

# Alfalfa resistance to post-harvest *Aspergillus* species: Combining ability analyses

C. A. Kimbeng<sup>1</sup>, S. R. Smith, Jr.<sup>2,4</sup>, V. Babij<sup>3</sup>, and K. M. Wittenberg<sup>3</sup>

<sup>1</sup>Department of Agronomy, Louisiana State University Agricultural Center, 104 M. B. Sturgis Hall, Baton Rouge, LA 70803, USA; <sup>2</sup>Crop and Soil Environmental Sciences, 424 Smyth Hall (0403), Virginia Tech, Blacksburg, VA 24061, USA; and <sup>3</sup>Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. Received 12 December 2003, accepted 10 June 2004.

Kimbeng, C. A., Smith, S. R., Jr., Babij, V. and Wittenberg, K. M. 2004. **Alfalfa resistance to post-harvest *Aspergillus* species: Combining ability analyses.** *Can. J. Plant Sci.* **84**: 1193–1197. Hay making is still the most widespread technique of forage preservation. Saprophytic fungi, such as *Aspergillus repens*, can reduce forage quality during the post-harvest period by causing hay molding. Methods to control molding have yielded variable results, but genetic resistance has yet to be exploited. The objective of this study was to determine the mode of inheritance of resistance to post-harvest fungal growth in alfalfa. Seven parental genotypes representing a range of resistance to fungal growth (*A. repens*) were crossed in a complete diallel including reciprocals for a total of 42 crosses. Parental genotypes along with their progeny were screened for resistance to *A. repens*. The relative importance of general combining ability (GCA) and specific combining ability (SCA) effects in controlling resistance to post-harvest *A. repens* was compared. The results revealed a preponderance of GCA effects. The parent that supported the least amount of fungal growth produced progeny with the least amount of fungal coverage. The reverse was true for the more susceptible parents. The results indicate that breeding procedures that maximize additive gene effects, such as recurrent selection, could be effective in improving resistance to post-harvest fungal growth in alfalfa.

**Key words:** Alfalfa, breeding, combining ability, resistance, *Aspergillus repens*, post-harvest fungi

Kimbeng, C. A., Smith, S. R., Babij, V. et Wittenberg, K. M. 2004. **Résistance de la luzerne aux espèces d'*Aspergillus* après la récolte : analyse des aptitudes à la combinaison.** *Can. J. Plant Sci.* **84**: 1193–1197. Le fanage reste la technique la plus largement répandue pour préserver les fourrages. Les champignons saprophytes tel *Aspergillus repens* peuvent néanmoins réduire la qualité du foin après sa récolte en causant le moisissement. Les méthodes de lutte contre la moisissure sont plus ou moins efficaces, mais on ne s'est guère attardé à la résistance génétique. Cette étude devait établir le type de résistance aux moisissures post-messianiques inhérente chez la luzerne. Les auteurs ont croisé sept génotypes parentaux de résistance très variable aux cryptogames (*A. repens*) dans le cadre d'un croisement diallele incluant des croisements réciproques, pour un total de 42 croisements. Ils ont ensuite examiné les parents et leur descendance pour établir leur degré de résistance à *A. repens*. Ils ont déterminé dans quelle mesure l'aptitude générale à la recombinaison et l'aptitude spécifique à la recombinaison interviennent dans la résistance à *A. repens* après la récolte. Les effets de l'aptitude générale à la recombinaison sont les plus importants. Le parent qui résiste le mieux à la contamination par le cryptogame donne la progéniture la moins attaquée par ce dernier. L'inverse est vrai pour les parents les plus sensibles. Les résultats laissent croire que les méthodes d'hybridation maximisant les effets génétiques additifs comme la sélection récurrente pourraient accroître la résistance de la luzerne aux moisissures après la récolte.

**Mots clés:** Luzerne, *Aspergillus repens*, aptitude générale à la recombinaison, résistance

Hay making is still the most widespread technique of forage preservation. Hay molding, caused by saprophytic fungi, reduces forage quality during the post-harvest period (Breton and Zwaepoel 1991; Undi et al. 1997; Wittenberg et al. 1998) and represents a major loss in alfalfa (*Medicago sativa* L.) production. Mohanty et al. (1969) estimated that the feed value of alfalfa decreases by 25 to 30% due to moldy hay. In addition, prolonged exposure to spores from moldy hay can result in severe lung allergies to farmers and livestock (Lacey 1975). Deterioration of forage can be reduced to a certain extent by reduction of field wilting time, storage at optimal conditions and use of preservatives or antifungal agents such as organic acids, ammonia compounds and bacterial inoculants (Wittenberg et al. 1998).

However, such methods rely heavily on harvest management, weather conditions, timing of application and product efficiency. The development of alfalfa cultivars with host-plant resistance to post-harvest fungi could enhance the efficacy of other preventative measures and lower the risks associated with hay molding. However, this option has not been actively pursued in alfalfa, probably because of the difficulty associated with screening for host-plant resistance during the post-harvest period and the complexity of the problem.

Although screening for host-plant resistance has traditionally been carried out on plants prior to harvest, there is evidence to suggest that resistance mechanisms operate post-harvest. For example, excised pea pods inoculated with

**Abbreviations:** GCA, general combining ability; SCA, specific combining ability

<sup>4</sup>To whom correspondences should be addressed

macro conidia of fungi pathogenic to peas showed a rapid increase in chitinase and  $\beta$ -1,3 glucanase activity after a lag phase of 4–8 h (Mauch et al. 1984). Chitinase,  $\beta$ -1,3 glucanase and other enzymes are believed to be positively associated with expression of disease resistance in plants. In alfalfa, Ferullo et al. (1996) noted major changes in the post-harvest period, including new polypeptides being synthesized up to 48 h after harvest. Also in alfalfa, divergent selection for resistance to sclerotinia crown and stem rot (*Sclerotinia trifoliorum* Eriks.) using excised stems revealed significant differences in disease reactions between the selected populations (Halimi et al. 1998). In addition, certain physical properties such as stem cell wall content and cuticle thickness may contribute to post-harvest resistance in alfalfa, as suggested by Babij (1997).

A laboratory screening procedure has been developed to assess alfalfa genotypes for resistance to post-harvest fungal growth (Wittenberg et al. 1998). This procedure identified genotypes that supported varying levels of fungal mycelial growth following an initial screening of 1144 genotypes from 22 cultivars with a mixture of *Aspergillus* species (Babij 1997). A subsequent screening of a portion of these genotypes and their maternally derived half-sib progenies, using *A. repens* (Corda) Saccardo (var. *columbaris* ATCC 557129; American Type Culture Collection (Rockville, MD)), revealed lower levels of fungal mycelial growth on the resistant compared to the susceptible group of genotypes (Kimbeng et al. 2000). The practical application of this screening procedure was also evaluated by incubating mini-bales composed of resistant or susceptible genotypes under conditions that simulated commercial hay storage (Babij 1997). Chitin (a polymer of N-acetyl-glucosamine) was used to quantify mold invasion as it is found in fungal spores and hyphal cell walls but is absent in higher plants (Teng and Whistler 1973). Chitin was measured as its hydrolysis product, glucosamine (Glu) (Wittenberg et al. 1998). Resistant genotypes had significantly lower chitin levels in comparison to susceptible genotypes after the mini-bale incubation period. These results suggest that variability exists among alfalfa genotypes for resistance to post-harvest fungal growth by *Aspergillus* species, that the laboratory screening procedure may predict response during hay storage, and that it may be possible to utilize breeding, along with other preventative measures to minimise hay molding. The objective of this study was to determine the mode of inheritance of resistance to post-harvest fungal growth in alfalfa.

## MATERIALS AND METHODS

### Plant Material

Seven alfalfa genotypes were selected from a previous screening study (Babij 1997) to represent a range of responses to post-harvest fungal growth (Table 1). The selected genotypes were grown in a controlled environment chamber with a 15-h photoperiod and 23°C/15°C day/night temperatures. The seven genotypes were crossed in a complete diallel. Seeds from each of the 42 F<sub>1</sub> progenies were planted into a potting soil mixture consisting of 1:1:1 ratio of topsoil:sand:peat in the

**Table 1. Characteristics of the seven alfalfa parental genotypes used to study inheritance of resistance to post-harvest growth of *Aspergillus repens***

Genotype (cultivar source)	% leaf area covered with <i>A. repens</i> <sup>z</sup>
A (Arrow)	26.4
B (Cimmaron)	52.9
C (Dominator)	49.7
D (Arrow)	33.1
E (Proof)	57.9
F (Rambler)	80.5
G (Cimmaron)	47.6
LSD	8.8

<sup>z</sup>Averaged across three screening trials.

greenhouse. Approximately 60 seeds of each cross were planted into four pots (replicates), or 15 seeds per pot. To ensure uniformity of growth within each pot, less vigorous seedlings were removed, leaving eight to ten plants per pot.

Stem cuttings were taken from each parental plant and used to establish clonal propagules. For each parent, five clonal plants per pot were transplanted to each of four pots (replicates) containing a soil mixture of 1:1:1 ratio of topsoil:sand:peat and maintained in the greenhouse.

The greenhouse-grown plants (parents and progenies) were cut once at the 10% bloom stage (no screening performed) and subsequent regrowth was harvested and used to evaluate post-harvest fungal resistance. The parents were screened in three runs (1997 Dec. 02, 1998 Jan. 12 and 1998 Feb. 17) while the progenies were screened in four runs (1997 Oct. 14 and the same three dates as the parents).

Plant material was harvested from all the greenhouse pots when most of the plants were at late bud to early bloom stage. Harvested plant material was bulked from all plants within each pot to give a total of four replicates per parent or progeny. Leaves from each bulk were plated on a petri plate for a total of four petri plates (replicates) per parent or progeny. During the plating of progenies, efforts were made to sample leaves from each of the 8 to 10 plants in a pot; however, a slightly different set of progeny plants may have been screened in each sampling.

The fungal species *A. repens* was used in this study. Past research on screening for post-harvest fungal resistance in alfalfa used a combination of *Aspergillus* species, since mold in hay usually involves several fungal species (Undi et al. 1997). Although these earlier screening studies identified resistant and susceptible genotypes, there was often a high degree of variability in the results, possibly due to different species dominating each trial. To simplify the procedure and decrease screening variability caused by interspecific competition within each petri plate, *A. repens* was chosen for this research. *A. repens* is the predominant fungal species causing molding in hay bales (Undi et al. 1997) and grows over a wide range of temperatures and moisture levels. Babij (1997) also showed that *A. repens* gave the widest range of resistance and susceptible responses of five *Aspergillus* species in experiments with four alfalfa genotypes.

### Inoculum Culture and Storage

The isolate of *A. repens* (var. *columbaris* ATCC 557129) used for this study was acquired from American Type Culture

**Table 2.** Analysis of variance for percentage of leaf area with post-harvest fungus (*Aspergillus repens*) coverage among progenies derived from a diallel cross among seven parents

Source of variation	Degrees of freedom	Mean squares	Probability	Proportion relative to sum of squares of crosses
Progenies	41	308.48	0.001	100.00
GCA	6	1457.82	0.001	69.15
SCA	14	104.87	0.283	11.61
Reciprocals	21	115.84	0.171	19.23
Error	123	88.14		—

Collection (Rockville, MD). The freeze-dried isolate was revived by preparing a conidial suspension in a 0.1% Tween 80 (polyoxyethylenesorbitan monooleat) (Mallinckrodt Specialty Chemicals, Paris, NY) 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. These cultures became the base stock inoculum from which subcultures were grown for each screening trial.

### Inoculum Preparation

Inoculum for each experiment consisted of conidia prepared by adding 10 mL of a sterile 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 PDA 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) and 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 and 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 then the pellet resuspended in 0.1% Tween 80 solution to a concentration of approximately  $1-3 \times 10^5$  spores mL<sup>-1</sup>, as determined by haemocytometer counts.

### Screening Procedure

Details of the screening procedure were described by Wittenberg et al. (1998). Plates containing dichloran (2,6-dichloro-4-nitro-aniline) with 18% glycerol agar (DG 18) were prepared in advance with 18–20 mL of the medium per plate. Harvested plants were placed in bags and transported to the laboratory. Fully developed trifoliate leaves (without insect bites, blemishes or tears) were removed from individual plants and placed onto the DG 18 medium (adaxial side down) using the intact petiole as a “handle” to cover the plate without leaf overlap (four to six per plate). The leaves were placed so 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 a suspension of  $1-3 \times 10^5$  conidia per plate using a Nalgene aerosol bottle. The concentrations varied ( $1-3 \times 10^5$  conidia per plate) between the screening trials depending on the number of conidia harvested.

The plates were recapped and incubated at 25°C in the dark. Plates were monitored daily for fungal growth, and leaves were visually assessed for the percentage of leaf area covered by mycelia using the James (1971) scale. The 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 both vegetative and reproductive mycelia with active conidial production.

### Experimental Design and Statistical Analysis

Plates were randomly distributed within replicates in the laminar flow hood during inoculation and in the incubation chamber. Thus, the experimental design during inoculation and incubation was a randomised complete block with four replicates. Analysis of variance (ANOVA) was performed using the Proc GLM procedure in SAS (SAS Institute Inc. 1998). The parents and progenies were analyzed separately. For both the parents and progeny, there were significant run by entry interaction effects ( $P < 0.05$ ), but a graphical analysis of the results showed that interaction effects were mainly due to scaling differences between runs rather than to changes in rank among entries. Therefore, for combining ability analyses, results were averaged across runs and analyzed following Griffing's (1956) Model I, Method III using software written by Burow and Coors (1994). We used Model I, wherein the parents were considered as a fixed effect, because the parents had undergone prior selection, and Method III because we were not interested in estimating heterosis.

## RESULTS AND DISCUSSION

There were significant differences among progenies in the percentage of leaf area covered by *A. repens* mycelial growth (Table 2). Partitioning of the variation among progenies into general combining ability (GCA) and specific combining ability (SCA) components revealed a preponderance of additive compared with nonadditive genetic effects for this trait. The GCA sum of squares accounted for two-thirds of the variation among crosses, while the sum of squares due to SCA and reciprocal crosses together accounted for only one-third of the variation (Table 2). Furthermore, the mean square term for GCA was significant ( $P \leq 0.01$ ) and mean squares attributed to SCA and reciprocal crosses were not significant ( $P > 0.05$ ).

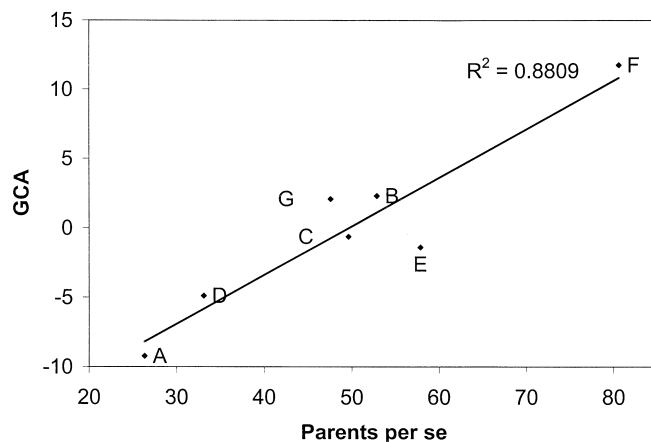
Estimates of GCA effects were compared to determine the relative contribution of each parent toward post-harvest fungal (*A. repens*) resistance or susceptibility among the progenies (Table 3). Negative GCA effects indicated a relative contribution toward resistance, while positive GCA

**Table 3. Mean and general combining ability (GCA) effects for percentage of leaf area with post-harvest fungus (*Aspergillus repens*) coverage for progenies among seven alfalfa parents**

Parents	Parents <sup>z</sup>							Mean	GCA effects of parents
	A	B	C	D	E	F	G		
A	–	46.13	40.36	35.87	33.34	44.29	42.62	38.42	–9.23**
B	–	–	48.16	44.91	50.03	57.23	50.45	49.97	2.31
C	–	–	–	42.24	42.61	60.14	46.01	47.02	–0.63
D	–	–	–	–	41.92	52.13	49.12	42.75	–4.90**
E	–	–	–	–	–	53.59	44.35	46.24	–1.41
F	–	–	–	–	–	–	68.05	59.43	11.77**
G	–	–	–	–	–	–	–	49.74	2.09

<sup>z</sup>Reciprocal crosses not presented because reciprocal effects were not significant (Table 2).

\*\*Significantly different from zero ( $P \leq 0.01$ ). LSD for comparing differences between means of the progenies are 13.23 ( $P = 0.05$ ) and 17.37 ( $P = 0.01$ ). LSD for comparing differences between means of progenies averaged across parents are 2.89 ( $P = 0.05$ ) and 3.77 ( $P = 0.01$ ).



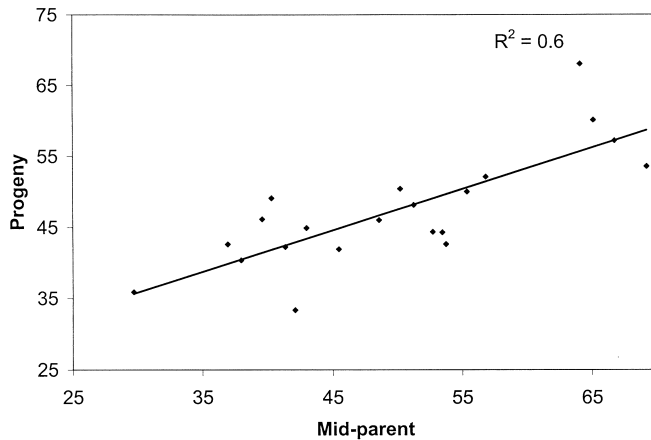
**Fig. 1.** Relationship between the general combining ability (GCA) and parent per se values for percentage leaf area covered with post-harvest *Aspergillus repens* in a 7 × 7 diallel cross of alfalfa.

effects indicated contribution toward susceptibility. Significant ( $P \leq 0.01$ ) GCA effects were found for parents A, D and F. Parent A was the most resistant of all the parents (Table 1) and its progeny supported the least fungal growth (Table 3). Conversely, Parent F was the most susceptible of all the parents, and progeny involving this parent supported the most fungal growth. The above trend was confirmed by the significant ( $P \leq 0.01$ ) positive relationship between the GCA and parental values (Fig. 1) and between the progeny and mid-parent values (Fig. 2) for percentage of leaf area covered by *A. repens* mycelial growth.

These results confirm previous reports of variation among alfalfa cultivars for resistance to post-harvest fungal growth (Babij 1997). They also suggest that the progress reported from selection for post-harvest *A. repens* growth in alfalfa may be due to heritable differences (Kimbeng et al. 2000). More importantly, the results indicate that breeding procedures designed to exploit additive genetic effects could be effective, at least for this set of parents, in developing alfalfa populations that support less fungal growth during the post-harvest period. That SCA effects were not significant is of little consequence, since a large number of parents are usually selected for synthesizing alfalfa cultivars to prevent inbreeding depression. Thus, it would be difficult to model SCA effects while developing synthetic alfalfa cultivars. Recurrent phenotypic mass selection, where genotypes are

selected after repeated screening, should be effective in improving resistance to post-harvest fungal growth. Initial screening could use *A. repens* as the fungal inoculum source, but it will be essential to screen the finished product against a combination of post-harvest fungal species and monitor fungal growth under actual hay storage conditions.

In this and other laboratory screening trials (Wittenberg et al. 1998; Kimbeng et al. 2000), inoculum rates were deliberately set higher than spore loads that naturally occur in alfalfa bales (Undi et al. 1997) to expedite the screening process. Consequently, although genotypes displayed significant differences in leaf area colonised on days 4 and 5 of incubation, colonization approached 100% for all genotypes after about 10–15 d of incubation. The practical application of this screening procedure was substantiated using mini-bales incubated under conditions that simulated commercial hay storage (Babij 1997). In the mini-bale study, mold growth was significantly higher on bales made from genotypes that allowed more fungal growth in laboratory leaf screening trials compared to genotypes that supported less growth. Little is known about the physical and/or chemical factors in the plant that influence the growth of the fungus, but fungal growth rate was consistently slower for some genotypes. Additionally, whole plant extracts from these genotypes impeded fungal growth in petri plates (Babij 1997; Kimbeng et al. 1997). In conclusion, culti-



**Fig. 2.** Relationship between the progeny and mid-parent values for percentage leaf area covered with post-harvest *Aspergillus repens* in a 7 × 7 diallel cross of alfalfa.

vars selected to support less post-harvest fungal growth, when used in conjunction with proper hay management techniques, could enhance the ability to harvest and store high-quality hay.

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**Babij, V. 1997.** Post-harvest resistance to fungal attack in alfalfa (*Medicago sativa* L.). MSc. thesis. University of Manitoba, Winnipeg MB.

**Breton, A. and Zwaepoel, P. 1991.** Succession of moist hay microflora during storage. *Can. J. Microbiol.* **37**: 248–251.

**Burow, M. D. and Coors, J. G. 1994.** Diallel: a microcomputer program for the simulation and analysis of diallel crosses. *Agron. J.* **86**: 154–158.

**Ferullo, J. M., Vezina, L. P., Castonguay, Y., Allard, G., Nadeau, P., Willemot, C. and Laberge, S. 1996.** Post-harvest alteration of *in-vitro* translatable mRNA population in alfalfa (*Medicago sativa* L.). *Crop Sci.* **36**: 1011–1016.

**Griffing, B. 1956.** Concept of general and specific combining ability in relation to diallel crossing systems. *Aust J. Biol. Sci.* **9**: 463–493.

**Halimi, E. S., Rowe, D. E. and Pratt, R. G. 1998.** Responses of alfalfa to stem-tip inoculations with five isolates of *Sclerotinia trifoliorum*. *Crop Sci.* **38**: 1180–1182.

**James, C. 1971.** A manual of assessment keys for plant diseases. Publication 1458. Canada Department of Agriculture, Ottawa, ON.

**Kimbeng, C. A., Babij, V., Smith, S. R., Jr. and Wittenberg, K. M. 1997.** Post-harvest fungal resistance in alfalfa: potential mechanisms and simulated bale studies. Page 42 in Proceedings of the 25th Central Alfalfa Improvement Conference, 1997 July 16–18, LaCrosse, WI.

**Kimbeng, C. A., Babij, V., Smith, S. R., Jr. and Wittenberg, K. M. 2000.** Alfalfa resistance to post-harvest *Aspergillus* species: response to selection. *Can. J. Plant Sci.* **80**: 755–763.

**Lacey, J. 1975.** Potential hazards to animals and man from microorganisms in fodder and grain. *Trans. British Mycol. Soc.* **65**: 171–184.

**Mauch, F., Hadwiger, L. A. and Boller, T. 1984.** Ethylene: symptom, not signal for the induction of chitinase and beta-1,3-glucanase in pea pods by pathogens and elicitors. *Plant Physiol.* **76**: 607–611.

**Mohanty, G. P., Jorgensen, N. A., Owens, M. J. and Voelker, H. H. 1969.** Effect of molding on the feeding value and digestibility of alfalfa hay. *J. Dairy Sci.* **50**: 990.

**SAS Institute, Inc. 1998.** SAS user's guide for personal computers. Release 6.12. SAS Institute, Inc., Cary, NC.

**Smith, J. E., Anderson, J. G., Dean, S. G. and Berry, D. R. 1981.** Biochemistry of microcycle conidiation. Pages 329–356 in G. T. Cole, and B. Kendrick, eds. *Biology of conidial fungi*, vol. 2. Academic Press, Toronto, ON.

**Teng, J. and Whistler, R. L. 1973.** Cellulose and chitin. Pages 249–269 in L. P. Miller, ed. *Phytochemistry*, vol. I. Van Nostrand Reinhold, New York, NY.

**Undi, M., Wittenberg, K. M. and Holliday, N. J. 1997.** Occurrence of fungal species in stored alfalfa forage as influenced by moisture content at baling and temperature during storage. *Can. J. Anim. Sci.* **77**: 95–103.

**Wittenberg, K. M., Smith, S. R., Jr., Katempa-Mupondwa, F. and Yang, J. F. 1998.** Screening methodology for post-harvest fungal resistance in alfalfa. *Can. J. Plant Sci.* **78**: 481–488.

