Interactions of Pseudomonas marginalis var. alfalfae, Erwinia amylovora var. alfalfae and an Unidentified Bacterium (WB-3) with Certain Root Pathogens of Alfalfa

P. A. Shinde and F. L. Lukezic

Graduate Student and Associate Professor, respectively, Department of Plant Pathology, The Pennsylvania State University, University Park 16802.

Financial support received by the senior author from the Agency for International Development of the United States Government, and Mahatma Phule Krishi Vidyapeeth, Maharashtra, India, is gratefully acknowledged.

Contribution No. 713, Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication 1-9-73 as Journal Series Paper No. 4369.

Accepted for publication 5 April 1974.

ABSTRACT

Interactions between *Pseudomonas marginalis* var. *alfalfae, Erwinia amylovora* var. *alfalfae* and an unidentified bacterium (WB-3) from alfalfa roots and three root pathogens of alfalfa were studied. The *P. marginalis* var. *alfalfae* and *Corynebacterium insidiosum* combination resulted in a reduction in top symptoms in cultivars DuPuits and Buffalo compared to top symptoms produced by *C. insidiosum* alone. A possible antagonistic relation between the two bacteria occurred. A similar reduction in top symptoms occurred in DuPuits, when *P. marginalis* var. *alfalfae* was combined with *Fusarium oxysporum* f. sp. *medicaginis*. However, an increase in top symptoms with the *P. marginalis* var. *alfalfae* and *F. oxysporum* combination in Buffalo suggests that the host has an important role in this interaction. The *F. tricinctum* and *P. marginalis* var. *alfalfae* combination produced a synergistic effect, which resulted in a significant increase in top symptoms in both cultivars.

In both cultivars, the *E. amylovora* var. *alfalfae* and *C. insidiosum* combination resulted in a reduction in top symptoms when compared to *C. insidiosum* alone. A similar reduction in top symptoms occurred in Buffalo when *E. amylovora* var. *alfalfae* was present in combination with *F. tricinctum*. However, the same combination in DuPuits resulted in a significant increase in top symptoms.

The unidentified bacterium (WB-3) in combination with *F. oxysporum* produced a synergistic effect in both cultivars. A similar effect was observed in Buffalo, but not in DuPuits, when the bacterium was present in combination with *F. tricinctum*. The results obtained in this study indicate that these bacteria as well as the hosts do have a definite role in the development of alfalfa diseases caused by *F. oxysporum* and *F. tricinctum* but not by *C. insidiosum* under the conditions of these tests.

Phytopathology 64:1169-1173

Selective media to inhibit growth of bacteria present in the diseased tissue are commonly used in isolation studies, on the assumption that bacteria associated with diseased tissue are saprophytes and are not involved in the disease syndrome. Preliminary work done at The Pennsylvania State University (15) strongly suggests that these bacteria influence the development of Fusarium root-rot of alfalfa (*Medicago sativa* L.). A review of literature showed that there are several reports (2, 12, 15, 16, 17, 18, 19, 20, 21, 22) of associated organisms in synergistic or antagonistic relationship with soil-borne plant pathogens.

Since *Pseudomonas marginalis* var. *alfalfae* (23) and *Erwinia amylovora* var. *alfalfae* (24) from the discolored roots of alfalfa were consistently present in association with species of *Fusarium,* an attempt was made in this study to determine what role they may have in the development of Fusarium root-rot and wilt diseases.

MATERIALS AND METHODS.—Possible interactions between *Corynebacterium insidiosum* (McCull.) H. L. Jens., the causal agent of bacterial wilt of alfalfa and *Pseudomonas marginalis* var. *alfalfae* (P-1), and *Erwinia amylovora* var. *alfalfae* (E-2) from the discolored alfalfa roots, were investigated in two tests. A culture of *C. insidiosum* (Ci-2) was increased on beef-lactose agar (BLA) plates and incubated for 6 days at 21 C. *Pseudomonas* and *Erwinia* isolates were increased on King's B and A (13) plates respectively and incubated at 27 C for 4 days. Inoculum, prepared by gently washing the surfaces of cultures with sterile water was diluted to equal turbidity (2.2×10^8 cells/ml). Equal volumes of the suspension were used to inoculate ten-day-old DuPuits and Buffalo seedlings, grown in nutrient solution and sand, and maintained under sterile conditions in tubes (25 × 200 mm). In the combination with *C. insidiosum* and other test bacteria, half the volume of each bacterial suspension was mixed (1:1, v/v) just prior to inoculation. The controls were treated in the same manner except sterile water was used. In the first tube-inoculation test, an isolate of *Erwinia* (E-2) and *C. insidiosum* were used alone and in combination. The seedlings were inoculated using the wounded-cotyledon inoculation method of Kreitlow (14). A total of 15 seedlings, one per tube, were inoculated with each inoculation treatment. Ten weeks after inoculation, the plants were individually evaluated for the top symptoms using this scale: 1 = healthy; 2 = slight reduction in height and leaf size; 3 = moderate reduction in height, leaf size and chlorosis of top trifoliolates; 4 = more than 40% reduction in height, severe reduction in leaf size and chlorosis of trifoliolates; 5 = dead plant.

In the second tube-inoculation test, the isolate *Pseudomonas* (P-1) alone and in combination with *C. insidiosum* (Ci-2) was used for inoculation. Roots of 5-wk-old DuPuits and Buffalo seedlings were injured with a fine, sterile, straight needle (3). The inoculum was standardized to a concn of 2.2×10^8 cells/ml with a spectrophotometer and each tube received a total of 2.2×10^8 cells. A total of 48 seedlings of each cultivar (four seedlings/tube) was inoculated with each treatment. Five weeks after inoculation, the plants were individually evaluated for the top symptoms.

Three greenhouse-inoculation experiments were conducted. The cultures of the fungi and one unidentified bacterium (WB-3) were grown separately on potato-dextrose agar (PDA); one isolate each of *Pseudomonas* (P-1) and *Erwinia* (E-5) were grown on King's B and A medium, respectively. All cultures were incubated at 27 C for 4-6 days. Inoculum was prepared by gently washing the surfaces of cultures with sterile water. Equal volume of bacterial suspension diluted to about 2.2×10^8 cells/ml was used for inoculation. In fungal and bacterium combination treatments, half the volume of the bacterial and fungal spore and mycelium (about 100,000 propagules/ml) suspension was mixed (1:1, v/v) just prior to inoculation.

Greenhouse-grown plants of different age groups of either DuPuits or DuPuits and Buffalo were inoculated by the bare-root soak method of Cormack et al. (4). The control plants were treated similarly except that sterile water was used. After inoculation four or five plants were planted in each 1064.5 ml plastic container of a pasturized (3:1:1, v/v) soil, peat, perlite mixture. The inoculated seedlings were transferred to a greenhouse bench 24 h after inoculation.

The plants in each experiment were individually evaluated for the top symptoms using the following scale: 1 = healthy; 2 = leaves pale green or chlorotic, with or without slight stunting; 3 = leaves pale yellow and stunted growth; 4 = severe yellowing of leaves and drying; 5 = dead plant.

In the first greenhouse experiment, 110-day-old plants (DuPuits) were used for inoculation. Cultures of *F. oxysporum* f. sp. *medicaginis* (Weimer) Snyd. and Hans. (F.916) the causal agent of wilt and one unidentified bacterium (WB-3), isolated earlier from alfalfa roots, were used. The inoculation treatments consisted of: (i) check; (ii) F.916 (alone); (iii) F.916 + WB-3 (1:1); (iv) WB-3 (alone). A total of twenty-five plants (five per pot) were inoculated with each treatment. Individual plants were evaluated 2 wk after inoculation for: (i) general top symptoms using the scale 1 to 5 described above; (ii) external root discoloration using the scale: 1 = clean, white roots; 2 = light yellow roots; 3 = brown roots, without necrosis; 4 = brown roots, with necrosis; and 5 = dead roots.

In the second greenhouse-experiment, 42-day-old seedlings of DuPuits and Buffalo were inoculated with the following treatments: (i) check, (ii) F.916 (alone), (iii) F.916 + P-1 (1:1), and (iv) P-1 alone. A total of forty plants of each variety (four per pot) were inoculated with each treatment. Three weeks after inoculation the plants in the pots were individually evaluated for the top symptoms using scale 1 to 5, described above. The plants were then carefully washed to remove soil from the soil and blotted with paper towels to remove the excess moisture. The plants were cut just below the first node and the fresh wt of shoots and roots determined and recorded.

In the third greenhouse-experiment, the interaction of three bacterial isolates with *F. tricinctum* (Cda.) Snyd. and Hans. (F.95) one of the causal agents of alfalfa root-rot was studied. Three bacterial isolates included were P-1, WB-3, and E-5 (an isolate of *Erwinia* from alfalfa roots). Fifty-six day-old seedlings were inoculated with the following treatments: (i) check, (ii) F.95 (alone), (iii) F.95 + P-1 (1:1), (iv) P-1 (alone), (v) F.95 + E-5 (1:1), (vi) E-5 (alone), (vii) F.95 + WB-3 (1:1), and (viii) WB-3 (alone). A total of 40 plants of each of DuPuits and Buffalo (five per pot) were inoculated with each treatment. Three wk after inoculation, the plants in pots were individually evaluated for the top symptoms, using the 1 to 5 scale described earlier.

The experimental design followed in all the inoculation experiments was a completely random design with a single pot, or test tube used as a replication. The data obtained in each experiment were separately subjected to an analysis of variance. The differences among treatment means were compared by Duncan's modified (Bayesian) least significant difference test (7). Isolations of the fungus and the bacterium from freshly killed plants and those showing yellowing and stunted growth were made to verify the presence of the different organisms.

RESULTS.—After an incubation period of 10 wk, no difference in general symptoms was observed between treatments which received *C. insidiosum* alone and those

TABLE 1. General top symptom ratings of alfalfa seedlings inoculated with *Corynebacterium insidiosum* and *Erwinia amylovora* var. *alfalfae* alone and in combination, 10 wk after inoculation

Treatment	DuPuits	Buffalo
	Index of top symptoms[a]	Index of top symptoms[a]
1. Controls	1.00 A[b]	1.00 A
2. *C. insidiosum*	3.06 A	2.46 A
3. *C. insidiosum* + *E. amylovora* var. *alfalfae*	2.73 A	2.00 A
4. *E. amylovora* var. *alfalfae*	1.53 A	1.40 A

[a]Mean of 15 replications each with a single seedling, and based on a scale of 1 to 5: 1 = healthy; 5 = dead plant.

[b]Means followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's modified (Bayesian) least significant difference test.

TABLE 2. Influence of *Fusarium oxysporum* and a bacterium (WB-3) on general top symptoms and external root discoloration of DuPuits plants, 15 days after inoculation

Inoculation Treatments	Index of general top symptoms[a]	Index of external root discoloration[b]
1. Controls	2.84 A[c]	2.96 A
2. *F. oxysporum* (F.916) alone	4.20 B	3.96 B
3. *F. oxysporum* + bacterium (WB-3)[d]	4.72 C	4.64 C
4. Bacterium (WB-3)[d] alone	3.92 B	2.92 B

[a]Mean of five replications, each with five plants, and based on a scale of 1 to 5: 1 = healthy; 5 = dead plant.

[b]Mean of five replications, each with five plants and based on scale 1 to 5: 1 = clean, white roots; 5 = dead roots.

[c]Means followed by the same letter are not significantly different from eahc other, $P = 0.05$, according to Duncan's modified (Bayesian) least significant difference test.

[d]Unidentified bacterium from alfalfa roots.

which received *C. insidiosum* and *E. amylovora* var. *alfalfae*. This observation was confirmed by a statistical analysis of the evaluation of individual plants (Table 1). In fact there was a slight reduction in general symptoms in *C. insidiosum* + *E. amylovora* var. *alfalfae* treatment compared to *C. insidiosum* alone in both the cultivars. In the second tube-inoculation test, with the inoculum concn (2.2×10^8 cells/tube), no difference was observed in general symptoms among the different treatments in both the cultivars, 5 wk after inoculation. Hence the experiment was not evaluated further.

In the greenhouse experiment, plants inoculated with *F. oxysporum* alone, and in combination with the unidentified bacterium (WB-3), showed symptoms that ranged from yellowing of leaves to wilting, within 10 days from inoculation. Two weeks after inoculation, when severe top symptoms had developed, the plants were harvested and evaluated. Analysis of data (Table 2) showed that the *Fusarium oxysporum* and bacterium combination resulted in significant increase in top symptoms, when compared to those produced by these organisms, alone. Compared to the controls all the inoculation treatments produced significantly more response in general symptoms. However, differences in general symptoms between the *F. oxysporum*-alone and the unidentified bacterium-alone treatments were nonsignificant. A similar response of all the inoculation treatments to external root discoloration was observed (Table 2).

Initial symptom development was evident 7 days after inoculation in the greenhouse experiment using *F. oxysporum* and *P. marginalis* var. *alfalfae,* alone and in combination. The symptoms ranged from pale-green leaves to a severe yellowing and wilting. Symptom expression was more pronounced in the *F. oxysporum-P. marginalis* var. *alfalfae* combination, than other treatments in both the cultivars. At the end of 3 wk, 50% or more of the plants inoculated with the *Fusarium*-bacterium were wilted. Typical *Fusarium* wilt symptoms developed in treatments where *F. oxysporum* was present. However, in both cultivars, necrosis and soft-rot of the roots were more severe in *Fusarium*-bacterium combination, than *F. oxysporum*-alone. In both cultivars, *Fusarium,* both alone and in *Fusarium*-bacterium treatments produced significantly more severe top symptoms, than occurred in the control or bacterium-alone treatments (Table 3). In the cultivar Buffalo, the *F. oxysporum-P. marginalis* var. *alfalfae* combination produced significantly more top symptoms than the *Fusarium*-alone treatment, indicating synergism between *F. oxysporum* and *P. marginalis* var. *alfalfae*. However, in DuPuits no significant difference in top symptoms of *Fusarium*-alone and *Fusarium*-bacterium treatment was observed. The bacterium treatment produced slightly stunted plants with small pale-green leaves in DuPuits, but had little (if any) adverse effect on Buffalo plants. The *Fusarium*-alone as well as the *Fusarium*-bacterium combination in both the cultivars caused a significant reduction in fresh wt of shoots and roots when compared to the check and bacterium-alone treatment (Table 3). However, differences between check and bacterium-alone treatments as well as the *Fusarium*-alone and the *Fusarium*-bacterium treatments were nonsignificant so far as fresh wt of shoots and roots in DuPuits and shoots in Buffalo were concerned. The bacterium-alone treatment in Buffalo produced significantly more fresh wt of roots than the other treatments including the check.

Development of symptoms was evident 10 days after inoculation in the experiment using *F. tricinctum, P. marginalis* var. *alfalfae, E. amylovora* var. *alfalfae* and an unidentified bacterium from alfalfa roots (WB-3), alone and in combination. Compared to the leaves of the check plants, leaves of inoculated plants, especially treatments in which *F. tricinctum* was present, appeared small and pale green. A few plants had light-yellow, cupped leaves. The *Fusarium* and bacterium combination in both cultivars, produced more root necrosis and in some cases soft-rot, than the other inoculation treatments (Table 4). *F. tricinctum* and *P. marginalis* var. *alfalfae* in combination produced significantly more response than treatments in which these organisms were present, alone (Table 4). There were no significant differences between the inoculations with *F. tricinctum*-alone and *P. marginalis* var. *alfalfae*-alone in DuPuits. However, in Buffalo the latter treatment produced significantly more response in general symptoms than the former. The *F. tricinctum* and *E. amylovora* var. *alfalfae* treatment produced significantly more severe symptoms than did

TABLE 3. Influence of *Fusarium oxysporum* and *Pseudomonas marginalis* var. *alfalfae* on disease severity, and fresh weight of shoots and roots of two alfalfa cultivars, 3 wk after inoculation

Inoculation Treatment	DuPuits			Buffalo		
	Mean disease severity index[a]	Average fresh weight of tissue (g)[b]		Mean disease severity index	Average fresh weight of tissue (g)	
		Shoots	Roots		Shoots	Roots
1. Check	1.20 A[c]	6.73 C	3.09 GI	1.50 A	5.21 E	2.90 I
2. *F. oxysporum* (alone)	3.75 B	2.38 D	1.35 HK	3.57 B	2.05 DF	1.32 HK
3. *F. oxysporum* + P-1[d] (1:1)	3.62 B	3.01 D	1.79 H	4.47 C	1.17 F	0.74 K
4. P-1 alone	1.82 A	5.93 CE	3.48 GI	1.52 A	6.18 CE	3.75 G

[a]Average of ten replications and based on a scale of 1 to 5: 1 = healthy; 5 = dead plant.

[b]Average of ten replications, each with four plants.

[c]Means followed by same letter are not significantly different from each other, $P = 0.05$, according to Duncan's modified (Bayesian) least significant difference test.

[d]*Pseudomonas marginalis* var. *alfalfae*.

the *F. tricinctum*-alone treatment in DuPuits, but not in Buffalo (Table 4). However, differences in symptoms between *E. amylovora* var. *alfalfae* alone and in combination with *F. tricinctum* were nonsignificant in both the cultivars. In Buffalo, the *F. tricinctum* and the unidentified bacterium combination produced significantly more response in symptoms than *F. tricinctum* or bacterium-alone treatments. However, in DuPuits the *F. tricinctum* and unidentified bacterium combination did not result in a significant increase in general symptoms, over those caused by *F. tricinctum* alone. In fact, the WB-3-alone treatment resulted in a significant increase in general top symptoms, over those resulting from the bacterium in combination with *F. tricinctum*.

Isolations made from freshly wilted plants or those showing severe symptoms, in a majority of cases yielded the test organism(s). However, in greenhouse experiments, occasional cross-contamination was detected.

DISCUSSION.—The absence of a significant difference in the symptom development in seedlings inoculated with *C. insidiosum* alone, and in combination with *E. amylovora* var. *alfalfae* or *P. marginalis* var. *alfalfae,* shows the lack of a additive interaction between these bacteria and *C. insidiosum*. However, a reduction in disease severity in the *C. insidiosum* and *E. amylovora* var. *alfalfae* combination compared to disease response in *C. insidiosum* alone treatment in both the cultivars (Table 1) does suggest an antagonism interaction. The mechanism responsible for the reduction of general symptoms was not determined. Hsieh and Buddenhagen (10) reported an inhibition of leaf blight symptom development when mixed inoculum of *Xanthomonas oryzae* and saprophytic bacteria isolated from rice, were used as inoculum. They observed that saprophytic bacteria, both in vitro and in vivo, have a shorter lag phase and a shorter generation time than *X. oryzae*. Further, no delay in symptom development was observed when the growth of saprophytic bacteria was completely inhibited or the rate of multiplication of saprophytic bacteria was reduced by streptomycin. In vitro, both *P. marginalis* var. *alfalfae* and *E. amylovora* var. *alfalfae* are fast growers compared to *C. insidiosum*. Because of this, *C. insidiosum* may be either competing for nutrition or affected by antagonistic materials. The existence of antagonism between pathogens or pathogens and nonpathogens have been reported in literature (5, 10, 12, 17, 20).

Enhancement of severe top symptoms as well as severe necrosis of roots observed in the *F. oxysporum* and unidentified bacterium combination (WB-3) (Table 2) in DuPuits indicates an additive effect between these two organisms. The significantly higher response produced by *P. marginalis* var. *alfalfae* and *F. oxysporum* combination in Buffalo also suggests an additive effect (Table 3). A similar effect was also observed in both DuPuits and Buffalo with the *F. tricinctum* and *P. marginalis* var. *alfalfae* combination; with *F. tricinctum* and *E. amylovora* combination in DuPuits, and *F. tricinctum* and an unidentified bacterium (WB-3) combination in Buffalo (Table 4). In Buffalo, the nonsignificant difference in top symptoms between

TABLE 4. Disease severity ratings of alfalfa (cultivars DuPuits and Buffalo) seedlings subjected to *Fusarium tricinctum* and different bacteria, alone and in combination, 3 wk after inoculation.

Inoculation Treatment	Disease severity[a]	
	DuPuits	Buffalo
Check	1.30[a] A[b]	1.60 AB
F. tricinctum (alone)	2.20 BCD	1.97 BCD
F. tricinctum + P-1[c]	3.30 GH	3.57 H
P-1 (alone)	2.60 DEF	2.85 EFG
F. tricinctum + E-5[d]	3.07 FGH	1.82 ABC
E-5 (alone)	2.45 CDEF	2.20 BCD
F. tricinctum + WB-3[e]	2.27 CDE	2.85 EFG
WB-3 (alone)	3.25 GH	2.00 BCD

[a]Mean of eight replications, each with five plants and based on a scale of 1 to 5: 1 = healthy; 5 = dead plant.

[b]Means followed by the same letter are not significantly different from each other, $P = 0.05$, according to Duncan's modified (Bayesian) least significant difference test.

[c]*Pseudomonas marginalis* var. *alfalfae* (P-1).

[d]*Erwinia amylovora* var. *alfalfae* (E-5).

[e]Unidentified bacterium from discolored alfalfa roots.

control and *F. tricinctum* alone (Table 4) suggests that the *Fusarium*-alone is weakly pathogenic on this cultivar. However, the significant increase in top symptoms when *P. marginalis* var. *alfalfae* or unidentified bacterium (WB-3) were present in combination (Table 4) shows that the bacteria aid in increasing the severity of the disease. These results support the observations of Shal'tanyanis and Puipene (22). They reported that when *Pseudomonas radiciperda, Pectobacterium carotovorum,* and *Pseudomonas fluorescens,* isolated from diseased alfalfa roots, were combined with *Fusarium oxysporum* and *F. javanicum* var. *radicola* more plants became diseased than when the organisms were used alone. At this time it is not known if the bacteria reported by Shinde and Lukezic (23, 24) and these bacteria are the same. There are major physiological differences between organisms (21) that suggest they are different.

In wilt diseases caused by bacteria and fungi, resistance to flow of water through xylem vessels brought about by number of factors is the basis of the "the plugging theory" (6). According to this theory plugging of xylem vessels by compounds of high molecular weight polysaccharides is one of the major factors responsible for the dysfunctioning of xylem vessels in diseased plants. Husain and Kelman (11) demonstrated that *Pseudomonas solanacearum* produces an extracellular polysaccharide and that the pathogenicity of different strains was correlated with their ability to produce polysaccharide. The unidentified bacterium (WB-3) produces large amounts of extracellular polysaccharide in vitro (authors, *unpublished*). It is possible that the polysaccharides could be produced in vivo and liberated into the transpiration stream by the bacterium (WB-3) which could be responsible for the observed differences in symptoms when this bacterium was present in combination with either *F. oxysporum* (Table 2) or *F. tricinctum* when on Buffalo (Table 4).

Numerous investigations have indicated the correlations or lack of correlations between the pathogenicity of an organism and its ability to produce in

vitro pectic enzymes and to utilize pectic substances (1). A few attempts have been made to determine whether an alteration in the ability of a pathogen to produce pectic enzymes results in a concomitant change in its pathogenicity. Friedman and Ceponis (9), using ultraviolet light, induced mutants of *P. marginalis,* and found that mutants with reduced virulence towards lettuce or witloof chicory also had a reduced ability to produce pectic enzymes or utilize pectic substances as sole source of carbon. *P. marginalis* var. *alfalfae* is capable of producing pectic enzymes and can utilize sodium polypectate (23). The pectic enzymes produced by *P. marginalis* var. *alfalfae* might have been responsible for the observed significant differences in general symptoms when in association with *F. oxysporum* (Table 3) and *F. tricinctum* (Table 4). These observations support the work that has been done in pathogen-pathogen or pathogen-nonpathogen associations which produce an additive relationship that results in more severe disease than either organisms could produce singly (2, 8, 15, 16, 18, 20).

The results obtained in this study and the fact that the frequent association of *P. marginalis* var. *alfalfae* and *E. amylovora* var. *alfalfae* with species of *Fusarium* in the discolored roots of stunted alfalfa plants, with small pale-green leaves strongly indicate that these bacteria have a definite role in the disease syndrome.

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