Protection of Douglas-Fir Seedlings Against Fusarium Root Rot by a Mycorrhizal Fungus in the Absence of Mycorrhiza Formation

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ABSTRACT

Douglas-fir seedlings inoculated in a controlled environment chamber with *Fusarium oxysporum* from a forest nursery developed root rot, had a high mortality rate, and grew poorly. When basidiospores of the mycorrhizal fungus *Laccaria laccata* were placed between seedling roots and inoculum of *F. oxysporum* in a mixture of soil, sand and perlite or vermiculite, less mortality and root rot occurred than in treatments receiving only *F. oxysporum* inoculum or *F. oxysporum* inoculum placed between roots and *L. laccata* spores. Mycelium of *L. laccata* prevented detrimental effects of *F. oxysporum* when placed in the soil mixture near seedling roots 1-3 weeks before introduction of *F. oxysporum*. Formation of mycorrhizae was not a prerequisite for the protective influence of the mycorrhizal fungus.

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Fusarium oxysporum Schlecht. causes root rot, stunting, and death of first-year Douglas-fir [*Pseudotsuga menziesii* (Mirb.) France] seedlings in nurseries in the Pacific Northwest (1). Severe disease depends upon inoculation during the first month of seedling growth, although symptoms may not be expressed until several weeks later (2).

As early as 1942, Davis et al. (3) suggested that ectomycorrhizae may protect feeder roots of nursery seedlings against pathogens. Since then, root protection by mycorrhizae or mycorrhizal fungi has been demonstrated in several cases (9). Douglas-fir seedlings begin to form ectomycorrhizae during the second or third month after germination. All seedlings are usually mycorrhizal by the end of the first growing season (19), and the number of ectomycorrhizae per unit of root weight increases rapidly during the forepart of the second season (12). Common mycorrhizal fungi in Douglas-fir nursery beds include *Laccaria laccata* (Scop. ex Fr.) Berk. & Br., and *Inocybe lacera* (Fr.) Kummer (18).

The purpose of this study was to determine whether associations between Douglas-fir seedlings and ectomycorrhizal fungi could protect the seedlings against Fusarium root rot before the normal onset of mycorrhiza formation. A preliminary report has appeared (14).

MATERIALS AND METHODS.—*Fungus isolates.*—One isolate of *F. oxysporum* (T801), obtained in 1970 from a necrotic Douglas fir seedling root, was used. In preliminary pathogenicity tests that included isolates of *F. oxysporum* known to cause root rot and "corky root" (1), isolate T801 behaved as the "corky root" isolates. Single isolates of *L. laccata* (T816) and *I. lacera* (T831) were used. These were obtained from sporophores in 1970, and were shown to be capable of forming ectomycorrhizae with Douglas-fir seedlings (W. A. Sinclair and A. O. Larsen, *unpublished*). All fungi were maintained on modified Melin-Norkrans (MMN) agar (8). In paired-culture tests on this medium, neither of the mycorrhizal fungi inhibited the growth of *F. oxysporum*.

Inocula.—Grain spawn of *F. oxysporum* was prepared by autoclaving moistened wheat grain for 2 hours and infesting this with plugs from agar cultures (10). After allowing 3 weeks for colonization by *F. oxysporum*, the grain was dried slowly and stored in closed, but not airtight, jars until used.

Inocula of *L. laccata* and *I. lacera* were produced by growing these fungi in a substrate mixture containing 165 cm^3 shredded peat moss and 335 cm^3 vermiculite moistened with 250 ml of MMN solution in 950-ml jars. This medium was autoclaved for 2 hours on each of two successive days. Plugs from agar cultures of *L. laccata* or *I. lacera* were then added. The fungi colonized the peatlite within 1-2 months, and could be recovered for up to 10 months if bits of substrate were transferred to MMN agar.

Basidiospores of *L. laccata* were collected, preserved by lyophilization, and rehydrated as previously described (15). Inoculum was prepared by mixing one volume of rehydrated basidiospores with 20 volumes of talc (USP) and mixing this in turn with 10 volumes of fine white sand. The mixture was used immediately.

Seed treatment and soil preparation.—Seeds of Douglas-fir were surface-sterilized in 30% hydrogen peroxide for 90 minutes (17) and germinated on sterilized filter paper in petri dishes at 22 C until radicles were 1-2 cm long. They were planted in soil mixtures consisting of equal parts of loam topsoil, sand, and either vermiculite or perlite. Mixtures were either autoclaved 4 hours or pasteurized at 75 C for 1 hour; they were then stored in closed, but not airtight, containers until used.

Growing conditions.—Seedlings were grown in a filtered-air chamber having transparent vinyl plastic walls, within a controlled environment (CE) chamber. Air was forced through the filtered-air chamber after passing

sequentially through two electrostatic precipitators (Honeywell F46A-Y503B Electronic Air Cleaner) and an activated charcoal filter. The filtered-air chamber was maintained at slightly greater air pressure than the ambient in the CE chamber. This arrangement allowed control of light, temperature and relative humidity (RH) in an open growing area while excluding spores of mycorrhiza-forming fungi. The following conditions were maintained at plant level within the chamber: a 14-hour photoperiod (21 klx of warm white fluorescent light supplemented by incandescent light); 24 C and 68% RH, alternating with a 10-hour dark period at 18 C and 78% RH.

Seedlings were grown either in drainable plastic pans or in BC-CFS Styroblocks which are molded styrofoam blocks 35 × 51 × 18 cm having 80 planting holes 4 cm in diameter and of 120 ml capacity. The holes are grouped in four sets of 20, each set surrounded by a 1 cm rim. The Styroblock was developed for growing seedlings of forest trees (13). One seedling was planted in each hole.

RESULTS.—*Seedling growth and root form as influenced by timing of inoculation with F. oxysporum and presence of mycorrhizal fungi.*—A factorial experiment was done to learn how the timing of inoculation with *F. oxysporum* affected disease development, and whether inoculation with *L. laccata* or *I. lacera* could prevent or suppress the disease. Peat-lite inocula of the mycorrhizal fungi were either omitted (control) or added to synthetic soil mixture in Styroblocks at the time of planting (20 germinated seeds per treatment or control). Grain spawn inoculum of *F. oxysporum* was either omitted (control) or applied at 1, 3, 6, or 11 weeks after planting. For each treatment, about 0.5 cm^3 of the appropriate inoculum was placed at one side of the planting hole about 4 cm below the soil surface. Where both mycorrhizal and *F. oxysporum* inocula were used, they were placed on opposite sides of the hole. The seedlings were fertilized biweekly with a dilute solution of complete fertilizer. After 20 weeks, the mean numbers of living seedlings in the various treatments ranged from 11 to 19. Those with healthy appearing shoots were counted; then all seedlings were removed from the containers and analyzed for root form, presence of mycorrhizae, and dry weight. Isolations on MMN agar were made from a few typically symptomatic roots where *F. oxysporum* had been inoculated.

Weight gain by the seedlings was suppressed significantly if *F. oxysporum* was added at 1 or 3 weeks, but not if it was added at 6 or 11 weeks. When *L. laccata* was present, *F. oxysporum* did not reduce the growth of the seedlings (Table 1). No consistent protection was afforded by *I. lacera*.

Much variation in seedling weight occurred within treatments. Coefficients of variation (CV) for seedling weight were generally greater in treatments where seedling growth was suppressed, than where it was not (Fig. 1). This correlation between mean dry weight and CV for weight ($r = 0.91$, $P = 0.01$) reflected severe growth suppression in some seedlings, while others in the same treatments grew at rates comparable to the controls.

The greatest reductions in number of seedlings with healthy tops occurred where *F. oxysporum* was introduced after planting (Table 1). The proportions of

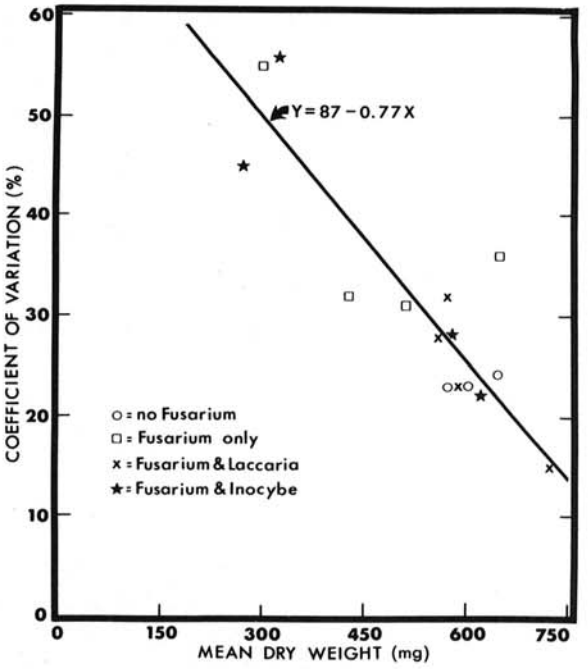

Fig. 1. Regression of coefficient of variation for weight on mean dry weight of 20-week-old Douglas-fir seedlings grown in Styroblocks.

TABLE 1. Dry weights, aerial symptoms, and root form scores for 20-week-old Douglas-fir seedlings noninoculated, or inoculated at planting time with mycorrhizal fungi, and at subsequent intervals with *Fusarium oxysporum*.

Fusarium oxysporum inoculation (weeks after planting)	Mycorrhizal inoculum: None			*Laccaria laccata*			*Inocybe lacera*		
	Dry wt. (mg)[a]	Healthy tops (%)[b]	Root form[c]	Dry wt. (mg)[a]	Healthy tops (%)[b]	Root form[c]	Dry wt. (mg)[a]	Healthy tops (%)[b]	Root form[c]
No inoculation	641 vw	100	4.2	579 w	100	4.5[d]	601 vw	93	4.3
1	427 xy	93	3.5	569 w	95	4.3	315 yz	73	2.7
3	307 yz	78	3.1	547 wx	86	4.0	552 wx	94	4.2
6	647 v	94	4.1	592 vw	100	4.4	256 z	80	3.2
11	508 wx	89	4.3	722 v	100	4.6[d]	621 v	100	4.4

[a]Means having any letter in common are not significantly different at $P = 0.05$.
[b]Number appearing healthy at end of experiment per total seedlings at time of inoculation with *F. oxysporum* × 100.
[c]Mean score where 1 = diseased, severely stunted and 5 = healthy, much branched and dense.
[d]Some trees in these treatments had fully formed ectomycorrhizae.

seedlings remaining healthy were significantly correlated with mean dry weights ($r = 0.84$, $P = 0.01$).

The root system of each seedling was scored 1 (stunted) to 5 (large, branched, and dense). Seedlings inoculated with *F. oxysporum* alone 1 or 3 weeks after planting had lower mean scores for root form than those inoculated 6 or 11 weeks after planting. If *L. laccata* was present, root development was not suppressed (Table 1). Six seedlings in the *Laccaria*-only treatment and two that received *Laccaria* plus *Fusarium* at 11 weeks had fully formed ectomycorrhizae. Other than these, no mycorrhizae were found. *I. lacera* had no consistent influence on root form, and formed no mycorrhizae. *F. oxysporum* was consistently isolated from the selected root pieces. Attempts to reisolate the mycorrhiza-forming fungi were not successful.

Survival of Douglas-fir seedlings in relation to placement of inoculum.—The possibility of root protection early in the life of the seedlings, before mycorrhizae could form, was tested in an experiment similar to that of Hyppel (4). A layer (7-cm thick) of pasteurized soil mixture was placed in plastic pans. This layer was followed by layers of inoculum, soil mixture (3-cm thick), inoculum, and soil mixture (3-cm thick). *F. oxysporum* inoculum was applied as grain spawn at a rate of three grains per cm^2. *L. laccata* inoculum was applied as basidiospore-talc-sand mixture at a rate of 2×10^4 spores per cm^2. Thus, the pans contained soil mixture approximately 13 cm thick with layers of inoculum 3 and 6 cm below the soil mixture surface. Control pans were identical, except that inoculum layers were ommitted. Treatment pans contained soil mixture infested with *F. oxysporum* alone at upper (F/O) or lower (O/F) position, *L. laccata* alone at upper (M/O) or lower (O/M) position, *F. oxysporum* above *L. laccata* (F/M) and *L. laccata* above *F. oxysporum* (M/F).

Twenty-four germinated seeds were planted in each pan; there were three replicate pans per treatment or control. The numbers of living and of healthy seedlings in each pan were recorded at intervals for 60 days, at which time the trees in two replicates were removed from the pans and the roots were examined. Seedlings in the remaining replicate were allowed to grow for 3 months more, then were examined for ectomycorrhizae.

Seedlings died rapidly when inoculum of *F. oxysporum* occupied the upper position; 80% were dead 16 days after planting. This occurred whether or not *L. laccata* was present in the lower position. When inoculum of *F. oxysporum* was in the lower position with no inoculum in the upper position, death was delayed; cumulative mortality 21 days after planting was about 60%, equivalent to that caused by *F. oxysporum* in the upper position after 10 days. Even after 60 days cumulative mortality did not reach 80% (Fig. 2).

When only *L. laccata* was present in either position the rate of seedling mortality was not different from the

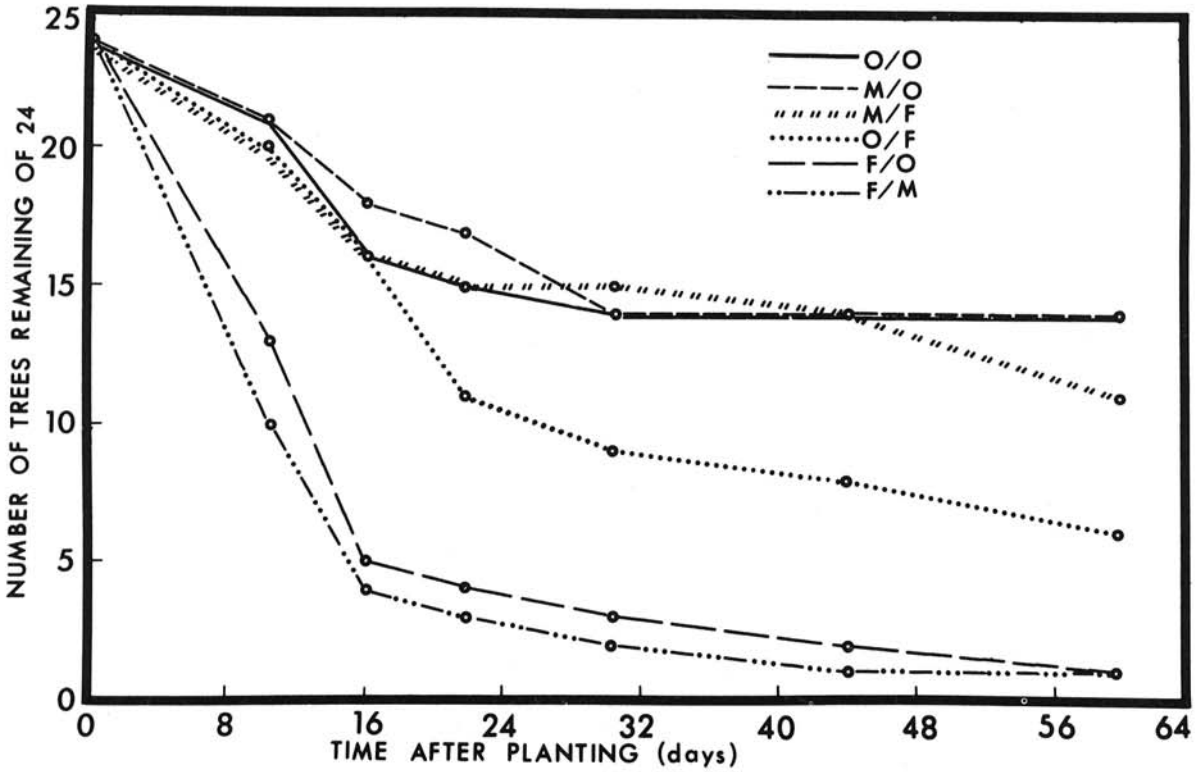

Fig. 2. Cumulative mortality of Douglas-fir seedlings grown in a soil mixture noninfested or infested with *Fusarium oxysporum* and/or *Laccaria laccata*; each point represents the mean of three replicates. The soil mixture was infested at 3 and/or 6 cm below the surface, as indicated by numerators and denominators, respectively, in the codes; O = no inoculum; M = *L. laccata*; F = *F. oxysporum*. Data from the treatment O/M are not included here; it gave the same result as M/O.

noninfested control. When *L. laccata* was present above *F. oxysporum* (M/F), mortality was the same as the noninfested control (O/O); when *F. oxysporum* was alone in the lower position (O/F), significantly more mortality occurred. This pattern continued for the first seven weeks. Observations 60 days after planting, however, showed increasing mortality in the M/F treatment (Fig. 2).

Differences between treatments in the number of seedlings judged to be healthy were similar to those for mortality. At 21 days after planting, for example, the mean numbers of healthy seedlings per replicate in treatments F/O, O/F, M/F, and O/O were 0.3, 5.0, 11.7 and 11.7, respectively; all differences were significant, $P = 0.05$. The number of healthy seedlings 21 days after planting approximated the number surviving at 60 days.

Representative seedlings from four pertinent treatments (O/O, O/F, M/O, M/F) 60 days after planting are shown in Fig. 3. The roots of seedlings in the *F. oxysporum*-only treatment were stunted. Roots in the other treatments appeared healthy. No mycorrhizae were seen, nor did mycorrhizae form in the remaining replicate after an additional 3 months.

DISCUSSION.—Our results confirmed the findings of Tint (16) and Bloomberg (2) that *F. oxysporum* causes the most severe disease when introduced early in the life of a seedling. The symptoms observed in tops and roots of plants grown and inoculated in a growth chamber were similar to those caused by *F. oxysporum* in the field. The strong correlations between dry weights and measures of seedling health and root condition indicate that the pathogen was also responsible for the growth reductions observed.

Some symptomatic seedlings were found in all *F. oxysporum*-inoculated treatments in Styroblocks, but there were fewer when *L. laccata* was also present in the soil mixture. In view of the variability of mycorrhiza formation in Douglas-fir seedlings (12), it is not surprising to find variability in degree of root protection by a mycorrhizal fungus as well, particularly in these experiments where so few mycorrhizae formed.

The high coefficients of variation (CV) associated with *F. oxysporum*-inoculated treatments (Fig. 2) suggest that the pathogen may have different effects on different seedlings; the increases in CV associated with weight accretion in field-grown seedlings (11) may be due to such

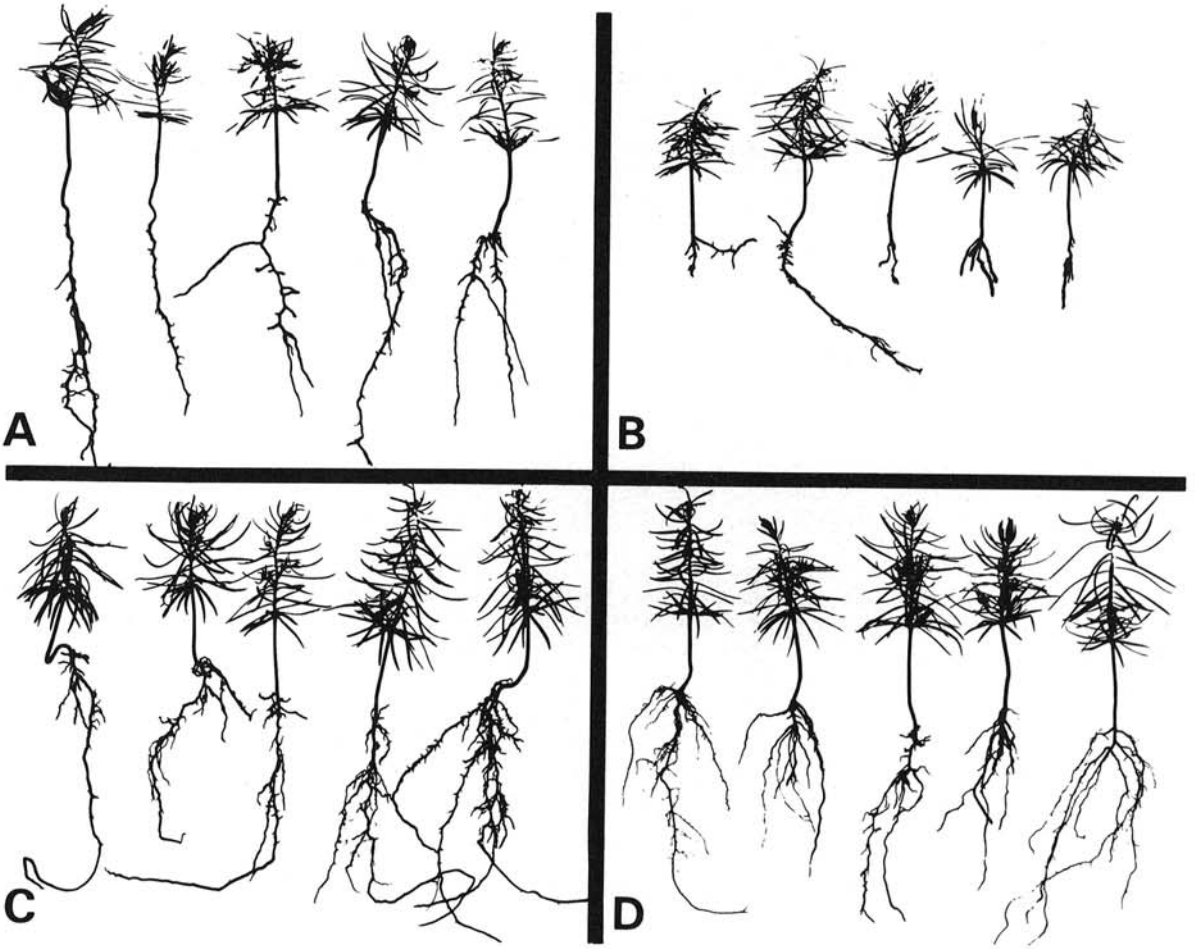

Fig. 3-(A to D). Representative seedlings of Douglas-fir 60 days after planting in a soil mixture infested at two depths with inoculum of *Fusarium oxysporum* and/or *Laccaria laccata*. **A)** Noninoculated control; **B)** *F. oxysporum* alone 6 cm deep; **C)** *L. laccata* alone 3 cm deep; **D)** *L. laccata* at 3 cm deep and *F. oxysporum* at 6 cm deep.

differential pathogenesis. The high CV's may also be a consequence of phenotypic variability in growth rate; slow-growing seedlings may be more susceptible to the pathogen than vigorous ones.

Our finding of root protection where *L. laccata* was introduced in Styroblocks prompted the "layer" experiment. Hyppel (4), in a similar experiment, showed protection of spruce seedlings up to 4 weeks of age by *Boletus bovinus* against *Fomes annosus*. The protective influence occurred before any mycorrhizae formed. A critical difference between Hyppel's system and ours was that *Boletus bovinus* is fungistatic to *F. annosus* in culture (5), while *L. laccata* is not antagonistic to *F. oxysporum* in culture.

The "layer" experiment confirmed that a protective influence of *L. laccata* on roots of Douglas-fir seedlings was not dependent on mycorrhizae. The roots of seedlings too young for mycorrhiza formation were protected by placing spores of *L. laccata* between suscept and pathogen. Moreover, no mycorrhizae formed after protection was evident.

The mechanism of protection is unknown. However, of the mechanisms for root protection by mycorrhizal fungi proposed by Zak (20) and Marx (8), that involving a physical barrier (the intact mantle) can be eliminated since protection was observed in the absence of a mantle. Antibiosis is unlikely since *L. laccata* showed no antagonistic influence on *F. oxysporum* in our preliminary paired culture tests or in previously reported tests (8). Protective effects of a rhizosphere population either antagonistic to or competitive with, *F. oxysporum* and stimulated by *L. laccata* are possible since the experiments were done in a soil-containing mixture that supported a rich microflora as shown by plating soil on various media.

An *L. laccata*-induced alteration in the suscept-pathogen interaction is another possible explanation for root protection. Many root pathogens are stimulated by suscept root exudates. Rapid colonization of seedling roots by *L. laccata* might reduce the amounts of stimulatory substances reaching propagules of *F. oxysporum* in the soil. Also, some mycorrhizae contain or secrete higher levels of volatiles and extractives which are inhibitory to pathogens than do nonmycorrhizal roots (6, 7). It would be interesting to know if roots under protective influence of *L. laccata*, especially before mycorrhiza formation, contain or secrete more fungistatic substances than roots not associated with a mycorrhiza-forming fungus. Experiments should be done to determine if prior exposure of roots to *L. laccata* or prior formation of ectomycorrhizae affect the ability of root exudates to influence growth of *F. oxysporum*.

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