Prevention of Soybean Root Nodulation by Tetracycline and Its Effect on Soybean Root Rot Caused by an Alfalfa Strain of Fusarium oxysporum

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ABSTRACT

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Tetracycline at or above 40 μg/ml was shown to be bactericidal to *Rhizobium japonicum*. In contrast, colony growth of *Fusarium oxysporum* from alfalfa was little affected by tetracycline at concentrations as high as 160 μg/ml. Soybean plants grown in soil containing *R. japonicum* and *F. oxysporum* were watered twice daily with 250 ml of Hoagland's solution containing tetracycline at 160 μg/ml of solution. Soybeans grown in the tetracycline-treated soil were not nodulated and were only minimally affected by the drug treatment. Even in the absence of nodulation, soybeans were infected with *F. oxysporum*; Fusarium root rot was more severe in the plants grown in the tetracycline-treated soil than in those grown in the non-treated soil. The results confirmed previous observations that inoculation with rhizobia reduces the severity of root rot in soybeans and that infection of soybean roots by *F. oxysporum* may develop independent of root nodulation.

Aside from the well-known role of inducing nodulation in legumes, rhizobia recently have been shown to interact with root rot fungi. Chou and Schmitthenner (3), using soybeans and *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb., showed a higher incidence of Phytophthora root rot in steamed soil than in soil containing *Rhizobium japonicum* (Kirchner) Buchanan or *Endogone mosseae*. Their findings are supported by the recent reports of Tu (14, 15) using soybean [*Glycine max* (L.) Merr. 'Amsoy'] plants and several root rot fungi including *Phytophthora megasperma*. These results show that the extent of experimentally-induced root rot in soybean plants was inversely proportional to the amount of rhizobia (*Rhizobium japonicum*) in the soil. In addition, experiments showed that rhizobia (i) grow around the hyphal tips of *P. megasperma*, (ii) occur inside the hyphae as parasites, and (iii) reduce fungal sporulation (15).

Gray and Hine (6) showed that early root infection by *P. megasperma* was associated with rhizobial root nodules in alfalfa in the field, and suggested that nodules may serve as early infection sites for the fungus. Orellana and Worley (11) demonstrated that although the hyphae of the root rot fungus, *Rhizoctonia solani*, penetrated both inter- and intracellularly into young nodules on soybean roots, penetration was restricted to the outer cortex of the nodules. Further spread of the fungal hyphae appeared to be impeded by a layer of thick-walled sclerenchymatous cells. The interaction of rhizobia and root rot fungi appears to be rather complex and needs further investigation.

In experiments in which soybeans were grown in soil to which rhizobia were not added, one cannot be certain that the soil was free of rhizobia. Seeds also may carry rhizobia. Such contamination could have contributed to the root infection in the work of Chou and Schmitthenner (3), and Tu (14), if root nodules serve as early infection sites.

Tetracycline was selected to obtain a rhizobia-free system. It is a wide-spectrum bacteriostat (and in high concentrations, bactericidal) which inhibits protein synthesis in both Gram-positive and Gram-negative bacteria (4, 5, 9). The effects of tetracycline on plant pathogenic fungi are varied (12) and depend on the species studied. An experimentally determined concentration of tetracycline, known to be bactericidal to rhizobia, was applied to potted soil artificially infected with *F. oxysporum* Schlecht. and *R. japonicum*. *Fusarium oxysporum* was selected for these experiments because it is resistant to high concentrations of tetracycline.

The present experiments were an attempt to study the interaction of rhizobia and the soybean root rot fungus, *Fusarium oxysporum*, under conditions in which the plants and soil are completely free of indigenous rhizobia and to assess whether root nodulation is a prerequisite for infection by *F. oxysporum*.

MATERIALS AND METHODS

Effective tetracycline concentration for suppression of Rhizobium japonicum.—The following procedure in vitro was used: *R. japonicum* was grown in liquid yeast mannitol (YM) medium (7) until the optical density (OD) at wavelength 660 nm (OD_{660nm}) reached 0.10 determined

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in a Beckman Spectronic-20 colorimeter. One ml of this suspension was added to 60 ml of YM medium in a 150-ml flask. The medium contained 0, 2.5, 5, 10, 20, 40, or 80 μg/ml tetracycline. After inoculation, the flasks were incubated in a 22 ± 1 C Gyrotory water-bath shaker (New Brunswick Instruments, New Brunswick, NJ 08903) for 24 hr. The growth rate of *R. japonicum* was assessed by the change in OD. All cultures containing less than 40 μg/ml of tetracycline were diluted 10× for OD measurements. Cultures containing more than 40 μg/ml of tetracycline were measured for OD without dilution because no apparent rhizobial growth was evident and the cultures were nearly transparent. The effective tetracycline concentration was taken at the concentration in which no rhizobial growth was apparent.

Selection of a tetracycline-tolerant fungus for interaction studies.—Fungi differ in tolerance to antibiotics (12). For these studies, a root rot fungus with a high tolerance to tetracycline was desirable. Four fungi, *Phytophthora megasperma*, *Pythium ultimum* Trow, *Fusarium oxysporum*, and *Ascochyta imperfecta* Pk., were screened for their resistance to tetracycline. The first two fungi are known to cause root rot in soybean plants (2), whereas *A. imperfecta* causes foot rot or base rot in alfalfa (15). The *F. oxysporum* used in this study was initially isolated from a rotted root of alfalfa collected near Hinton, Alberta, and was proven to be pathogenic to soybean according to Koch's postulates. *Fusarium oxysporum* is known to cause root rot of soybean (13).

Each fungus was grown on potato-dextrose agar (PDA) plates containing nine different concentrations of tetracycline (i.e., 0, 10, 20, 40, 60, 80, 100, 120, and 160 μg/ml). The extent of growth was assessed by measuring colony diameters daily for 7 days. *Fusarium oxysporum* was chosen for the interaction study, in part, because of its high resistance to tetracycline demonstrated in this study. Detailed results are given below.

Effect of tetracycline on interaction of Rhizobium japonicum and Fusarium oxysporum.—*Rhizobium japonicum*, *F. oxysporum*, and soybean were used throughout this interaction study. The rhizobial inoculum was obtained from Nitragin Sales Corporation, Milwaukee, WI 53209.

The interaction study was conducted in a soil mixture consisting of sand, loam, and peat (4:2:1, v/v). The soil mixture was steamed twice, 2 days apart, for 6 hr each time. The pH of the soil mixture was between 6.9 to 7.2 in 10 random samples.

The fungal inoculum was propagated in sand and oatmeal media (sand, 370 g; oatmeal, 10 g; and 80 ml H_2O) for 1 mo. The culture was shaken three times a week to loosen the clump.

Amsoy soybean seeds were surface-sterilized in 5% sodium hypochlorite for 5 min, rinsed with 70% ethanol, and then rinsed with sterilized water. The seeds were germinated in petri plates on wetted filter paper. After germination they were planted in 5-cm-diameter pots filled with a perlite-vermiculite mixture. These plants were watered with Hoagland's solution (8) twice daily, and were maintained in a controlled environment at 21 ± 1 C with a 12-hr light period. The light was supplied by cool-bean fluorescent tubes with an intensity of 25,000 lux at bench level. Seedlings in the unifoliate stage were transplanted (two seedlings/pot) to 20-cm-diameter pots containing soil mixtures detailed below.

The levels of infestation with *F. oxysporum* were prepared, one with 1:10 and the other with 1:100 fungal inoculum:soil (v/v). Four soil samples (5 g each) were taken from each of the two infested soil mixtures and from the noninfested soil mixture. These samples were plated according to the method of Nash and Snyder (10). Numbers of propagules of *F. oxysporum* per gram of soil averaged 9,680, 1,050, and 12 in soil with 1:10, 1:100, and zero in mixtures of soil and the prepared inoculum.

Sixteen pots each were prepared from each soil mixture. To half of each of these groups of pots, rhizobia were added at a final concentration of 10^4 rhizobia/cm^3 of potted soil. Thus, six subgroups of eight pots each were formed. The subgroups were halved again, one set of four pots was watered with Hoagland's solution containing 160 μg/ml tetracycline, the other four pots received Hoagland's solution alone (Table 1). The plants were watered (250 ml/pot) twice a day at 0800 hours and 1700 hours. All plants were kept in the same greenhouse previously described.

Four wk later, the root system of each of the plants was soaked in a tank of water, rinsed, and wiped dry with several layers of paper towels. The roots were examined for root rot and scored on a scale that ranged from zero (no root rot) to 9 (very severe). Root nodules were counted, and fresh weights of whole plants were recorded.

RESULTS

Effective tetracycline concentration for controlling growth of Rhizobium japonicum.—Rhizobial growth

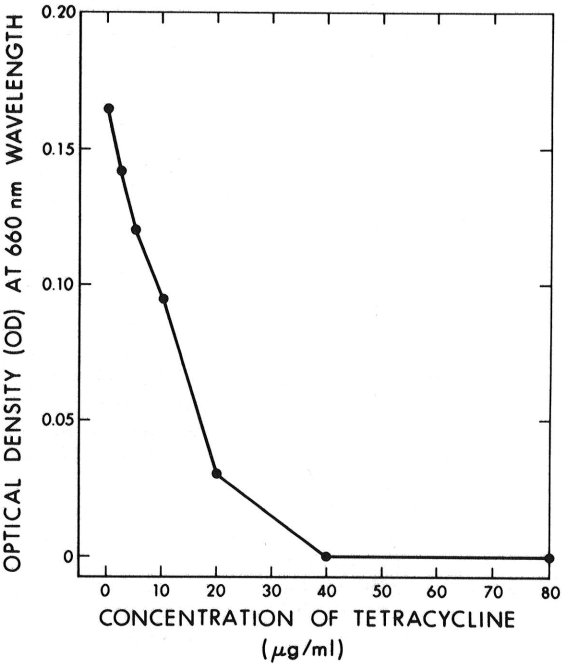

Fig. 1. Growth of *Rhizobium japonicum* in liquid yeast-mannitol medium (YM) containing a range of concentrations of tetracycline. Readings were taken after 24 hr of incubation on 10× diluted or nondiluted suspension of *R. japonicum* prepared from YM cultures incubated to $OD_{660nm} = 0.10$ (Beckman Spectronic 20 spectrophotometer).

declined as the concentration of tetracycline increased (Fig. 1) and was prevented with tetracycline above 40 or 80 μg/ml, as judged by the lack of OD change even in nondiluted rhizobial cultures. Forty μg/ml was taken as the effective concentration for prevention of growth of *R. japonicum*.

Selection of a suitable root rot fungus for the interaction study.—*Phytophthora megasperma* and *P. ultimum* had little tolerance to tetracycline, since no growth was observed for these fungi on PDA with a tetracycline concentration higher than 20 μg/ml and 40 μg/ml, respectively (Fig. 2). In contrast, *Fusarium oxysporum* and *A. imperfecta* were little affected by tetracycline even at concentrations as high as 160 μg/ml (the upper limit of these tests) (Fig. 2).

Effect of tetracycline application on soybean plants.—Soybean plants watered with Hoagland's solution containing 160 μg/ml of tetracycline showed a slight general yellowing of the leaves which was more apparent on younger leaves. In addition, a few yellowish spots approximately 0.5 to 0.7 cm in diameter developed on some of the leaves. The fresh weights of plants grown in soil without fungal inoculum and with or without rhizobia, but watered with tetracycline, were only slightly less than comparable plants watered with Hoagland's solution alone. This suggests that tetracycline did not have a major effect on the growth rate of the soybeans (Table 1). Necrotic lesions and leaf burns were not observed. These observations are in accord with the findings of Barton and MacNab (1), who found that oxytetracycline (500 μg/ml) causes a slight chlorosis in cabbage, tomato, carrot, and snapdragon plants.

Effect of tetracycline on nodulation and Fusarium root rot.—The plants watered with Hoagland's solution containing tetracycline produced no root nodules, including pots to which *R. japonicum* was added. Plants grown in soil without addition of *R. japonicum* and watered with tetracycline-free Hoagland's solution produced a few nodules and the number of root nodules per plant ranged from zero to eight. These results show

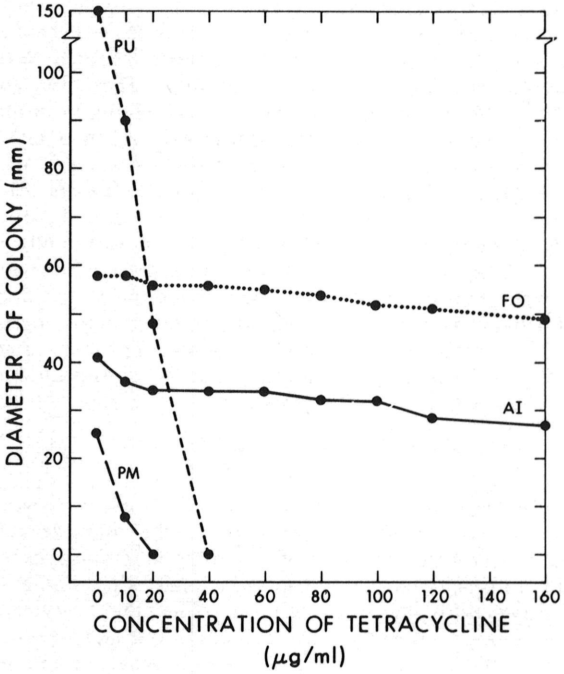

Fig. 2. Growth (after 7 days) of *Fusarium oxysporum* (FO), *Ascochyta imperfecta* (AI), *Phytophthora megasperma* (PM), and *Pythium ultimum* (PU) in potato-dextrose agar containing a range of concentrations of tetracycline.

TABLE 1. Effect of tetracycline on the interaction of soybean plants, *Fusarium oxysporum*, and *Rhizobium japonicum* 4 wk after transplanting[a]

Observed parameters	Initial Rhizobium population in soil (CFU/cm³)	Parameters observed for plants watered with Hoagland's solution and grown in soil artificially infested with *F. oxysporum* (CFU/gram of soil)[b]					
		Hoagland's + tetracycline[c]			Hoagland's only		
		9680 CFU	1050 CFU	12 CFU	9680 CFU	1050 CFU	12 CFU
Root rot severity index[d]	0	8.5	5.0	0.2	8.5	4.5	0.2
	10⁴	8.5	5.5	0.2	6.2	2.5	0.2
Rhizobium nodules per plant	0	0	0	0	2	3	6
	10⁴	0	0	0	17	48	136
Whole-plant weight (g)	0	5.5	7.8	11.2	5.7	9.7	12.3
	10⁴	5.4	7.3	11.5	8.2	10.4	12.9

[a] Plant age at transplanting was 10 days.

[b] Describe inoculum culturing and give 1/10, 1/100, and 0 mixtures that resulted in 9,680, 1,050, and 12 CFU g soil.

[c] The concentration of tetracycline was 160 μg/ml of Hoagland's solution.

[d] Root rot severity was indexed according to a scale of 0 to 9 in which 0 represents little or no root rot and 9 represents roots entirely rotted.

that tetracyline at 160 μg/ml effectively prevented root nodulation in soybeans.

Plants grown in fungus-free soil with added rhizobia and watered with tetracycline-free Hoagland's solution had abundant nodulation (an average of 136 nodules per plant) and were free from root rot. Plants grown in soil with both rhizobia and *F. oxysporum* showed that plants watered with Hoagland's solution containing tetracycline developed more root rot than those watered with tetracycline-free Hoagland's solution. The root rot severity generally increased with increasing concentration of fungal inoculum in the soil. However, at a given concentration of *F. oxysporum*, root rot severity was consistently higher in tetracycline-treated plants. The increased root rot severity in tetracycline-treated plants does not seem to be attributable to a direct weakening of the plants by tetracycline, since the fresh weight of nonfungal infected plants was little affected by tetracycline. Furthermore, little difference was observed in the degree of root rot severity in tetracycline-treated or nontreated plants grown in the soil infested with *F. oxysporum* but without supplemental rhizobia.

DISCUSSION

Tetracycline-treated plants showed a slight yellowing and occasional light yellow spots on the young leaves, which indicated an effect of this compound on soybean. Phytotoxicity has been observed in many kinds of plants sprayed with 500 μg/ml of tetracycline (1). This type of yellowing apparently is not caused by nitrogen or other nutrient deficiency, since plants which were grown in the soil without added rhizobia and without tetracycline treatment did not develop any yellowing, suggesting that the yellowing was caused solely by tetracycline.

Although tetracycline could induce foliar yellowing, it did not appear to predispose the plants to root rot caused by *F. oxysporum*. The growth rate of control plants watered with and without tetracycline was quite similar. In addition, no apparent differences in root rot severity were observed in plants grown in soil infested with *F. oxysporum* with or without tetracycline treatment. These results suggest that the increase in root rot severity in tetracycline-treated plants probably was related to the elimination of rhizobia. These results are consistent with the observations that certain root rot fungi are less pathogenic on nodulated plants, that nodulated plants are more resistant to certain root rot fungi, or both (3, 14).

Nodulation decreased with increasing population of *F. oxysporum*. This can be attributed to the increased incidence and severity of root rot paralleling the increase in concentration of inoculum of the pathogen; severe root rot drastically reduces the number of surviving roots available for symbiotic nodulation.

In this study, a few root nodules consistently were found in the nontetracycline-treated plants. The number of root nodules varied from one plant to another and ranged between none and eight. This may have resulted from rhizobia carried on the pit of the seeds which were beyond the reach of the surface disinfectant, or it may reflect the presence of rhizobia indigenous in the soil mixture and not eliminated by steam treatment.

The results of these experiments show that tetracycline inhibits rhizobia in the soil without affecting the infectivity of *F. oxysporum*. It may be concluded that nodulation of soybean plants by rhizobia is not a prerequisite for infection by *F. oxysporum*. On the contrary, a protective effect of rhizobia against root rot induced by *F. oxysporum* was demonstrated in soybeans.

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