

Alfalfa resistance to post-harvest *Aspergillus* species: Response to selection

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¹Sugar Experiment Station., Te Kowai, PMB 57, Mackay Mail Centre, Queensland, 4741 Australia; ²Department of Plant Science, and ³Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. Received 17 June 1999, accepted 14 April 2000.

Kimbeng, C. A., Smith, Jr., S. R., Babij, V. and Wittenberg, K. M. 2000. **Alfalfa resistance to post-harvest *Aspergillus* species: Response to selection.** Can. J. Plant Sci. **80**: 755–763. Alfalfa (*Medicago sativa* L.) cultivar development programs routinely incorporate resistance to fungal diseases that limit forage yield, quality and stand longevity. “Hay molding”, caused by saprophytic fungi, reduces forage quality during the post-harvest period, but genetic resistance has not been reported. The objective was to determine the response to selection for post-harvest fungal resistance in alfalfa. Initially, 1144 genotypes from 22 alfalfa cultivars were screened for post-harvest fungal resistance with a combination of *Aspergillus* species. Forty resistant and 40 susceptible genotypes were selected by measuring percentage of leaf area with fungal coverage. The selected genotypes and their maternally derived half-sib families were screened for resistance to *Aspergillus repens*. Mean leaf area with fungal coverage was significantly ($P < 0.05$) lower for resistant than susceptible parental genotypes and maternal half-sib families. Percent leaf area with fungal coverage ranged from 13.2 to 70.5% (mean, 41.7 ± 1.7) among resistant and 28.2 – 83.4% (mean, 50.2 ± 1.7) among susceptible parental genotypes. Among the maternal half-sib families, resistant families ranged from 14.0 to 51.3% (mean, 32.1 ± 1.8) and susceptible families from 18.8 to 60.1% (mean, 40.3 ± 1.9). These results show a positive response to selection for post-harvest fungal resistance in alfalfa.

Key words: Alfalfa, *Medicago sativa*, post-harvest fungi, *Aspergillus* species, breeding, divergent selection

Kimbeng, C. A., Smith, Jr., S. R., Babij, V. et Wittenberg, K. M. 2000. **Résistance de la luzerne aux infestations à *Aspergillus* après la récolte.** Can. J. Plant Sci. **80**: 755–763. Les programmes de sélection variétale de luzerne (*Medicago sativa* L.) comprennent généralement la résistance aux maladies fongiques qui risquent d’abaisser le rendement et la qualité du fourrage ainsi que la longévité de la culture. Le «moississement du foin» causé par des champignons saprophytes abaisse la qualité fourragère durant la période qui suit la récolte, mais on ne connaît pas encore de résistance génétique envers ces espèces d’organismes. Nous avons donc mesuré la réponse à une sélection axée sur la résistance aux infestations fongiques de conservation. Au départ, 1 144 génotypes provenant de 22 cultivars de luzerne étaient testés sur leur résistance envers un cocktail d’espèces d’*Aspergillus*. Quarante génotypes résistants et 40 sensibles étaient sélectionnés en mesurant le pourcentage de surface foliaire colonisée par les champignons. Les génotypes retenus et leurs descendance maternelles étaient ensuite évalués sur leur résistance à *Aspergillus repens*. La surface foliaire moyenne colonisée était significativement ($P < 0,05$) plus petite chez les génotypes parentaux résistants et leurs descendance maternelles que chez les génotypes et descendance vulnérables. Le pourcentage de surface foliaire colonisée allait de 13,2 à 70,5 (moyenne $41,7 \pm 1,7$) pour les génotypes parentaux résistants et de 28,2 à 83,4 (moyenne $50,2 \pm 1,7$) pour les génotypes sensibles. De même chez les descendance maternelles, il allait de 14,0 à 51,3 (moyenne $32,1 \pm 1,8$) pour les résistantes et de 18,8 à 60,1 (moyenne $40,3 \pm 1,9$) pour les vulnérables. Ces observations font ressortir d’une réponse positive de la luzerne à la sélection sur la résistance aux affections fongiques de conservation.

Mots clés: Luzerne, *Medicago sativa*, infection fongique de conservation, espèces d’*Aspergillus*, amélioration génétique, sélection divergente

Breeding for disease resistance has played an important role in the successful production of alfalfa in North America. Beginning from the mid-1970s, breeding for disease resistance in alfalfa became increasingly important in cultivar development (Barnes et al. 1988). Cultivar development programs routinely incorporate field resistance to one or more diseases that are known to limit forage yield, quality and stand longevity (Pratt and Rowe 1994; Halimi et al. 1998; Certified Alfalfa Seed Council 1998).

Fungal invasion, by a number of saprophytic genera including *Absidia*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium* and *Phoma*, is one of the major causes of post-harvest losses in alfalfa production (Breton and Zwaepoel 1991; Undi et al. 1997; Wittenberg et al. 1998). Mohanty et al. (1969) estimated that the feed value of alfalfa decreases by 25 – 30% due to moldy hay. Such losses may be minimized by reducing the field wilting time through mechanical conditioning and by using chemical additives and optimum storage conditions. However, the effectiveness of these practices is variable and depends on harvest management, weather conditions, timing of applica-

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tion and product efficiency. The development of alfalfa cultivars with resistance to post-harvest fungi would lower the risk for post-harvest quality losses, but genetic resistance to these saprophytic fungi has not been reported in alfalfa.

Although screening for disease resistance has generally been carried out on whole, living plants, there is evidence to suggest that genes that confer resistance to pathogens are expressed post-harvest in excised plant parts. For example, excised pea pods inoculated with macro conidia of fungi pathogenic to peas, showed a rapid increase in chitinase and β -1,3 glucanase activity after a lag phase of 4-8 h (Mauch et al. 1984). Chitinase, β -1,3 glucanase and other enzymes are believed to be associated with expression of disease resistance in plants. In alfalfa, Ferullo et al. (1996) noted considerable changes during the post-harvest period, including new polypeptides being synthesized up to 48 h after harvest. Divergent selection for resistance to sclerotinia crown and stem rot (*Sclerotinia trifoliorum*) using excised stems revealed significant differences in disease reaction between the selected populations (Halimi et al. 1998).

Aspergillus spp. and other post-harvest fungi are assumed to attack alfalfa in swaths and bales as saprophytic rather than pathogenic organisms. Babij (1997) conducted a series of experiments to determine the nature of observed resistance response reactions of *Aspergillus* spp. on alfalfa. Results of experiments designed to determine potential in situ or induced chemical inhibition of fungal growth were inconclusive, but there was a positive association between slower leaf drydown rate and reduced rate of leaf fungal growth. These preliminary results suggest that post-harvest fungal resistance in alfalfa may be due to preformed cell structures such as increased thickness of the leaf cuticle or epidermal cell walls.

If variability for post-harvest fungal decomposition exists in alfalfa, populations that are resistant to post-harvest fungal invasion could be developed. Recently, Wittenberg et al. (1998) developed a procedure to screen alfalfa for resistance to post-harvest fungal invasion. Babij (1997) evaluated the practical application of the screening procedure by incubating mini-bales comprised of resistant or susceptible genotypes under conditions that simulated commercial hay storage. Chitin (a polymer of N-acetyl-glucosamine) was used to quantify mold invasion because it is found in fungal spore and mycelium cell walls, but is absent in higher plants (Teng and Whistler 1973). Chitin was measured as its hydrolysis product, glucosamine (Wittenberg et al. 1998). Resistant genotypes had significantly lower levels of chitin than susceptible genotypes during the mini-bale incubation period. These results indicated that it may be possible to select for post-harvest fungal resistance in alfalfa.

The objective of this study was to determine response to selection for post-harvest fungal resistance in alfalfa.

MATERIALS AND METHODS

Babij (1997) screened 22 cultivars for reaction to post-harvest fungi using the procedure of Wittenberg et al. (1998) and identified resistant genotypes that showed reduced fungal growth, and susceptible genotypes that supported greater amounts of fungal growth. This study compared the perfor-

mance of plants in these two groups of parental genotypes and the maternal half-sib families derived from them.

Genetic Materials

The screening procedure and criteria used in selecting the parental genotypes was described in detail by Babij (1997) in an MSc thesis. Briefly, a total of 1144 genotypes (i.e., 52 randomly chosen plants from each of 22 registered cultivars) were initially screened using a mixture of three *Aspergillus* species (*A. flavus*, *A. glaucus*, and *A. fumigatus*). Originally, *A. repens* and *A. versicolor* were a planned component of this mixture, but insufficient inoculum at the time of screening prevented their inclusion in the mixture. The genotypes that ranked as most resistant (87) and most susceptible (88) to fungal mycelium growth on excised leaves were selected. These genotypes were then screened with a mixture of five *Aspergillus* species (*A. flavus*, *A. glaucus*, *A. repens*, *A. versicolor* and *A. fumigatus*) to further select the 40 most resistant and 40 most susceptible genotypes. Although using only three *Aspergillus* species in the initial screening may have resulted in preferential selection, the final 40 resistant and susceptible genotypes were selected based on reaction to a five-species inoculum mixture.

Parental Genotypes

The 40 resistant and 40 susceptible parental genotypes selected by Babij (1997) were transplanted to the field in the summer of 1995 in separate, but adjacent isolation plots. Each group was planted in five rows spaced 60cm apart, with eight plants per row on 45 cm spacings. In the fall of 1996 stem and/or crown bud cuttings (clones) were taken from each genotype and used to establish vegetative propagules. The cuttings were rooted in sterile soil less mix in root trainers in a growth chamber. Five vigorously growing plants (clones) per genotype were transplanted to pots containing a soil mixture of 1:1:1 topsoil:sand:peat (five cuttings per genotype per pot) and maintained in a greenhouse. Multiple plants (clones) per genotype provided sufficient vegetative material for screening. Plant mortality occurred during vegetative propagation and transplanting. Consequently, only 36 resistant and 28 susceptible parental genotypes were available for subsequent screening experiments. The number of resistant and susceptible parental genotypes selected from each of the original cultivars is listed in Table 1.

Plants in the growth chamber were cut once at the 10% bloom stage (no screening performed) and the subsequent regrowth was harvested and evaluated for post-harvest fungal resistance using *Aspergillus repens*. The five plants (clones) from each genotype were evaluated in a series of three trials (Exp. I) with greenhouse sampling occurring on 22 April, 22 May, and 27 June 1997. Total vegetative material from all plants (clones) per genotype was bulked for each of the screening trials. In a fourth trial, sampled 18 July 1997, field-grown plants from the initial isolation plots (described above) were used.

Past research in screening for post-harvest fungal resistance in alfalfa used a combination of *Aspergillus* species

Table 1. Alfalfa cultivars, screened for post-harvest fungal resistance (Babij 1997), and the number of genotypes per cultivar that comprise the resistant and susceptible parental groups and that were used to derive the maternal half-sib families

Source	Resistant	Susceptible
	(No. of plants)	
Algonquin	—	3
Alouette	3	1
Allegro	2 (3) ^z	3
Arrow	6	—
Beaver	1	—
Cimmaron	3	2 (3)
Class	—	3 (4)
DK 133	3	—
Dominator	3	—
GH 787	—	2
Greenfield	2	—
OAC Minto	2 (3)	1
Pick Seed 8920MF	—	2
Proof	3	— (1)
Rambler	—	5 (7)
Rushmore	1	4
Sterling	1	1
Ultra Leaf	1	— (1)
Venture	1	1
WL 32HQ	4 (5)	—
Total	36 (39) ^y	28 (34)

^zGenotypes in the resistant and susceptible parental groups were used to derive the maternal half-sib families except where additional genotypes were used as indicated in parentheses.

^yBabij (1997) originally selected 40 resistant and 40 susceptible genotypes from a collection of 1144 genotypes from 22 cultivars. Winter mortality in the field reduced the resistant genotypes to 39 and the susceptible genotypes to 34 and mortality during vegetative propagation further reduced numbers to 36 and 28, respectively.

since mold in hay is usually the result of several fungal species (Babij 1997; Wittenberg et al 1998). Although these earlier screening studies identified resistant and susceptible genotypes, there was often a high degree of variability in the results. There was a concern that one species may be dominating during one trial and another species dominating in another trial. Therefore, *Aspergillus repens* was chosen for this research because it is the predominant fungal species causing molding in hay bales (Undi et al. 1997) and grows over a wide range of temperature and moisture levels. Babij (1997) also showed that *A. repens* gave the widest range of resistance and susceptible response reactions in experiments where four alfalfa genotypes were separately inoculated with each of the five previously mentioned *Aspergillus* species. Additionally, it was also felt that use of one fungal species would simplify the procedure and decrease screening variability caused by interspecies competition within each petri plate.

Maternal Half-sib Families

In the summer of 1996, isolation cages (3 m × 5 m) were erected over the field isolation plots of resistant and susceptible parental genotypes, and the plants were randomly inter-pollinated (within each group) using alfalfa leafcutter bees

(*Megachile rotundata* F.). Approximately 1200 bees were released over three release dates (2 wk intervals) when the majority of the plants were at the 50% flowering stage (early July). Mortality over the winter of 1995–96 reduced field plant numbers to 39 resistant and 34 susceptible parental genotypes (Table 1). Seed harvested from each plant was used to establish approximately 10 genotypes per maternal half-sib family in each of two pots in the greenhouse in January 1997. The seeds were planted into a potting soil mixture consisting of 1:1:1 of topsoil:sand:peat. To insure uniformity within each pot, less vigorous seedlings were discarded, leaving approximately six genotypes per pot. Based on this criteria, 39 and 33 maternal half-sib families were available for subsequent evaluation.

Plants were cut once at the 10% bloom stage (no screening performed) and the subsequent regrowth was harvested from each maternal half-sib family and evaluated for post-harvest fungal resistance using *Aspergillus repens* in a series of three trials for exp. II (screening procedure described in subsequent section). Harvested plant material was bulked from the six maternal half-sib genotypes per pot on 29 May, 3 July and 1 August 1997. Bulk plant material from each pot was maintained separately so that each half-sib family was represented by two bulked samples. Efforts were made to subsample leaves for plating from each of the six maternal half-sib genotypes per pot however, a slightly different set of genotypes may have been screened during each trial of exp. II.

Inoculum Culture and Storage

The isolate of *Aspergillus repens* (var. *Columnaris* ATCC 557129) used for this study was acquired from American Type Culture Collection, Rockville, MD. The freeze-dried isolate was revived by forming a conidial suspension in 0.1% Tween 80 (Mallinckrodt Specialty Chemicals, Paris, New York) solution, and inoculating 10 mL of potato dextrose agar (PDA) slants in 50 mL culture tubes. After about 3 wk of incubation at 25°C, the tubes were sealed with parafilm (American National Can, Chicago, IL) and stored at 5°C. This became the base stock inoculum from which subcultures were grown for each screening trial in exp. I and II.

Inoculum Grow-out and Harvesting

Inoculum for each experiment consisted of conidia prepared by adding 10 mL of 0.1% Tween 80 solution to a well-sporulated stock slant, and dislodging the conidia by gently scraping the agar surface using sterile applicator sticks. The conidial suspension was added to 10–20 mL of potato dextrose agar in 50 or 100 mL screw cap flasks. The conidia were incubated at 35°C for 24 h to break dormancy (Smith et al. 1981), then the temperature was lowered to 25°C for an additional 3–4 wk. The conidia were harvested by adding 10 mL of 0.1% Tween 80 solution and 10–30 sterilized glass beads to each flask, then agitating the flasks for 30 min at 300 rpm in a controlled-environment incubator shaker (New Brunswick Scientific Co. Inc.). The suspension was strained through eight layers of sterilized cotton gauze, centrifuged at 3400 rpm for 15 min and the

Table 2. Mean squares (MS) from analysis of variance (ANOVA) for percent leaf area with post-harvest fungal coverage in four experiments and summed over four experiments for resistant (RP) and susceptible (SP) populations of alfalfa

Source of variation ^z	df	Total	Exp. I	Exp. II	Exp. III	Exp. IV
		(%)				
Rep (R)	1	8141.518*	5.281	7825.787*	185.040	0.845
Block (B)	3	4234.917*	8122.944*	637.059	199.187	445.739*
Parents (P)/B	60	4432.775*	957.967*	909.649*	316.739*	481.925*
Among RP (RP)/B ^y	32	3909.212*	861.009*	1066.959*	167.139*	431.667*
Among SP (SP)/B ^y	24	4105.974*	826.769*	698.387*	374.937*	398.783*
(RP vs. SP)/B ^y	4	10582.080*	2520.821*	918.750	1164.359*	1382.837*
Error (R × PB)	63	734.811	259.901	377.105	73.937	87.984
Total	127					
CV (%)		14.9	31.6	25.8	33.7	17.3

^zError (R × PB) was the error term used for all *F* tests.

^yTo test the differences among parents within each population and the single degree of freedom contrast between populations, MSs for among parents and the contrasts were obtained from ANOVAs for each population separately and divided by the appropriate error MS (R × PB) from this combined ANOVA.

* Significant at $P \leq 0.05$.

resulting pellet resuspended in 0.1 % Tween 80 solution to a concentration of approximately $1-3 \times 10^5$ spores mL⁻¹, determined using a haemocytometer.

Screening procedure

Details of the screening procedure were reported by Wittenberg et al. (1998). Dichloran with 18% glycerol agar plates were prepared in advance with 18–20 mL of medium per plate. This medium is commonly used for enumeration of xerophilic fungi (Pitt and Hocking 1985), which have the ability to grow on low-moisture products such as hay (Babij 1997). The medium contains 2 µg L⁻¹ dichloran (2, 6-dichloro-4-nitroaniline), which inhibits growth of *Aspergillus* or *Eurotium* colonies (Beauchat 1992) and 18% glycerol (vol/vol), which reduces available water to 0.96 to reduce bacterial growth (Hocking 1991). Chloramphenicol was also added to reduce bacterial growth at 100 mg L⁻¹ (Hocking 1991).

Plants were harvested for inoculation when 10% of the plants in a population were in the early to late bloom stage, then placed in sterile bags and transported to the laboratory. Fully developed trifoliate leaves (without insect bites, blemishes or tears) were randomly removed from individual plants and placed onto the 18% glycerol agar medium (adaxial side down) using the intact petiole as a “handle.” The surface area of each plate was covered with no leaf-to-leaf contact (4–6 leaves per plate). The leaves were placed such that the entire surface made full contact with the agar. The plates containing the excised leaves were placed in a laminar flow hood (Biological Safety Cabinets, Plymouth, MN) and sprayed with $1-3 \times 10^5$ conidia per plate using a nalgene aerosol bottle.

The plates were recapped and placed at 25°C. Plates were monitored daily for fungal growth and leaves visually assessed for percentage of leaf area with fungal coverage using the James (1971) scale. The actual assessment occurred when approximately 70% of the plates exhibited colonization on at least 20% of leaf area (usually on day 5) (Wittenberg et al. 1998). Fungal coverage included vegetative mycelium growth and reproductive mycelium with active conidia production.

A serial dilution and spread plate technique was used to determine inoculum potential for each trial. The harvested $1-3 \times 10^5$ conidia mL⁻¹ solution was serially diluted (in duplicate) to 10⁻⁶ concentration by adding aliquots of 1 mL of the solution to 9 mL of 0.1% Tween 80 solution. Aliquots of 0.1 mL of each concentration were spread using a bent glass rod over the surface of sterile plates containing 18–20 ml of potato dextrose agar. The plates were capped and incubated at 25°C. Colony counts were made on the serial dilution plates to determine inoculum potential.

Experimental Design and Statistical Analysis

The experimental design during leaf incubation of the parental genotypes (exp. I) was an incomplete block design (blocks/replications) with two replications and four blocks. Each genotype was represented by duplicate petri plates, which were analyzed as replications. Blocks were used to reduce microenvironmental heterogeneity (Gomez and Gomez 1984). A block consisted of nine resistant and seven susceptible genotypes and represented the number of plates that were inoculated at a time in the laminar flow hood. Entries were randomly distributed within each block (in the laminar flow hood). The experimental design during leaf incubation of maternal half-sib families (exp. II) was an incomplete block design (blocks/replications) with two replications (each pot per family representing a replication) and three blocks. A block consisted of 13 and 11 maternal half-sib families from the parental resistant and susceptible genotypes, respectively.

Percentage of leaf area with fungal coverage was analyzed for normality with the SAS procedure UNIVARIATE (SAS Institute, Inc. 1989). The data for maternal half-sib families were transformed to the square root scale since this provided the smallest coefficient of variation and largest probability of a normal distribution (SAS Institute, Inc. 1989). Analysis of variance was used to determine the significance of observed differences between the resistant and susceptible parental genotypes and the resistant and susceptible maternal half-sib families. Variation due to parental genotypes or maternal half-sib families was partitioned among the resistant parental or maternal family group and

Table 3. Mean squares (MS) from analysis of variance (ANOVA)^z for percent leaf area with post-harvest fungal coverage in three experiments and summed over three experiments for maternal half-sib families derived from resistant (RP) and susceptible (SP) populations of alfalfa

Source of variation ^y	df	Total	Exp. I	Exp. II	Exp. III
			(%)		
Rep (R)	1	0.635	3.867	0.090	4.358*
Block (B)	2	18.459*	36.027*	4.826*	3.130*
Family (F)/B	69	4.281*	4.987*	2.024	1.287*
Among RF (RF)/B ^x	36	3.068*	2.918	1.840	1.363
Among SF (SF)/B ^x	30	4.379*	6.012*	2.011	0.939
(RF vs. SF)/B ^x	3	17.845*	19.567*	4.368*	3.849*
Error (R × FB)	71	1.771*	2.764	1.484	0.852
Total	143				
CV (%)		13.0	30.2	28.1	12.8

^zData transformed to the square root scale was used in the ANOVA.

^yError (R × FB) was the error term used for all *F* tests.

^xTo test the differences among progenies within each population and the single degree of freedom contrast between populations, MSs for among progenies and the contrasts were obtained from ANOVAs for each population separately and divided by the appropriate error MS (R × FB) from this combined ANOVA.

* Significant at $P \leq 0.05$.

among the susceptible parental or family group. A pre-planned single degree of freedom contrast was also conducted between the resistant and susceptible parental groups and between the half-sib family groups. In this analysis, the sums of squares and degrees of freedom were pooled over blocks. Statistical significance was assigned at $P < 0.05$ level. Phenotypic correlation coefficients were computed between trials to determine whether genotypes performed consistently within each experiment.

RESULTS AND DISCUSSION

Combined over trials within experiments, analysis of variance for percentage of leaf area with fungal coverage showed significant differences ($P < 0.05$) between the resistant and susceptible parental genotype groups and between the resistant and susceptible maternal half-sib family groups (Table 2 and 3). Within-group differences were also detect-

ed for the parental resistant and susceptible genotypes over all trials (Table 2). However, for the maternal half-sib families, the only significant within-group difference occurred in the susceptible family group in trial 1 (Table 3). Mean comparisons between resistant and susceptible groups were significant over all parental and maternal half-sib trials, with the exception of trial 2 for the parental genotype evaluation (Figs. 1 and 2).

The range and frequency distribution of *A. repens* growth in the resistant and susceptible parental and maternal half-sib family populations are shown graphically in Fig. 3 and 4, respectively. Percent leaf area with fungal coverage among the parental genotypes ranged from 13.2 to 70.5% (mean, 41.7 ± 1.7) in the resistant population and from 28.2 to 83.4% (mean, 50.2 ± 1.7) in the susceptible population (Fig. 3). Among the maternal half-sib families, the range was 14.0 to 51.3% (mean, 32.1 ± 1.8) and 18.8 to 60.2%

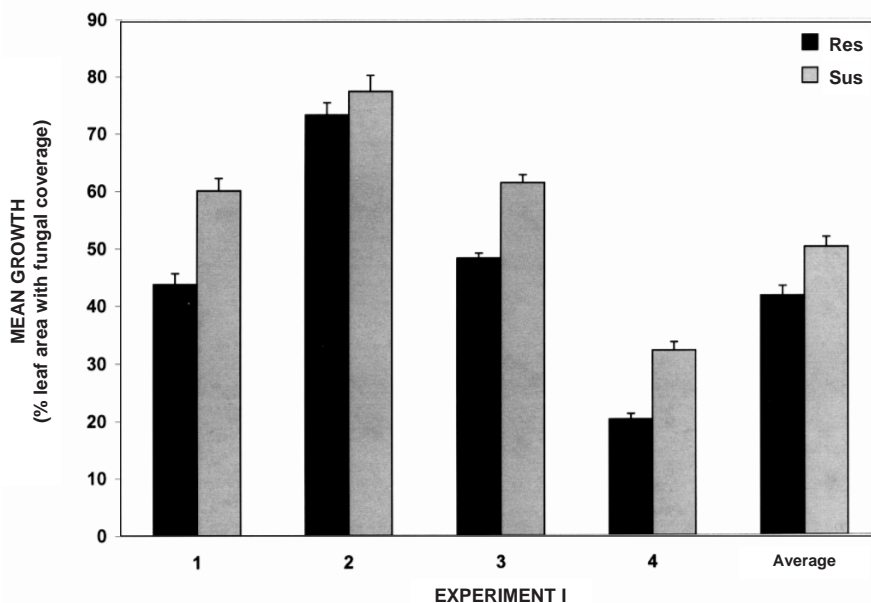


Fig. 1. Percentage of alfalfa leaf area with fungal coverage (*Aspergillus repens*) of resistant (Res) and susceptible (Sus) parental genotype groups in four trials (Exp. I). Bars indicate standard error.

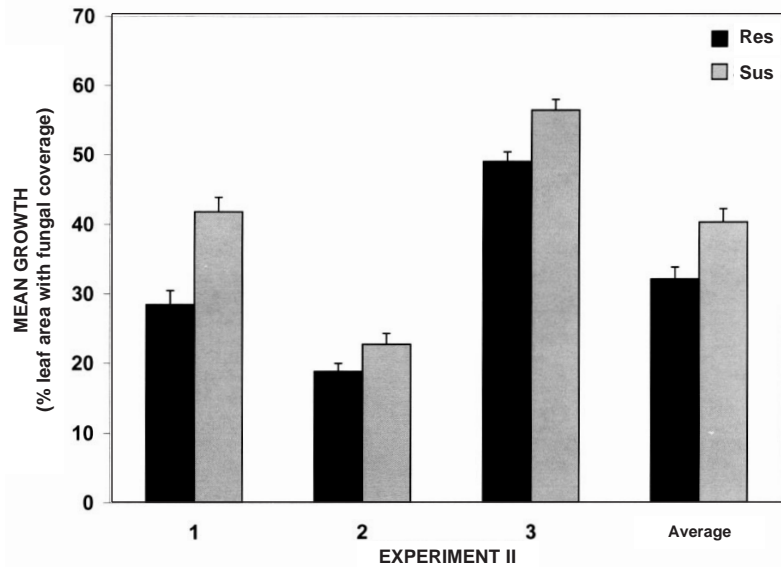


Fig. 2. Percentage of alfalfa leaf area with fungal coverage (*Aspergillus repens*) of resistant (Res) and susceptible (Sus) maternal half-sib families (derived from resistant and susceptible parental genotypes) in three trials (Exp. II). Bars indicate standard error. Means and standard error based on absolute values, while interpretation in the results and discussion based on transformed data.

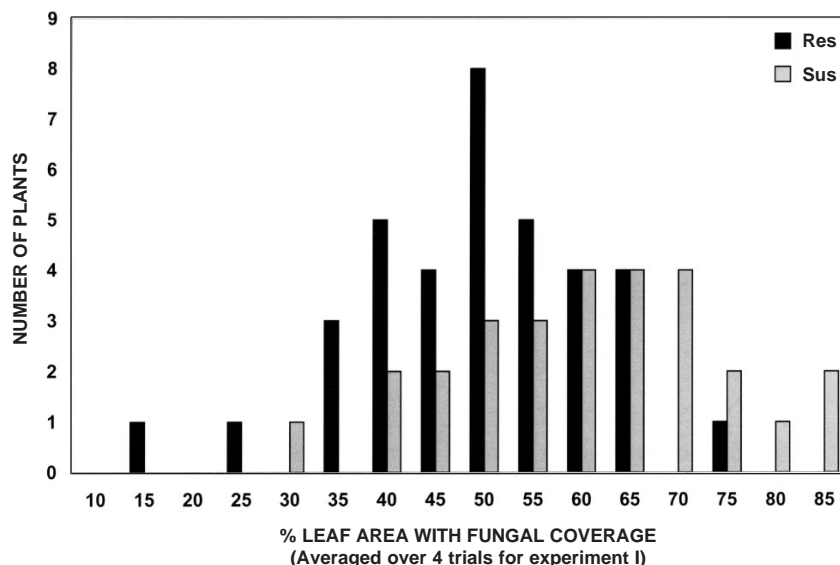


Fig. 3. Frequency distribution of leaf area with fungal coverage (*Aspergillus repens*) of resistant (Res) and susceptible (Sus) parental genotype groups averaged over four trials in Exp. I.

(mean, 40.3 ± 1.9) in the resistant and susceptible populations, respectively (Fig. 4). Both graphs depict a higher proportion of entries showing reduced leaf fungal coverage in the resistant relative to the susceptible population. The graphs, as well as the ranking of the top 12 genotypes (Table 4), show that some entries in the susceptible populations performed better than entries from the resistant populations. This may be due to the methodology used for selecting the parents where a mixture of three to five *Aspergillus* species was used, whereas, in the present experiments, only *A. repens* was used.

Although every attempt was made to use consistent methodology from trial to trial within each experiment, ini-

tial inoculum concentration and rate of fungal growth made that difficult. As a result, inoculum potential (number of viable conidia) and incubation period (days) were different between trials within each experiment. In the parental genotype evaluation (exp. I), inoculum potential at 10^{-5} dilution and the incubation period for the four trials were as follows: 38, 5 d; 48, 6 d; 67, 4 d; and 61, 4 d. Similarly, inoculum potential and incubation periods for the three trials in the maternal half-sib family evaluation (exp. II) were 48, 5 d; 61, 4 d; and 56, 4 d. Initial inoculum potential for trial 2 in exp. I was comparable with that in the other three trials, but the plates were not evaluated until 6 d of incubation, by which time more than 20% of leaf area had been colonized

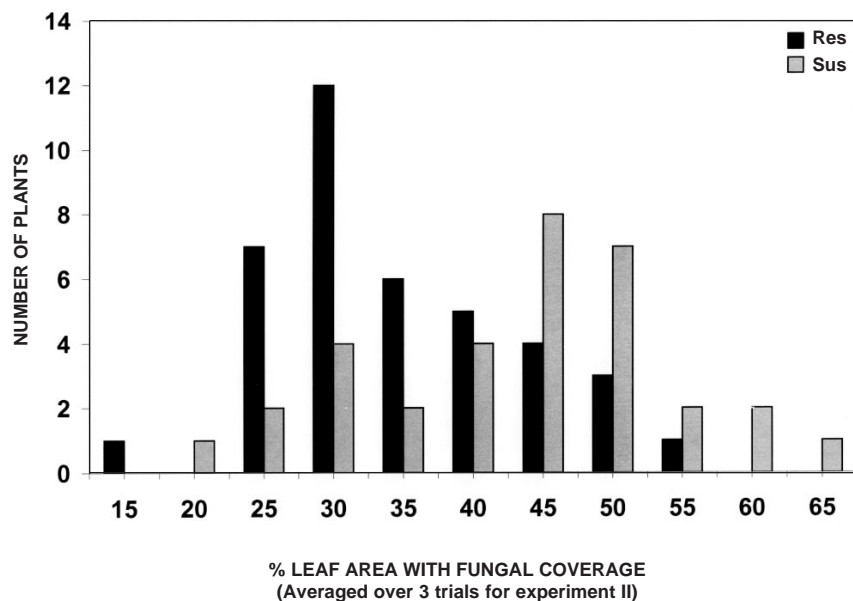


Fig. 4. Frequency distribution of leaf area with fungal coverage (*Aspergillus repens*) of resistant (Res) and susceptible (Sus) maternal half-sib families (derived from resistant and susceptible parental genotypes) averaged over three trials in Exp. II.

Table 4. Ranking of the 12 alfalfa genotypes that showed the highest mean post-harvest fungal resistance from parental and maternal half-sib family groups. (1) represents the genotype that showed the highest level of resistance (ie. the lowest percent leaf area with fungal coverage)

Genotype	Genotype ranking				
	Total	Exp. I	Exp. II	Exp. III	Exp. IV
		<i>Parents^x</i>			
R 16	1	7	2	1	2
R 25	2	4	5	7	22
S 28	3	15	4	5	33
R 33	4	28	1	20	14
R 6	5	11	9	18	19
R 26	6	2	7	52	31
R 5	7	6	35	9	9
S 14	8	3	11	26	42
R 9	9	1	20	31	12
R 3	10	8	54	8	11
R 2	11	12	22	12	10
R 19	12	25	36	3	1
		<i>Maternal Half-sib families^y</i>			
R 6	1	8	1	1	
S 3	2	9	6	8	
R 7	3	17	9	5	
R 9	4	7	28	10	
R 1	5	1	34	12	
R 3	6	6	2	30	
R 31	7	15	20	7	
S 6	8	14	12	15	
R 28	9	11	15	24	
R 13	10	10	17	25	
R 8	11	3	19	41	
R 5	12	29	50	2	

^xRanked out of the 64 genotypes that comprised the resistant (R) and susceptible (S) parental groups. Mortality during vegetative propagation reduced the original 80 selected genotypes to 64 (36 resistant and 28 susceptible).

^yRanked out of the 72 maternal half-sib families from the resistant and susceptible groups. Winter mortality in the field reduced the number of original parental genotypes, used to derive the maternal half-sib families, to 72 (39 resistant and 34 susceptible).

in the majority of the plates. This may explain why there was no difference between the resistant and susceptible groups for trial 2. When trial 2 was omitted from the analysis, the mean difference in percentage of leaf area with fungal coverage between the resistant and susceptible parental genotype groups increased dramatically (resistant group mean 37%; susceptible group mean 51%). Wittenberg et al. (1998) suggested that plates should be scored when 70% of the plates exhibit colonization on at least 20% of leaf area. These results confirm that evaluators must pay careful attention to the incubation and leaf colonization period during screening.

As a result of the high inoculum potential used in our screening procedure, the proportion of leaf area with fungal coverage approached 100% after about 7 d of incubation for all trials in both experiments. Nonetheless, significant differences were found for percentage of leaf area with fungal growth on days 4 and 5 of incubation between parental genotypes and half-sib families within each group and between groups. Similar results were reported when mini-bales of resistant and susceptible genotypes were incubated under conditions that favor mold growth (Babij 1997). Fungal growth was significantly lower on mini-bales of resistant genotypes than on susceptible genotypes for up to 11 d. The mechanism of resistance is not well understood, but the rate of fungal growth is definitely delayed on resistant genotypes (Kimbeng et al. 1997). Therefore, when used in conjunction with proper hay management techniques, the development of resistant cultivars should enhance the ability to harvest and store high quality hay.

Genotype by trial interactions were significant (Tables 2 and 3) and correlation coefficients between trials were low (Table 5). Random genetic drift cannot account for this variation since repeated observations were made on the same genotypes. Inoculum potential differed slightly between trials, which in turn affected the rate of growth of the fungus

Table 5. Phenotypic correlation coefficient matrix between experiments for percent leaf area with post-harvest fungal coverage for alfalfa plants in the resistant (upper diagonal) and susceptible (lower diagonal) parental populations and in the maternal half-sib families derived from these parents.

Experiment	I	II	III	IV
		<i>Parents^a</i>		
1	–	0.14NS	0.18NS	0.40*
2	0.54*	–	0.35NS	0.31NS
3	0.31NS	0.44NS	–	0.22NS
4	0.48*	0.33NS	0.42NS	–
		<i>Maternal half-sib progenies^b</i>		
1	–	0.53**	0.34NS	–
2	0.18NS	–	0.26NS	–
3	0.53**	0.28NS	–	–

^aNumber of entries for parents: resistant, 36; susceptible 28.

^bNumber of entries for maternal half-sib families: resistant, 39; susceptible 33.

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

as observed in the incubation period. The spray bottle technique also does not deliver a guaranteed amount of inoculum per plate. Furthermore, although most plants were grown in the greenhouse, environmental and cultural conditions varied across sampling dates. This may have caused fluctuations in individual plant maturity, leaf flora, fauna and metabolites at time of harvest. These conditions, acting either independently or in unison may have contributed to the significant genotype by trial interaction observed. These results suggest that several rounds of screening should be used to identify resistant genotypes. In this regard, three parental genotypes were identified (R6, R9 and R3), which ranked among the top 12 based on the parental and maternal half-sib family evaluations, and for which the trial-to-trial fluctuations were minimal (Table 4). These genotypes, along with others from a previous screening, will be incorporated into a recurrent selection program with the aim of increasing the level of resistance to *A. repens* in alfalfa.

In conclusion, the results of this study suggested that progress is possible in selecting for post-harvest fungal resistance in alfalfa in a recurrent selection program. Further genetic experiments are necessary to substantiate this screening procedure and to determine if the genotypic differences observed in these experiments are heritable. Refinement of the screening procedure, which should include screening of field-grown plants, may improve selection efficiency by minimizing experimental variability. Babij (1997) reported a reduction in mold growth on mini-bales made from resistant genotypes which were stored under conditions simulating a commercial hay stack, which suggests that this laboratory procedure may be able to predict response during hay storage. If germplasm or cultivars are developed using *Aspergillus repens* as the fungal inoculum source, it will be essential to screen the finished product with a combination of post-harvest fungal species and monitor fungal growth under actual hay storage conditions before commercial release.

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