Correlation Between Root and Stem Reactions of Alfalfa to *Phytophthora megasperma* f. sp. *medicaginis*

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ABSTRACT

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The mature root and stem reactions to *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) of 58 S_0 alfalfa plants from diverse sources were compared. Plants with highly susceptible roots always had highly susceptible stems, and plants with highly resistant roots never had highly susceptible stems. However, some plants with susceptible roots had resistant stems. The correlation between mature root and stem reaction to Pmm was highly significant ($r = 0.82$) for the 58 S_0 plants tested. Isolations of Pmm from stems showed that its colonization was restricted in resistant stems compared to susceptible stems. Both the roots and stems of S_1 plants from a highly susceptible and a highly resistant parent plant were all susceptible and resistant, respectively. The correlation between the mature root and stem reaction to Pmm on 44 F_1 plants of the cross of a resistant and a susceptible plant was highly significant ($r = 0.59$). When the stem inoculation method was used, no evidence was obtained for the presence of pathogenic specialization within North American and Australian isolates of Pmm. The main advantage of the stem inoculation method is that final disease development is evident 4 days after inoculation. However, only highly susceptible individuals can be reliably detected.

Screening for resistance in alfalfa (*Medicago sativa* L.) to *Phytophthora megasperma* Drechs. f. sp. *medicaginis* (Pmm) can be accomplished successfully by infesting the potting medium in which 8- to 12-wk-old plants (mature plants) are growing with the fungus and then scoring the roots for disease severity after 3–6 wk (1). This method has been used widely (3–5,11,12,14).

The major advantage of the mature root assay is that the plant organ on which disease severity is measured is also the organ which is naturally attacked by the fungus. However, one of the disadvantages of this assay is that considerable greenhouse space is required for a prolonged time period. Also, high soil moisture levels are necessary for optimum disease development and the problems encountered in providing consistently uniform soil moisture conditions from experiment to experiment are considerable. High soil moisture levels at temperatures above 30 C also cause the abiotic root disease "scald" (2).

The development of a reliable seedling assay for detection of mature root resistance to Pmm has provided a method which has increased the efficiency of routine screening (6). Strict control of both inoculum concentration and soil moisture level, however, is still required for effective disease evaluation with this method.

Inoculation of aerial plant parts with a root-rot pathogen can provide a rapid means of disease evaluation (12). Pratt et al (12) reported that resistant alfalfa cultivars selected by mature root reaction contained significantly more resistant seedlings than did susceptible cultivars detected by placing zoospores of Pmm on the cotyledons. However, tests were not made to determine whether individual plants, resistant in the cotyledon assay, were resistant at maturity. A rapid method of disease screening would be to inoculate alfalfa stems with mycelium of Pmm. Inoculation of aboveground organs has been used successfully to develop Phytophthora stem-rot-resistant lines of soybean (7), cowpea (13), and safflower (16).

The purpose of this report was to compare the stem and root reactions of alfalfa to Pmm. Plants, each with a known mature root reaction, were stem-inoculated with Pmm, given a stem disease rating, and then the root and stem disease ratings were compared statistically. In subsequent tests, the root and stem disease ratings were obtained for individual plants in S_1 and F_1 families from parents that had either highly resistant or highly susceptible stem and root reactions to Pmm. The stem inoculation method was used to determine whether pathogenic specialization was present in the USA and Australian isolates of Pmm. In this pathogenic specialization study, one susceptible and four resistant alfalfa plants, from diverse germplasm, were inoculated with five different isolates of the pathogen.

MATERIALS AND METHODS

Sources of the pathogen. All studies used a single-zoospore isolate (5b-4) of Pmm supplied by S. A. Miller (University of Wisconsin-Madison). The initial culture was obtained by baiting from soil collected from the Phytophthora root rot disease nursery at St. Paul, MN (10). In one study, four hyphal-tip isolates (isolates 19524, 20192, 21408, and 21498), which were obtained by direct isolation from diseased alfalfa roots collected in Queensland, Australia, by the senior author, were included.

Determination of mature root reactions of S_0 plants. The mature tap root reaction to Pmm was determined for tetraploid alfalfa S_0 plants from a range of winter-hardy cultivars and breeding lines (Table 1). Before inoculation, each plant was clonally propagated from stem cuttings, so that highly susceptible plants could be preserved. Four plants were grown to 8 wk of age in 10.3-cm-diameter clay pots which contained about 400 g dry weight of a peat-sand mix (1:1, v/v) (PS). The pots then were inoculated with Pmm (0.18 g dry weight of mycelial fragments per pot) by pouring 30 ml of inoculum uniformly over the mix, then the inoculum was incorporated into the upper 2 cm. Immediately after infestation, pots were placed in saucers kept filled with water for 2-day periods, then were allowed to drain for the next 2 days. This process of alternate wetting and drying was continued for 21 days, and then the roots were given a disease severity index (DSI) rating (see footnote a, Table 1). At least eight S_0 plants for each of the five root disease classes were identified.

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Determination of stem reaction of S_0 plants. Plants, or uninoculated vegetative propagules of highly susceptible plants (Table 1) which had been evaluated for their mature root reaction to Pmm, were transplanted into PS mix in 14-cm-diameter clay pots and grown in the greenhouse. The plants were fertilized weekly with a 20-20-20 water-soluble fertilizer (Peters soluble general purpose fertilizer; R.B. Peters Co., Inc.). Seven to 10 days prior to making stem inoculations, top growth of each plant was removed. Stem inoculations were made by puncturing a re-growing stem 1 cm below the axil of the third leaf below the shoot apex. A wisp of aerial mycelium, that was taken from the margin of a 5- to 8-day-old V-8 agar plate culture, was inserted into the wound. The wound then was covered with petroleum jelly to minimize fluid loss. At least two stems of uniform development were inoculated for each plant. The plants were grown in the greenhouse at 20 to 25 C for 4 days (symptoms did not change after 4 days) and then were rated by the following DSI: 1 = necrosis at inoculation point only, slight watersoaking up to 0.5 cm on either side of inoculation point; 2 = watersoaked lesion for 0.5–2.0 cm from inoculation point, no necrosis, or necrotic flecks only, which extended for 1 cm from either side of inoculation point; 3 = necrotic runner lesions, 1–2 cm long, which extended from either side of the inoculation point, and tissue was not collapsed completely; and 4 = long, necrotic lesion, which extended at least 3 cm from either side of the inoculation point, tissue collapsed, and shoot wilted.

Assessment of the degree of colonization of alfalfa stems by *P. megasperma* f. sp. *medicaginis*. Alfalfa plants that gave a range of stem disease severity scores when inoculated with Pmm were inoculated in the stems as described above. Four days after inoculation, two stems from each plant were rated for disease severity. The stems then were surface sterilized and sectioned into 1-mm lengths, beginning from the inoculation point and progressing toward the shoot apex. Each tissue piece was sequentially plated onto V-8 agar which contained 0.1 g/L neomycin sulfate. These plates were incubated at 20 C for 5 days. The distance between the tissue piece farthest from the inoculation point from which the fungus was recovered and the inoculation point was used to estimate the distance the fungus had moved in the stem.

Comparison between root and stem disease reactions of tetraploid alfalfa plants from S_1 and F_1 populations. S_1 seed was obtained by selfing plants M245 (root DSI = 5, stem DSI = 4) and M193 (root DSI = 1, stem DSI = 1); and F_1 seed was obtained from the cross of M30 (root DSI = 1, stem DSI = 2) × M245. Flowers were suction emasculated prior to hand pollination to obtain F_1 seed. Seed from the above S_1 or F_1 population were sown into 10.3-cm-diameter clay pots (one seed per pot). After 10 wk, top growth was removed, and 6 days later, two uniform stems on each plant were inoculated with Pmm. The inoculated stems were given a disease score, herbage was removed, and the stems of the 6-day regrowth were again inoculated and then scored.

After the stem reactions were determined, each plant was inoculated with Pmm to determine its mature root reaction. Thus, the root and stem reactions of each plant then could be compared within each of the two S_1 and F_1 families.

Screening of *P. megasperma* f. sp. *medicaginis* isolates for detection of pathogenic specialization. Isolate 5b-4 of Pmm and the Australian isolates of Pmm (19524, 20192, 21408, and 21498) were used to stem-inoculate the alfalfa clones M245 (from cultivar Saranac, USA; root DSI = 5), M14 (from cultivar Agate, USA; root DSI = 1); M188 (cultivar Hunter River P_3, a breeding line from Australia, root DSI = 1); and M193 (NAPB 0310, a North American Plant Breeders breeding line from USA, root DSI = 1). Two different stems were inoculated with the same isolate on each plant and all isolates were tested once.

RESULTS

Each S_0 plant that gave a highly susceptible root reaction (DSI = 5) when inoculated with Pmm also gave a highly susceptible stem reaction (DSI = 4) (Table 1). Plants with root DSI's of 1 or 2 (resistant) never gave a highly susceptible stem reaction. Variation among plants with root DSI's of 1 or 2 was not significant ($P = 0.05$) for stem DSI's as determined by the ratio of among-clone to within-clone variance (F-test). This would indicate that the genetic variation for stem reactions in root DSI classes 1 and 2 was not significant. One of the 12 plants with a root DSI of 3 (moderately resistant) had highly susceptible stems. The remaining plants in this class gave stem DSI's ranging 1–3. Among-plant variation was significant ($F = 10.68$, $P < 0.005$), which indicated the presence of genetic variation for stem reaction within this root disease class. Six of eight plants with a root DSI of 4 (susceptible) that were stem inoculated consistently gave stem DSI's of 4, whereas one of the remaining plants gave a score of 2 and the other a 3. Thus, variation among plants was highly significant ($P = 0.01$). The correlation between mature root DSI and stem DSI to Pmm for 58 S_0 plants tested was highly significant ($r = 0.82$). When only the data for the 25 S_0 plants with root scores of 1 and 5 were used, the correlation between characters was $r = 0.96$.

Isolations made from inoculated stems showed that the distance the fungus had colonized tissue was dependent on both the root and stem scores (Table 2). The growth of the pathogen in stems of plants with stem DSI's of 1 or 2 was restricted, when compared to that in stems with a DSI of 3, which in turn showed restricted colonization when compared to stems scoring 4.

All S_1 alfalfa plants from the highly susceptible parent M245 had susceptible roots and stems (Table 3). Of 28 clones tested, 27 consistently gave a stem rating of 4 on all four stems inoculated. The remaining plant gave a rating of 4 on three inoculated stems and a 3 rating on the remaining stem. The same S_1 plants had root DSI's of 4 or 5. All S_1 plants from the highly resistant parent M193 had a stem DSI of 1 and root DSI's of 1 or 2.

The correlation between stem and root reaction to Pmm was tested further on progeny of the cross M30 (resistant parent) ×

TABLE 1. Comparison of the root and stem disease severity indices obtained on S_0 tetraploid alfalfa plants after inoculation with *Phytophthora megasperma* f. sp. *medicaginis*

Mature tap root DSI[a]	No. of plants per root DSI class	Cultivar and breeding line sources of S_0 plants	Mean stem DSI[b] ± [c]	Range of stem DSI's
1	15	Apollo, Agate, NAPB 0310[d], Hunter River P_3	1.6 ± 0.4	1–2
2	13	Agate, Apollo, NAPB 72, NAPB 0321, NAPB 0310	2.1 ± 0.4	1–3
3	12	Apollo, Agate, NAPB 72, NAPB 0321, NAPB 0310	2.3 ± 1.1	1–4
4	8	Apollo, Agate, NAPB 0310, NAPB 72	3.6 ± 0.7	2–4
5	10	Saranac, Vernal, NAPB 0321	4.0 ± 0	

[a] Root disease severity index (DSI): 1 = tap root, secondary roots and fine feeder roots white (healthy); 2 = small lesions not encompassing more than 0.2 of the circumference of the tap root. They were present mainly at the junction of the tap and lateral roots. Girdling lesions permissible on roots up to 1 mm in diameter; 3 = lesions on the tap root encompassing 0.2–0.5 of the circumference. Girdling lesion permissible on all roots up to 2 mm in diameter; 4 = lesions completely girdling the tap root and/or larger lateral roots. Almost all smaller secondary roots destroyed; and 5 = entire tap root rotted, aboveground parts dead.

[b] Stem disease severity index (DSI) (see Materials and Methods): 1=slight watersoaking to 0.5 cm from inoculation point; 4=necrotic lesion at least 3 cm from inoculation point. Two stems of each plant were inoculated.

[c] s = standard deviation between mean stem ratings of S_0 plants within each root disease class.

[d] NAPB = North American Plant Breeders.

M245 (susceptible parent). The cross M193 (resistant parent) × M245 was not used for this study, because the F_1 population had a very low frequency of plants with susceptible roots. However, the root reactions of F_1 plants of the cross M30 × M245 were distributed more evenly throughout the five root disease classes. No F_1 plants of the cross M30 × M245 with root disease scores of 1, 2, or 3 gave a stem rating of 4. The two F_1 plants with root ratings of 5 consistently gave a stem rating of 4 (Table 4). However, some F_1 plants with root ratings of 4 consistently gave stem ratings of 2. The correlation between mature root and stem reaction was highly significant ($r = 0.59$) for the 44 F_1's tested. This correlation is lower than that obtained for the S_0 plants (Table 1). This is due mainly to fewer F_1 plants with a root DSI of 5 than among the S_0 plants. This is particularly important because our data indicate that a plant with a root score of 5 always gives a stem score of 4.

When the stem inoculation method was used, no evidence was obtained for pathogenic specialization among the USA and Australian isolates of Pmm. However, the method did allow the rapid detection of a loss of pathogenicity of isolate 19524, as it did not produce stem symptoms on the highly susceptible plant M245. This loss of pathogenicity subsequently was confirmed when the isolate did not produce root symptoms on cuttings of M245 after 21 days, whereas isolate 5b-4 killed all cuttings of this plant.

DISCUSSION

The inoculation of alfalfa stems with *P. megasperma* f. sp. *medicaginis* allows the ready detection of plants with highly susceptible roots (DSI = 5) because all plants that gave a stem rating of 4 always gave a root rating of 5. Plants with root DSI's of 1 or 2 never had stem ratings of 4. In routine disease evaluation, this stem inoculation method could not be the only procedure used, because a few plants with root DSI's of 4 had restricted stem lesions (DSI = 2). It, however, could be used initially to effectively eliminate many of the susceptible plants from a breeding line and then the remaining plants could be evaluated by the mature root assay.

The correlation between root and stem reactions will vary because the association depends on the relative frequencies of plants present in each root disease class. In populations of plants with root disease classes of only 1, 2, and 5, a high correlation could be expected; however, in a plant population with a relatively high frequency of plants with root DSI of 4, the coefficient value would be much lower due to some plants having resistant stem reactions.

The high value for the correlation coefficient ($r = 0.96$) between root and stem reaction for the 25 S_0 plants with root DSI's of 1 and 5 strongly indicates that the root and stem reactions to Pmm are controlled by the same genetic system. The probability of obtaining such a result in heterozygous S_0 clones if different genetic systems were involved would be very small. Even though the association between root and stem reaction of F_1 plants from the cross M30 ×M245 was $r = 0.59$, these results also support the hypothesis that the root and stem reaction to Pmm is controlled by the same gene(s). If different genes were operative, they would be expected to segregate independently and a much lower correlation would be observed. Although our S_1 plant reactions also support this hypothesis, we cannot conclude from them that the root and stem reactions to Pmm are controlled by the same gene(s), because it is possible that both clones could be homozygous for different loci that controlled these reactions. Our results are in contrast to those of Thomas and Hill (15) who found that different genetic factors condition resistance to *Phytophthora drechsleri* in roots, hypocotyls, and cotyledons of safflower. Also, Kilen et al (9) found that the soybean line CNS was susceptible when hypocotyls were inoculated, but resistant if grown in a liquid medium which contained the fungus. This resistance to root inoculation was under the control of a different genetic system from that which conferred the hypocotyl disease reaction (9).

Keen and Horsch (8) criticized the use of "unnatural" host organ-pathogen systems in the study of naturally occurring disease-resistance mechanisms in plants. They reported that the Rps gene for resistance to *P. megasperma* f. sp. *glycinea* in soybeans was expressed in hypocotyls but not in roots. Our results with plants with a root DSI of 4 may support these conclusions. An explanation for the poor correlation between stem and root reactions of plants with a DSI = 4 could be that the same gene(s) is operating in roots and stems to produce a phytoalexin, but that the rate of accumulation is much faster in the stems than in the roots of the individuals with a root DSI = 4 and a stem DSI = 2. The importance of the rate of accumulation of phytoalexin after infection on the progress of the host-pathogen interaction has been substantiated (17).

The stem inoculation method, due to its rapidity, has a use as a preliminary means of eliminating susceptible plants in a routine breeding program and for detecting pathogenic specialization and loss of pathogenicity of cultures. Inoculations can be made with relative ease, and the numerous stems produced by each plant

TABLE 2. Colonization of stems of tetraploid alfalfa plants 4 days after inoculation with *P. megasperma* f. sp. *medicaginis*

Plant number	Cultivar or breeding line source	DSI root[v]	DSI stem[w]	Mean distance (mm) colonized by fungus[x]
M245	Saranac	5	4	23.0 a[y]
M349	Saranac	5	4	23.5 a
M269	Vernal	5	4	19.5 a
M267	Vernal	5	4	19.5 a
M272	Vernal	5	4	25.0 a
M302	NAPB[z] 72	3	3	13.0 b
M307	NAPB 0321	3	3	10.5 b
M18	Agate	3	3	10.5 b
M12	Agate	2	2	2.5 c
M27	Agate	2	2	1.5 c
M30	Apollo	1	2	1.0 c
M193	NAPB 0310	1	1	0.5 c
M195	NAPB 0310	1	1	0.5 c
M16	Agate	1	1	0. c
M17	Agate	1	1	0 c

[v] Root disease severity index score (DSI) 1 = (see footnote a, Table 1).
[w] Stem disease severity index score (DSI) 1 = (see footnote b, Table 1).
[x] Isolations were made from two inoculated stems of each plant.
[y] Means within a column not followed by the same letter are significantly different ($P = 0.01$) as determined by the least significant difference test.
[z] NAPB = North American Plant Breeders.

TABLE 3. Disease reaction of the roots and stems of S_1 alfalfa plants from a resistant (M193) and a susceptible parent (M245) to *Phytophthora megasperma* f. sp. *medicaginis*

Parental plant[a]	No. plants tested per S_1 population	Mean DSI's of S_1 populations ± s[b]: Stem inoculations[c]	Root inoculations[d]
M245	28	3.99 ± 0.05	4.68 ± 0.47
M193	30	1.00 ± 0.0	1.36 ± 0.49

[a] The stem and root reactions of the parent plants M245 and M193 are given in Table 2.
[b] s = standard deviation between mean DSI's of S_1 plants.
[c] Stem disease severity index (DSI) 1 = (see footnote b, Table 1).
[d] Root disease severity index (DSI) 1 = (see footnote a, Table 1).

TABLE 4. Disease reaction of the roots and stems of F_1 alfalfa plants from a cross between a resistant (M30) and a susceptible parent (M245) to *Phytophthora megasperma* f. sp. *megicaginis*

Mature root DSI[a]	No. of F_1 plants per root DSI class	Mean stem DSI[b] ± s[c]
1	17	1.8 ± 0.7
2	12	1.9 ± 0.8
3	6	2.5 ± 0.6
4	7	2.8 ± 0.7
5	2	4.0 ± 0

[a] Root disease severity index (DSI) 1 = (see footnote a, Table 1).
[b] Stem disease severity index (DSI) 1 = (see footnote b, Table 1).
[c] s = standard deviation between mean stem ratings of F_1 plants within each root disease index class.

provide a ready source of replication. However, for evaluation of alfalfa for Phytophthora root rot resistance, our results indicate that stem inoculations alone will only allow the detection of highly susceptible clones.

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