

Rapid Screening for Bacterial Wilt Resistance in Alfalfa with a Phytotoxic Glycopeptide from *Corynebacterium insidiosum*

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ABSTRACT

A rapid bioassay for bacterial wilt resistance in alfalfa was developed using the phytotoxic glycopeptide from *Corynebacterium insidiosum*. The test involved the placement of young stem cuttings (12 cm long) in a 0.05% solution of the toxin at 18 C. The degree of wilting 6 h after exposure to the toxin was measured by

percentage wt loss of water. There was a significant correlation between wilt symptom production in the bioassay and the development of root discoloration in 18 clones of alfalfa inoculated with the pathogen.

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Bacterial wilt of alfalfa (*Medicago sativa* L.) caused by *Corynebacterium insidiosum* (McCull) H. L. Jens. is probably the most important malady of the crop in the United States (3). Ries and Strobel (6) showed that a culture of the organism produced a phytotoxic glycopeptide that has a molecular weight of 5×10^6 ; is blue due to the chelation of copper; and contains residues of L-fucose, mannose, glucose, and galactose. In addition, Ries and Strobel (5) demonstrated the presence of this toxin in alfalfa plants infected with *C. insidiosum* in quantities large enough to account for wilt symptom production in alfalfa stem cuttings.

In the United States, screening alfalfa for disease resistance usually involves inoculation of the roots of plants with suspensions of diseased plant parts (1, 3, 4). The plants are then put into the field, and after several mo evaluated for typical disease symptoms. Such a program is expensive and potentially inaccurate since individual plants may escape infection by the bacterium (4). Since the toxin has a major role in disease development, it might be feasible to use it in a more rapid, less expensive test for disease resistance. Thus, the purpose of this work was to evaluate the potential usefulness of the toxic glycopeptide produced by the pathogen to screen alfalfa clones for wilt resistance.

MATERIALS AND METHODS. — Alfalfa clones.

— Eighteen alfalfa clones from Ladak and New Mexico Common parentage, and from the population ACW3 and ACW5 (1) were obtained for this study. The plants were maintained in a greenhouse throughout the study.

Bacterial cultures and toxic glycopeptide. — The culture of *C. insidiosum* (courtesy of T. I. Frosheiser, University of Minnesota) was maintained on a medium containing 1.5% glucose, 2.0% agar, 1.0% yeast extract dialysate, and 0.5% calcium carbonate (7). The organism maintained its pathogenicity for over a 5-yr period. It was transferred every 3 mo to a fresh culture slant. It produced disease symptoms in 2-yr-old alfalfa plants when inoculated by a root dip technique (5). The toxin was obtained from 250-ml

batches of this medium, without the agar, after incubation for 4 days at 20 C on a Psychrotherm incubator shaker at 20 rpm. The purified toxic glycopeptide used in these studies was obtained according to the procedures of Ries and Strobel (6).

Determination of reaction to the pathogen. — Reaction of the alfalfa clones to the bacterium was determined by the root soak method (2). Six-wk old rooted cuttings (5-10 of each clone) were placed in a 0.05 M sucrose suspension of *C. insidiosum* containing 7.5×10^{10} cells per ml for 12 h. The inoculated cuttings were planted in soil in a greenhouse bench and watered daily with Hoagland's solution. Approximately 4 mo were allowed for maximum development of disease symptomatology, (including plant death) at which time each plant was evaluated for disease severity according to the system proposed by Cormack et al. (2). This system follows a numerical scale, rated 1 = a plant exhibiting no root discoloration; 2 = a plant with some root discoloration, but showing a walling-off response to the pathogen, and scores of 3, 4, and 5 assigned to an increasing amount of root and vascular system discoloration, respectively. The disease score for 5 to 10 plants representing each clone were averaged, and that value assigned to that particular clone. Clones having scores of 3.0 or greater were considered susceptible to alfalfa wilt; and those of less than 3, were evaluated as resistant.

Toxin wilt test. — Stem wilting induced by the toxin was determined by placing young stem cuttings (10-12 cm) in a 0.05% solution of the toxic glycopeptide. To obtain reproducible results, it was necessary to remove the stem sections from the treated plants by cutting them while they were completely immersed in H₂O. The cuttings were tested at 18 C under fluorescent light with an intensity of 1.15 ergs/cm²-sec from 4-6 h. The wilting of the plants was determined by measuring wt loss. An arbitrary score of 1 was assigned to plants having less than 10% wt loss, 2 to those in the 10-15% wt loss range, 3 for 15-20% loss, and 4 above 20% wt loss. The wt loss scores could be easily determined

visually (Fig. 1). This was useful in that large numbers of plants could be scored more quickly than by making wt measurements. Evaluations of the wilt response were made every 2 h and the final score made at the end of 6 h. Wilt scores for each clone represent an average obtained from three individual experiments with three individual cuttings per clone. The toxin solution was taken up by all cuttings tested. A statistical correlation was made between the scores from the root inoculation experiments and the stem wilt scores. In all wilt tests, cuttings placed in distilled H₂O were used as controls.

RESULTS AND DISCUSSION. — A broad range of response to root inoculation with *C. insidiosum* was noted in the alfalfa clones tested (Table 1). Only one clone showed no disease symptoms after inoculation with the bacterium. Several clones obtained from breeding programs and labeled as resistant were susceptible in our inoculation tests. This may be a reflection of the high concn of bacteria that were used in our inoculation experiments, or these clones may have represented "escapes" in the testing procedures commonly used by other investigators. (4).

The reaction of the various clones in the toxin-wilt test also differed. Those clones yielding wilt scores of 2.0 and above were considered strongly wilted. The disease scores and the wilt scores were significantly correlated ($r = 0.62$) (Table 1). Even though clones 9 and 14 (Table 1) yielded a rating of 3.0 - 4.0 in the inoculation tests (4), both had low wilt scores. Both of these clones had been evaluated as resistant in breeding programs of other workers. A closer examination of these clones revealed that they behaved in a unique manner. Both yielded a wilt reaction of 3.0 when exposed to a 1% solution of the toxin for 3 h as contrasted with a 1.0 reaction in a 0.05% toxin solution. When subsequently placed in distilled H₂O for 12 h, they became turgid as did most cuttings. However, upon being exposed to 1% toxin solution on a second occasion, no wilting occurred for up to 6 h. By contrast, the wilt score of

TABLE 1. The response of various alfalfa clones to inoculation with *Corynebacterium insidiosum* versus their response to being exposed to a 0.05% solution of a toxic glycopeptide from *C. insidiosum* for 6 h at 18 C

Alfalfa parentage and clone number	Disease score ^a	Wilt score ^b
Ladak		
1	1.0 (R)	1.4
2	3.1 (S)	2.3
3	3.4 (S)	2.0
4	2.7 (R)	1.3
5	2.6 (R)	1.7
6	2.0 (R)	1.5
7	3.1 (S)	3.0
New Mexico		
Common		
8	2.5 (R)	1.0
9 ^c	3.0 (S)	1.0
10	3.0 (S)	2.0
11	4.0 (S)	2.0
ACW3 and ACW5		
12	1.6 (R)	1.0
13	2.0 (R)	1.0
14 ^c	3.0 (S)	1.0
Ladak		
15	2.3 (R)	1.0
16	2.6 (R)	1.3
17	4.0 (S)	2.0
18	4.3 (S)	3.0

^a A score of 3.0 or less is resistant (R) and above 3.0 is susceptible (S). The score is an average value for 5 to 10 plants of each clone.

^b The wilt score for each clone represents an average of 3 experiments with 3 cuttings of each clone per experiment.

^c Clones 9 and 14 have a unique response to the toxin as pointed out in the text.

all other clones was virtually unchanged after the second exposure to the toxin. This suggests that an induction of wilt resistance in the first treatment protects these two unique clones from wilting in subsequent treatments. These results help to explain the discrepancy between the wilt score and the disease score in the test; and were it not for such a reaction in these few alfalfa clones, the correlation between the wilt score and the disease score is $r = 0.71$.

A critical point relative to the validity of the toxin test is the range of wilt ratings for cuttings from any individual clone. The range for any individual cutting did not deviate more than + 1.0 from the average wilt value assigned to that clone. It is recommended that at least three cuttings of any clone being evaluated should be tested at least twice. It is also suggested that two clones differing widely in their response to the toxin be used as indicators of test validity on any group of cuttings being screened.

Ideally, breeding for disease resistance to bacterial wilt would involve the development of a number of plants homozygous for wilt resistance. Because of the tetraploid nature of alfalfa, homozygosity is difficult

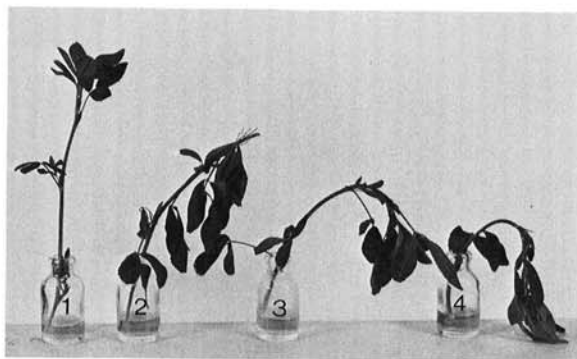


Fig. 1. Wilt ratings (1 to 4) assigned to alfalfa clones relative to their response to 0.05% solution of the glycopeptide toxin after 6 h. The rating scale ranges from 1 to 4 and is based on a visual estimate of the amount of wilting.

to obtain without large populations and a precise assay for resistance. This test not only permits the handling of a large number of plants in an early stage of development but lessens the time necessary to evaluate plants in a breeding cycle. Furthermore, the non-destructive nature of the test permits the breeder to propagate the tested cuttings by rooting them in a mist bench.

Although a correlation coefficient of $r = 0.62$ is not high, it does represent a better correlation than that currently obtained using the bacterial inoculation technique (2). The wilt test method is based on an artificial method of scoring which may influence the correlation. In a regular breeding program, the clones selected would be of the non-wilting type. Of hundreds of wilt tests made in our laboratory, plants having a high wilt reading have never been found resistant to our isolate of *C. insidiosum*. Although the unique clones 9 and 14 gave low wilt test readings and proved to be susceptible to the pathogen, this sort of difficulty could be tolerated in a mass screening test if the final test of selected stock is one that ultimately involves the pathogenic bacterium. The toxin wilt test is most valuable in that it decreases the total number of plants to be tested by the bacterial inoculation

procedure. Thus, if it is used in conjunction with bacterial inoculation, it saves time, expense, and effort.

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