pathogen growth (1,28), sporulation (10,25), and spore dispersal and motility (11,23–25). The effect of soil water on soil microorganisms also has been studied (6,7). However, there are few studies on the effect of soil water on host plants and all of them deal with the effect of water stress on the increased severity of plant diseases (9,12). Crist and Schoeneweiss (9) observed that the susceptibility of seedlings of *Betula alba* to canker formation and to colonization by *Botryosphaeria dothidea* increased with decreasing water potentials. Duniway (12) found that preinoculation water stress predisposed safflower plants to infection by *P. cryptogea* and increased the disease severity. There are few research reports on the effect of preinoculation soil saturation even though Ward (31) listed excessive soil water (soil saturation) as a predisposition factor in plant disease as early as 1901.

Knous postulated (20) that the lack of oxygen in flooded soil rather than the presence of the pathogen could be the principal cause of Phytophthora root rot of alfalfa. Knous and Maxfield (21) reported an increased concentration of squalene in the susceptible cultivar Saranac was associated with root tissue dissolution characteristic of Phytophthora root rot in a field irrigated every 4 days. On plots irrigated every 10 days, neither Phytophthora root rot nor increased squalene was found. They stated that, "This increase may be the result of root tissue predisposition due to oxygen stress and not as a direct result of flooding or inoculum concentration."

We conducted this study to determine the effect of excessive soil water (soil saturation) prior to inoculation on the disease severity of Phytophthora root rot of alfalfa and on the possible mechanisms involved.

## MATERIALS AND METHODS

**Treatments and inoculation.** *P. megasperma* f. sp. *medicaginis*, isolate P1057 (*Phytophthora* culture collection, Department of Plant Pathology, University of California, Riverside), was grown on V8 agar (V8A) at 24 C. The susceptible alfalfa cultivar Moapa 69 was used in all experiments. Twenty surface-sterilized alfalfa seeds were planted in two rows in each pot (10 cm × 10 cm × 10 cm) containing doubly steamed U.C. mix (peat moss: sand [1:1, v/v]). Three weeks after seeds were planted, half of the pots were watered from the top each day and saucers beneath these pots were kept full of water. The soil appeared to be saturated. For the unsaturated treatment the pots were watered from the top once a day, but since no saucers were used, excess water drained from the pots. At inoculation time the soil in the previously unsaturated pots was watered heavily. Saucers were then removed from all pots and pots were allowed to drain for 1 hr. The water content appeared to be similar in saturated and in nonsaturated pots when plants were inoculated. Fifty milliliters of minced mycelium from two plates of V8 agar, 5–7 days old, and blended 15 sec in a Waring blender in 1 L of distilled water was evenly distributed in three shallow trenches beside and between the two rows of plants in each pot. After inoculation all the pots, previously saturated and nonsaturated, were watered on the surface once a day. Three weeks after inoculation the severity of root rot was scored: 0 = no disease; 1 = small lesions on not more than 10% of the tap root surface; 2 = slightly larger lesions encompassing not over 20% of the root surface; 3 = lesions covering 20–50% of the root surface; 4 = lesions covering 50–100% of the root surface but the plant was still living; 5 = entire root rotted and the plant dead (16). Fresh weight and height of shoots, diameter of tap roots, and fresh weights of roots of uninoculated and inoculated plants grown in saturated and nonsaturated soil were recorded. The experiment was repeated five times with 12 replications of each treatment (each pot was a replicate).

Alfalfa seedlings also were tested. Ten alfalfa seeds were planted in each of two rows of each pot containing U.C. mix soil. Three days after planting, pots were kept in a water-filled container 10 cm in depth for 0, 1, 2, 3, 4, and 5-day periods. After the saturation period both nonsaturated and saturated pots were flooded and allowed to drain for 1 hr. After inoculation all the pots were watered on the top once a day. A suspension of zoospores, used as inoculum, was prepared as follows: 2- to 4-day-old cultures of P1057, started from minced mycelium at a concentration of 1 ml of minced mycelium distributed over a plate of V8A, were flooded with sterile distilled deionized water and shaken on a reciprocal shaker (60 excursions per minute) for 2 days. The water on the plates was replaced with fresh water and the cultures were chilled at

12 C for 14–16 hr. The zoospores were counted on a haemocytometer. The motility period of the zoospores was extended by maintaining the suspensions at 12 C before they were used as inoculum. The suspension of zoospores (10 ml at $2 \times 10^3$ zoospores per milliliter) was poured slowly into three trenches (20 mm in depth) beside and between the two rows of seedlings in each pot but was not allowed to contact the bases of the stems. Ten milliliters of water was added after inoculation. The uninoculated control pots received 20 ml of water. The zoospore suspension was checked before and after inoculation to assure that the zoospores were motile. All the pots before and after inoculation were kept in a Sherer growth chamber at 18 C (12 hr dark) and at 24 C (12 hr light) at 7.6 nanoeinsteins $cm^{-2} sec^{-1}$ intensity. The percentage of seedlings killed was recorded 3–6 days after inoculation. The same experiment was done with seedlings grown under axenic conditions as described below. The experiment was repeated four times with 12 replications of each treatment (each pot was a replicate).

**Growth of plants under axenic conditions.** To grow alfalfa seedlings under axenic conditions so that the effect of contaminant bacteria or fungi could be ruled out, plants were grown in paper cups containing soil that had been autoclaved in Mason jars. A paper cup (168 ml) with four drain holes at the bottom edge and containing 130 g of autoclaved U.C. mixed soil to which 145 ml of sterile distilled water was added, was placed in a 2-L Mason jar with a screw cap. The Mason jar containing a paper cup of soil, was autoclaved for 15 min on two successive days. One-hundred surface-sterilized alfalfa seeds were planted in each cup of soil. Seeds were surface sterilized with a solution containing 10% ethanol and 0.525% sodium hypochlorite for 5 min. After the seedling plants emerged the metal jar lid was replaced with the bottom of a sterile glass petri dish (9 cm) to allow light into the jar. Plants in each jar were incubated in the dark (18 C) for 12 hr and in the light (7.6 nanoeinstein $cm^{-2} sec^{-1}$, 24 C) for 12 hr. To provide saturated soil 3 days after planting, sterile distilled water was added to the jar so that the paper cup containing the soil was nearly submerged in water. To provide for normal watering the paper cup of soil was watered aseptically only from the top. Sterility was monitored by plating samples of soil, roots, and shoots on potato dextrose agar (PDA) and observed by scanning electron microscopy. The alfalfa seedlings grown under axenic conditions were inoculated with zoospores (10 ml at $2 \times 10^3$ zoospores per milliliter). Each Mason jar containing U.C. mix was considered to be a replicate.

**Chemotaxis.** After the alfalfa seedlings were grown in saturated and in unsaturated soil for various periods of time, the seedlings were removed carefully from the soil by immersing the roots and soil in water. A seedling from saturated soil and one from unsaturated soil was placed in a large petri dish (9 cm in diameter) containing 40 ml of a zoospore suspension ($4–6 \times 10^4$ zoospores per milliliter) for 45–60 min. Following migration of the zoospores to the roots, the width (at the widest point) of the mass of zoospore encystment on the root was measured. The experiment was repeated five times with six replications of each treatment.

**Scanning electron microscopy.** Roots, incubated in saturated and unsaturated soil under axenic and nonaxenic conditions, were fixed with 2% glutaraldehyde in phosphate buffer at pH 6.8 at 5 C overnight, washed with distilled water, and dehydrated by

TABLE 1. Reduction in weight of alfalfa plants inoculated with *Phytophthora megasperma* f. sp. *medicaginis* in soil saturated (S) or unsaturated (US) before inoculation[a]

| Parameter measured | Percentage of the uninoculated control[b] | |
| --- | --- | --- |
| | S | US |
| Shoot weight | 33 | 60 |
| Shoot height | 33 | 76 |
| Root weight | 12 | 72 |
| Tap root diameter[c] | 31 | 87 |

[a] Inoculum consisted of 50 ml of minced mycelium suspension (two 90-mm diameter plates of V8A blended in 1 L of water per pot) applied to each pot.
[b] The values for the presaturated and unsaturated controls were similar. The percentages are based on each uninoculated control.
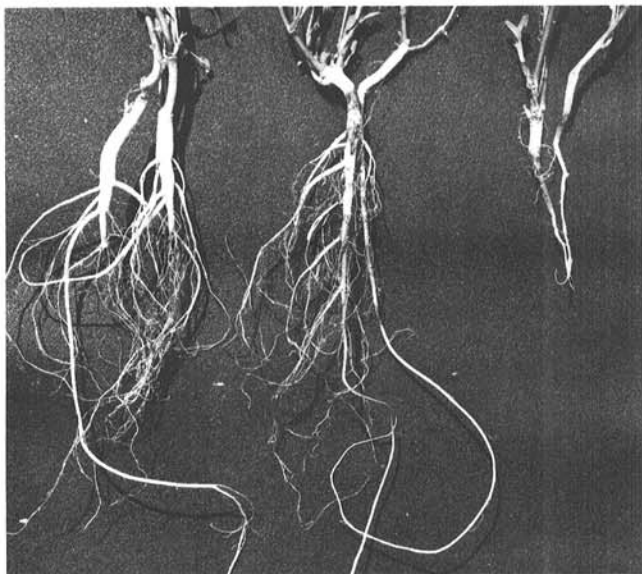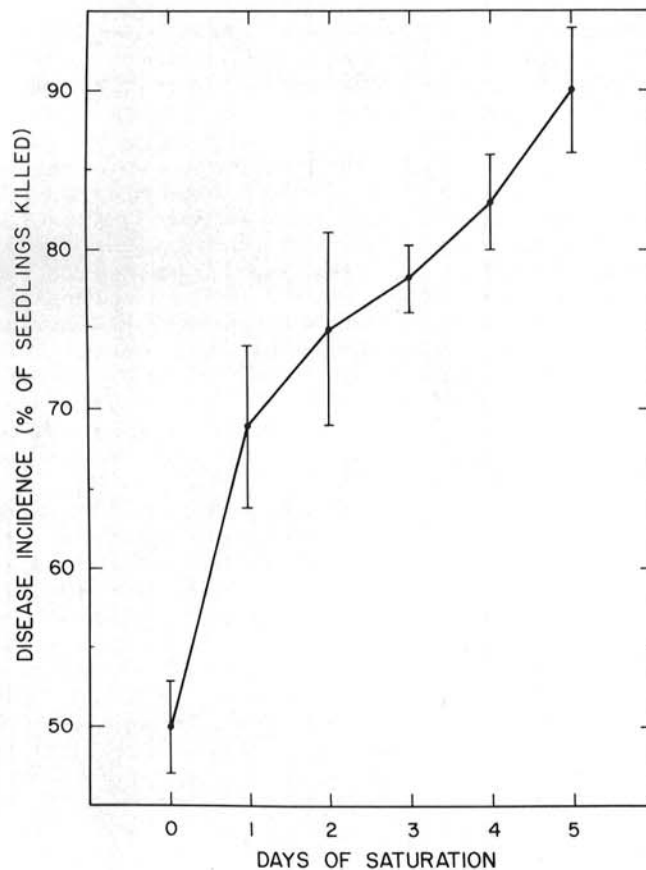[c] Diameter about 1 cm below the crown.



**Fig. 1.** Symptoms on alfalfa roots infected with *Phytophthora megasperma* f. sp. *medicaginis*. Left: uninoculated control; roots grown in unsaturated soil. Middle: inoculated roots grown in unsaturated soil prior to inoculation. Right: inoculated roots grown in soil saturated for 1 wk prior to inoculation with minced agar and mycelium of a 7-day-old culture on V8A. Uninoculated roots grown in unsaturated soil were identical in appearance to those in the control shown here.



**Fig. 2.** Incidence of Phytophthora root rot on alfalfa seedlings after different periods of time in saturated soil before inoculation with zoospores. Data were recorded 6 days later. Each point represents the mean of three replications and the bar represents the standard deviation.

transferring daily through increasing concentrations of glycerol (10, 20, 30, 50, 50, and 60%). The root tips were placed on "double stick" cellophane tape mounted on a scanning electron microscope (SEM) specimen holder, coated uniformly with gold vapor, and examined with a Jeolco Model JSM U3 scanning electron microscope.

Roots on which zoospores were to be observed 1 hr after being transferred from a zoospore suspension were fixed with 2% glutaraldehyde.

**Electrical conductivity and chemical analysis of root exudates.** Axenically grown alfalfa seedlings from water-saturated and unsaturated soil were gently removed from soil after submersion in water and washed. The roots of 400 seedlings were placed in a 100-ml beaker containing 20 ml of deionized distilled water, and the beakers were shaken on a reciprocal shaker (60 excursions per minute) for 1 hr at room temperature. Electrical conductivity of the root exudates was measured with a Serfass Conductance Bridge (Thomas). The experiment was repeated four times with three replications (400 seedlings composited from four paper cups of U.C. mix) of each treatment. The total amino acid content was measured by the ninhydrin test utilizing leucine as the standard

(29), and the sugar content by the anthrone method (3) utilizing glucose as the standard. The experiment was repeated three times with three replications of each treatment. Results were expressed as the percentage of the amount of amino acids or sugars contained in exudates from roots grown in saturated soil over those in exudates from roots grown in unsaturated soil.

## RESULTS

**Effect of preinoculation saturation of soil on severity of disease and yield.** Root rot was much more severe on plants grown in soil saturated with water prior to inoculation (disease index of 4.8) than on plants grown in unsaturated soil (disease index = 2.5) (Fig. 1). Yield of foliage also was reduced more in the soil saturated prior to inoculation than in nonsaturated soil (Table 1). The fresh weight of foliage from plants grown in presaturated soil was 33% that of the uninoculated control, but yield of plants from unsaturated soil was 60% of the uninoculated control. Shoot height, root weight, and diameter of tap roots were reduced more in saturated soil than in unsaturated soil (Table 1).

When seedlings in pots were inoculated with motile zoospores, more seedlings were killed in saturated soil prior to inoculation than in unsaturated soil. The percentage of seedling plants killed increased as the time of saturation increased (Fig. 2). Saturation of soil for 1 day resulted in 70% seedling mortality, whereas in unsaturated soil, only about 50% of the seedlings were killed. Similar results were obtained in the experiments in which the plants were grown axenically.

**Chemotaxis and germination of zoospores.** Roots of plants grown in saturated soil before inoculation attracted many more zoospores than did roots from plants in unsaturated soil (Fig. 3). Zoospores were chemotactically attracted preferentially to the zone of elongation and to the areas from which secondary roots emerged. Saturation for 1 day increased the width of the zoospore
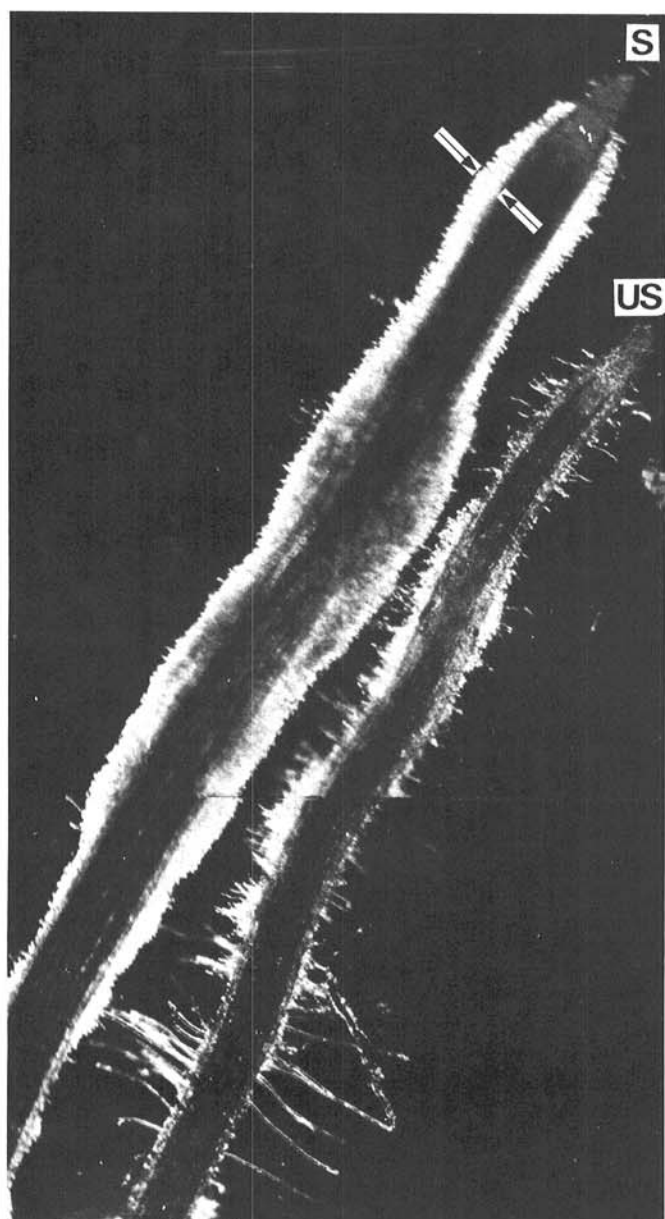


Fig. 3. Attraction of zoospores of *Phytophthora megasperma* f. sp. *medicaginis* to alfalfa roots grown in saturated soil (S) and in unsaturated soil (US). Area between arrows = zone of encysted zoospores.
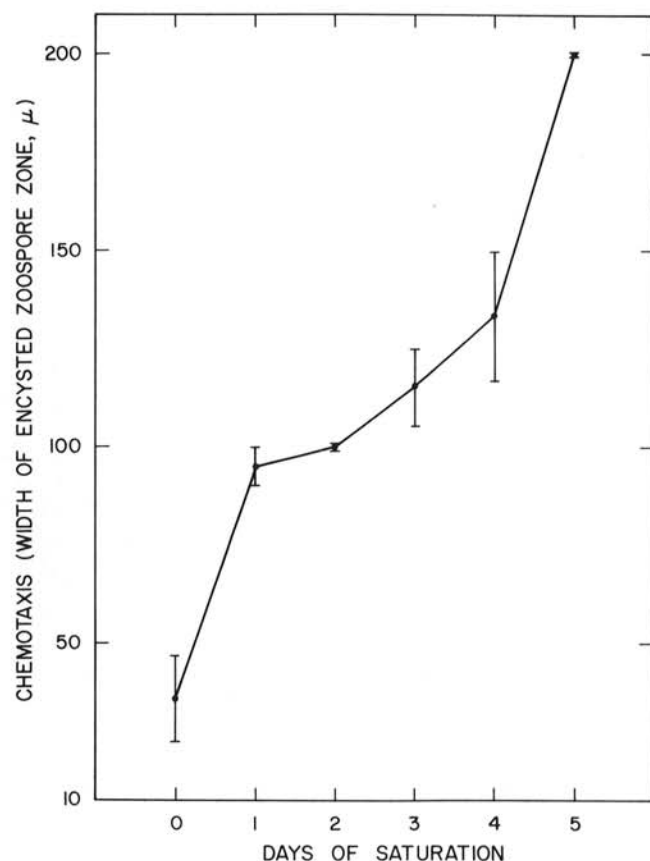


Fig. 4. Chemotaxis of zoospores of *Phytophthora megasperma* f. sp. *medicaginis* to alfalfa roots after different periods of time in saturated soil. Each point represents the mean of five replications and the bar represents the standard deviation.
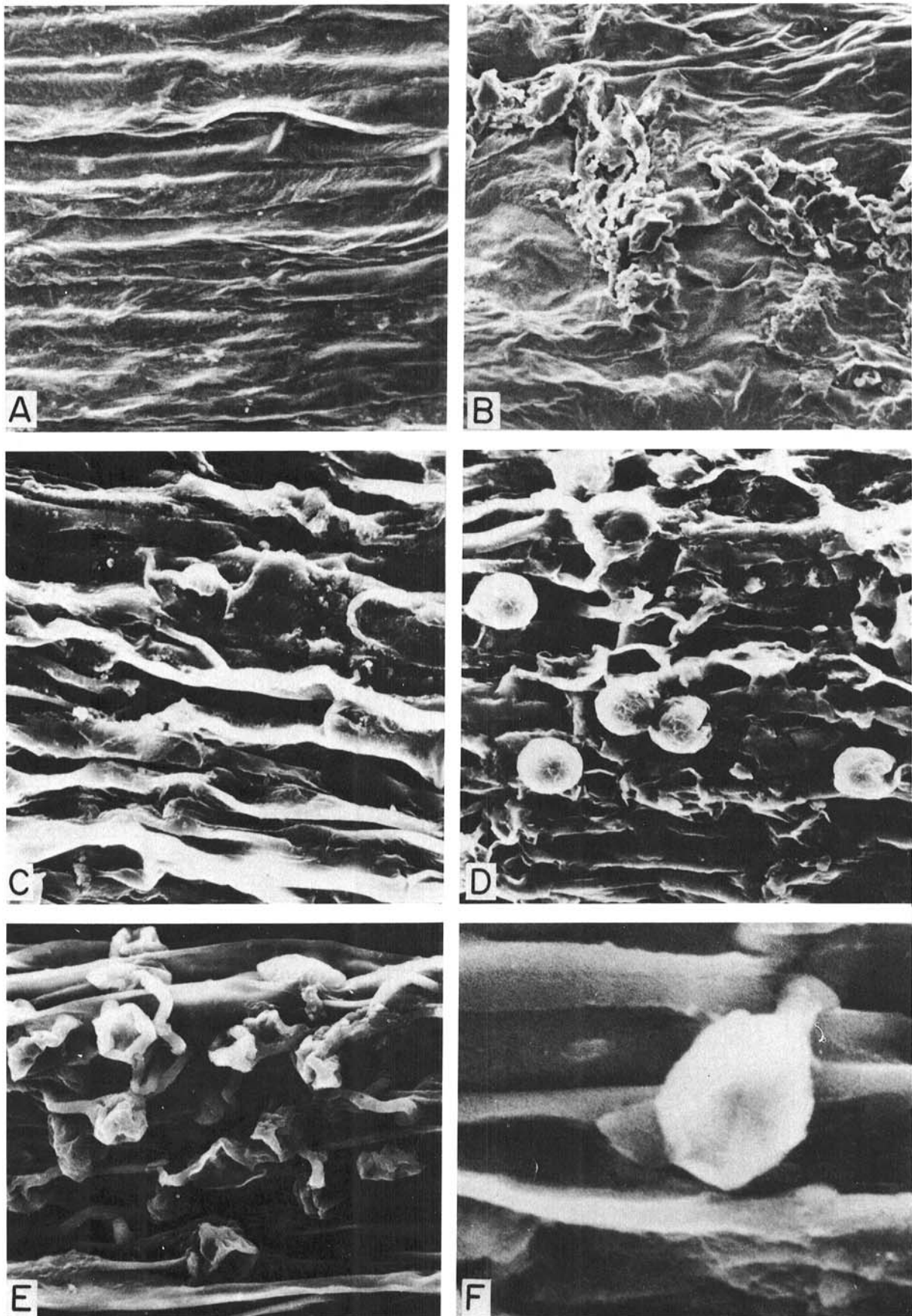
**Fig. 5.** Scanning electron micrographs of the surface of alfalfa roots. **A,** Alfalfa roots grown in unsaturated soil (× 1,000). **B,** Alfalfa roots grown in saturated soil for 1 day (× 1,000). **C,** Five days (× 1,000). **D,** Zoospores of *Phytophthora megasperma* f. sp. *medicaginis* penetrating a root from saturated soil (×1,000). **E,** Germinated zoospores on a root from saturated soil (× 1,000). **F,** Growth of the germinated zoospores into the break in the root surface (×3,000).

mass attracted to the root from 35 to 95 $\mu$m (Fig. 4). Similar results occurred in the experiment conducted under axenic conditions.

**Scanning electron microscopic observations of roots grown in saturated and nonsaturated soil.** Scanning electron microscopy revealed that breaks occurred on the surface of roots grown in saturated soil under axenic conditions, whereas the surface of roots from unsaturated soil remained smooth and intact (Fig. 5). Encysted zoospores germinated and the germ tubes tended to grow toward the breaks (Fig. 5F). When roots were grown in saturated soil exposed to aerial contamination, bacteria as well as germinated zoospores occupied the breaks on roots from saturated soil.

**Electrical conductivity and chemical analysis of root exudates.** The electrical conductivity of exudates collected from roots of plants incubated for 1 day in saturated soil was higher (22 $\mu$mhos/cm) than that of roots from unsaturated soil (15 $\mu$mhos/cm). As the period of saturation was increased to 5 days, the conductivity of the root exudate increased to a maximum of 30 $\mu$mhos/cm (Fig. 6).

The exudate from roots grown in saturated soils contained more sugars and amino acids than did that of roots from unsaturated soil (Fig. 7). After 1 day of saturation the amino acid content increased to 130% and after 7 days to 210% of the unsaturated control. The sugar content in exudates of roots grown in saturated soil for 1 day increased to 110% and after 7 days to 430% that of the unsaturated control.

## DISCUSSION

The results indicated that saturation of soil prior to inoculation predisposed roots of alfalfa to root rot caused by *P. megasperma* f. sp. *medicaginis*. In all experiments, the soil was saturated before inoculation; hence, the direct effect of saturation was not on the pathogen. Since the disease-increasing effect of soil saturation also occurred under axenic conditions, the effect of soil saturation must be on the physiology of roots rather than on soil microorganisms. According to Schoeneweiss (27), any factor which acts prior to infection and affects the susceptibility of the plants to disease is a predispositional factor. Results of this study demonstrate that saturation of soil, prior to infection, increases the susceptibility of alfalfa plants and thus it is a predispositional factor. Knous and Maxfield (21) previously presented the predisposition hypothesis and indicated that oxygen stress might be involved. This factor is probably one of the most likely to effect permeability of roots.

The finding by scanning electron microscopy that cracks developed in roots from saturated soil and an increased amount of amino acid and sugar exudates indicates that the root was predisposed. The predispositional effect of increased root exudates exerted a secondary chemotactic effect on zoospores. Thus this effect could be similar to that of increased inoculum concentration. Increased percentage of death of seedlings, pregrown in saturated soil and inoculated with motile zoospores (Fig. 2) supports the theory that the increased attraction of zoospores to roots grown in saturated soil accounts in part for the predisposition effect.

The tactic response of zoospores toward plant roots has been attributed to chemicals (5,18,32) of which amino acids may be the most important. Our results showed that roots grown in saturated soil exuded more amino acids than did roots grown in unsaturated soil (Fig. 7). It is reasonable to conclude that the increased amount of amino acids was responsible for the increased degree of chemotaxis observed.

Anaerobic conditions, caused by soil saturation, could lead to accumulation of ethanol which also has been demonstrated to be an attractant of zoospores (2,4). It is reasonable to suspect that ethanol, in addition to amino acids, might also have an effect on chemotaxis of zoospores of *P. megasperma* f. sp. *medicaginis* to alfalfa roots grown in saturated soil. The increased electrical conductivity of root exudates (Fig. 6), enhanced nutrient leakage (Fig. 7), and damage to the root surface (Fig. 5) suggested that membrane disruption had taken place during the saturation period. Kiyosawa (19) reported that ethanol exerted a "fluidizing" effect on the cell membrane and attacks the membrane constituents of the cells. In addition, squalene has been found to be increased in alfalfa

roots grown in waterlogged soil (21). An increase in squalene could affect directly or indirectly the plant steroids in cell membranes.

Erwin et al (17) reported that flooding soil at high temperature (40–42 C) caused injury to alfalfa roots and resulted in the physiological disease called "scald." However, in experiments reported here at day and night temperatures of 24 C and 18 C, respectively, the external symptoms on noninoculated alfalfa roots
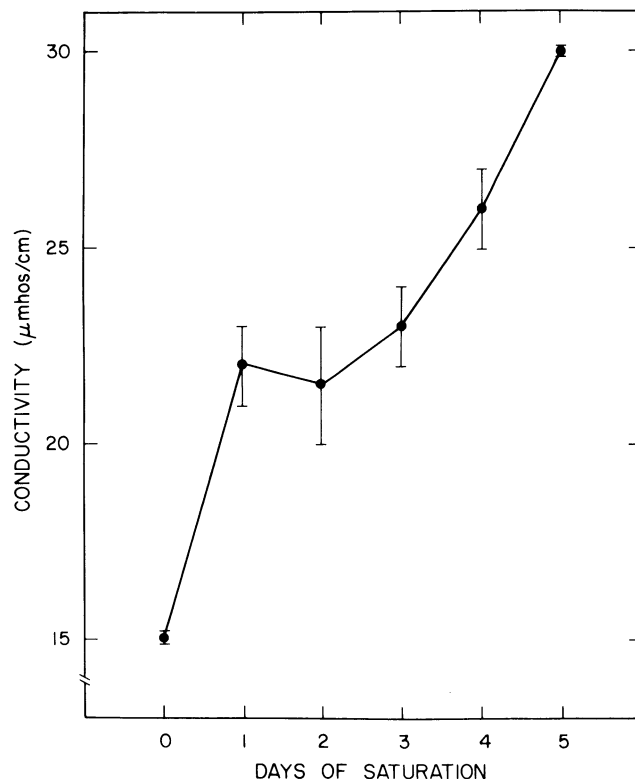


**Fig. 6.** Electrical conductivity of exudates from alfalfa roots grown in unsaturated and in saturated soil for different periods of time. Bars represent the standard deviation.
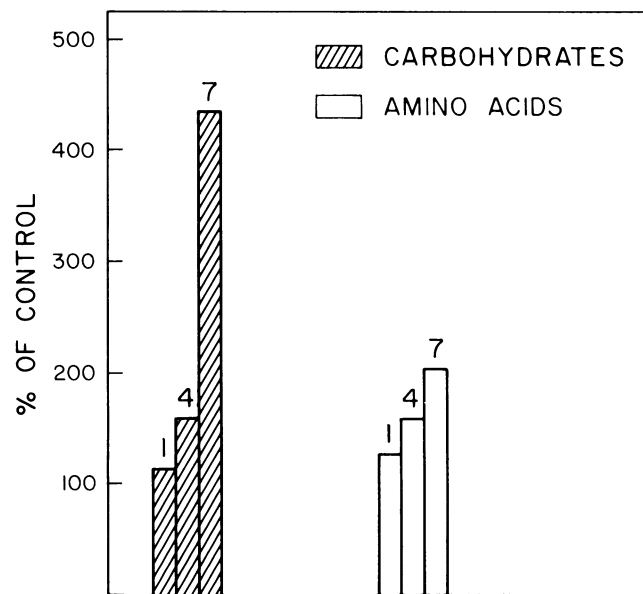


**Fig. 7.** Amino acid and sugar analyses of exudates from alfalfa roots grown in unsaturated soil and in soil saturated for 1, 4, and 7 days. Sugars were determined quantitatively by the anthrone test and amino acids by the ninhydrin test. The increase in quantity of sugars and amino acids is expressed as a percentage of the quantity from roots grown in unsaturated soil.

were not similar to "scald"; however, the root damage noted by scanning electron microscopy may be a milder version of the macroscopic symptoms noted on plants flooded at high soil temperature.

The tolerance (or resistance) of alfalfa roots to soil saturation might have a corresponding effect on their resistance to Phytophthora root rot. Alfalfa cultivar Moapa 69 used in this study is a susceptible cultivar. The comparative effect of saturation of soil on cultivars with different degrees of resistance should be studied. Tolerance to saturation of soil could be a useful parameter in breeding programs.

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