POST-HARVEST RESISTANCE TO FUNGAL ATTACK IN ALFALFA (*MEDICAGO SATIVA* L.)

A Thesis
Submitted to the Faculty
of
Graduate Studies
The University of Manitoba

by

Vivian Babij

In Partial Fulfilment of the
Requirements for the Degree
of
Master of Science
Department of Animal Science

July 1997

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is an important crop which is grown primarily for hay production. Weather does not often allow for proper drying conditions, and hay is frequently stored at moisture contents conducive for mold growth, which reduces hay quality. A leaf screening procedure was developed for the detection of alfalfa genotypes resistant or susceptible to fungal growth after harvest. The objectives of these studies were to verify the screening procedure; to determine characteristics associated with fungal growth; and to select a susceptible and a resistant population for future studies. Verification studies conducted to validate the screening procedure included comparisons of fungal growth when whole plants were cut, chopped and stored under warm humid conditions in the laboratory, and simulated field trials that evaluated mold accumulation during wilting and bale storage. Four genotypes that were previously identified as having low, variable and high susceptibility to fungal growth after harvest were used for the verification studies. The extent of fungal growth on plant material that was chopped and incubated under conditions conducive to molding was measured by glucosamine analysis. The glucosamine results supported plant resistance ratings as determined by the screening procedure. Minimal fungal growth across all genotypes in the simulated field wilting study made comparative evaluation impossible, however, lab-scale bales produced from plant material classified resistant to fungal attack had lower ($P<0.05$) glucoamine levels after 9 days of storage than did lab-scale bales produced from plant material identified to be susceptible using the screening procedure. Comparisons of five *Aspergillus*

species as inoculants in the screening procedure suggest that the monitoring of colonization by *Aspergillus repens* and *A.versicolor* on fresh leaf material can be useful for screening alfalfa genotypes for resistance to molding during harvest and storage. Post-harvest studies were conducted with alfalfa genotypes that had different susceptibilities to colonization by *Aspergillus* species, to investigate chemical, microbial and physical factors that may contribute to mold inhibition after harvest. The results of the chemical extraction studies were inconclusive, however, darkened zones observed in the disc diffusion assay studies indicated the presence of an inhibitory compound. The susceptible alfalfa genotype had higher ($P<0.05$) populations of lactic acid bacteria and total bacteria than the resistant genotypes. No difference between genotypes was observed in water activity of fresh plant material, and in the water-soluble carbohydrate concentration at cutting; however, drying rates were lower ($P<0.05$) for the more resistant genotypes. A screening of 1144 genotypes representing 22 cultivars of alfalfa for post-harvest resistance to fungal growth demonstrated that there is variation in their susceptibility to colonization by fungi. Three screening trials were conducted and a final population of three resistant and three susceptible genotypes were selected for future study.

FORWARD

The format of this thesis is prepared in manuscript style. Manuscript I discusses the studies conducted on verifying the screening procedure. Manuscript II examines factors related to post harvest decomposition, and Manuscript III describes the population selection using the screening procedure. The authors of of manuscripts I and II are Babij, V. I., Wittenberg, K. M. and Smith, S. R. Jr. Manuscript I will be submitted to Crop Science and manuscript II to Can. J. Plant Sci.

ACKNOWLEDGMENTS

I would like to take this opportunity to acknowledge a number of individuals who helped me throughout this endeavour. My sincere gratitude to my advisor, Dr. K. M. Wittenberg, for patience and assistance in completing my research and thesis. Special thanks to my committee members, Dr. S. R. Smith Jr. for advise throughout the project, and Dr. R. R. Marquardt for suggestions in constructing the final copy of the thesis. Appreciation is extended to Dr. G. Crow, and Dr. L. Onischuk for statistical assistance.

I would like to thank the Animal Science technical staff; Janice Haines and Peter Mills for completing the chemical analysis, and Angela Oswald for conducting the microbial studies. Thanks is extended to Matt Freuhm and the greenhouse staff for their assistance in maintaining the plants used in this project.

I would like to acknowledge that funding for this work was provided by the Canada/Manitoba Agreement on Agriculture Sustainability. Plants used for this project were supplied by Dr. S. R. Smith Jr. from Plant Science, and the fungal cultures by Dr. J. Mills at Agriculture Canada.

Thank you to the many students who offered support and assisted me in this venture.

I dedicate this thesis to my husband Gerry, and our sons, Markian and Chris, thank you for your support and encouragement.

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ABBREVIATIONS

A_w	water activity
cfu	colony forming units
cm	centimeter
°C	degrees Celsius
CV	cultivar
d	day
df	degrees of freedom
DM	dry matter
geno	genotype
Glu	glucosamine
h	hour
ha	hectare
HPLC	high performance liquid chromatography
kg	kilogram
l	leter
LAB	lactic acid bacteria
M	molar
MEA	malt extract agar
MC	moisture content
ml	millileter
mm	millimeter
MRS	media for evaluating lactic acid bacteria
MS	mean squared
N	normality
NIRS	near infrared reflectance spectroscopy
rep	replicate
res	resistant
RMI	relative mold index
SE	standard error
SS	sum of squares
susc	susceptible
ul	microliter
um	micrometer
UV	ultraviolet
WSC	water soluble carbohydrate

GENERAL INTRODUCTION

Alfalfa (*Medicago sativa* L.) which is grown primarily for hay production, is the most important forage crop in Canada. Well preserved hay is produced by drying the forage to 85% dry matter, however, poor wilting conditions often do not allow for proper drying. High moisture conditions during wilting and storage, support increased numbers of post-harvest decomposition organisms (Undi 1995) that reduce hay quality by lowering nutritive value, digestibility, and acceptability (Kaspersson et al. 1984; Undi 1995).

Development of alfalfa with fungal resistance is evident in the living plant, however, developing alfalfa cultivars with resistance to post-harvest fungal invasion is a new area of investigation. An effective method for screening alfalfa for post-harvest resistance would be essential in the development of resistant cultivars. Resistant cultivars could allow harvest of hay at a higher moisture content. A screening procedure for selecting alfalfa plants that show reduced fungal attack after harvest was recently developed by Wittenberg et al. (accepted). The procedure involves plating fresh alfalfa leaves onto agar, inoculating with a spore suspension consisting of five *Aspergillus* species, incubating at 25 °C, and assessing the area of fungal biomass coverage on the leaves.

Studies conducted by Wittenberg et al. (accepted) demonstrate that alfalfa (*Medicago sativa* L.) plants show variation in susceptibility to post-harvest fungal attack. Potential mechanisms that reduce molding after harvest include; chemical inhibitors, epiphytic microflora, substrate constituents, and physical characteristics. Plants have been naturally selected with physical characteristics that protect against attacking microorganisms; for example, the plant surface is difficult to penetrate. Microorganisms secrete enzymes that break down plant surfaces

(Thomashow and Weller 1995), however, some plants respond by producing compounds that inhibit microbial growth (Carr and Klessig 1989). The physical makeup of the plant tissue is important when forages are harvested for storage. Material that retains moisture prolongs conditions that are condusive for spoilage organisms, therefore, a forage that achieves a dry matter (DM) greater than 80% in a short time frame is preferred for hay production. The water-soluble carbohydrates are important in preservation as these molecules are water binding which reduces the amount of water available for microbial growth (Rockland and Nishi 1980). Also, microorganisms that coexist in nature are often beneficial to plants as they often produce chitanase an enzyme that inhibits fungal growth (Cherin et al. 1995). These factors are present in many living plants and may influence the amount of biodegradation that occurs in the harvested plants.

The objectives of this work were to verify the screening procedure developed by Wittenberg et al.(accepted), to determine factors that are associated with reduced mold growth in harvested alfalfa plants, and to select resistant and susceptible populations for further studies. The first objective involved verification trials to compare the results from the leaf screening procedure with actual fungal growth that occurs on leaf and stem material from the same plants when subjected to wilting and storage conditions conducive to molding. Three studies were conducted with four genotypes, ranging in sensitivity to post-harvest molding: a modified screening procedure which tested individual *Aspergillus* species as well as a combined inoculum; whole plant incubation which measured the actual mold accumulation at different stages of incubation; and a field wilting and bale storage trial which determined mold accumulation over time.

The second objective was to investigate possible mechanisms that could account for variation in susceptibility to fungal attack in cut alfalfa (*Medicago sativa* L) forage. Two populations of alfalfa, each having genotypes that range in susceptibility to post-harvest *Aspergillus* growth, were used to compare the presence of chemical inhibitors, epiphytic microflora populations, and whole plant a_w, WSC, and wilting rates.

The third objective was the use of the leaf screening procedure to select two populations of alfalfa genotypes that either showed resistance to post harvest invasion, and susceptibility to post-harvest fungal invasion. The selection was from an initial population of 1144 plants representing 22 cultivars. These populations were selected for a future study to determine trait heritability.

REVIEW OF LITERATURE

ALFALFA AN IMPORTANT CROP IN CANADA

Alfalfa, *Medicago sativa* L, is the most important forage species grown in Canada and USA (Barnes et al. 1988). French colonists first introduced alfalfa to Ontario in 1871, and its use gradually spread throughout eastern Canada. A winter-hardy strain, Grimm, was first grown in Alberta in 1918, and was soon cultivated throughout western Canada (Smoliak et al. 1990).

Alfalfa cultivation has increased from less than 0.4 million ha in 1941 to 1.8 million ha in 1961 in Canada (Barnes et al. 1988). Today alfalfa is grown for seed, pasture, the dehydration industry, silage and green manure. Approximately 20,851 ha of alfalfa was grown for pedigreed seed in 1996 (Canadian Seed Growers' Association, 1996), and according to the 1991 census 5.8 million ha of tame hay was grown in Canada, much of which consisted of alfalfa (Statistics Canada 1992).

Exported hay, mostly alfalfa, was valued at $25,476,896 in 1994, representing a small fraction (approximately 0.2%) of the total forage produced (Statistics Canada, 1995). The majority of hay produced is still used by the producer as livestock feed or sold within Canada. Alfalfa is preferred for hay production as it dries quicker than most legumes (Macdonald and Clark 1987).

Development history

Nine distinct sources of *Medicago* germplasm were introduced into North America from different regions of the world. The winter hardy sources descended from Siberia, Russia, India, Peru, Turkey, and Europe, and the non dormant sources originated from Africa, Chile, India

and Peru (Kidwell et al. 1994). All varieties grown in North America are classified as *M. sativa* because they readily intercross. Varieties with winter hardy characteristics that are typical of the species *M. falcata* would be classified as *M. sativa* spp. *falcata*.

Early alfalfa improvement was primarily for development of winter hardy strains from one germplasm. Between 1941 and 1955 emphasis was on development of bacterial wilt resistance and winter hardiness with two or three of the original germplasms. Alfalfa breeding has been an active area of research resulting in improved strains for forage yield, rangeland and pasture use, forage quality, and multiple disease and pest resistance. During the mid-1970s resistance to Phytophthora root rot caused by the fungus *Phytophthora megasperma* Drechs f.sp.*medicaginis*, and anthracnose caused by *Colleotrichum trifolii* Bain was routinely incorporated into new cultivars (Barnes et al. 1988). Resistance to Fusarium wilt caused by *Fusarium oxysporium* Schl. f. sp. *medicaginis* Weiner, and Verticillium wilt caused by *Verticillium albo-atrum* Reike and Berth, were the main focus in alfalfa development in the 1980s (Barnes et al. 1988). Page et al. (1992) reported that 100 cultivars are available with varying levels of resistance to Verticillium wilt. Development of fungal resistance is evident in the living plants, however, developing alfalfa cultivars with some resistance to post harvest fungi is a new area of investigation.

The number of recognized cultivars in Canada and USA increased from 33 in 1955 to more than 250 in 1985 (Barnes et al. 1988). Development of alfalfa strains for reduced post harvest fungal growth has not been considered in the past. Breeding varieties of plants that would resist saprophytic organisms during field wilting and during storage would be beneficial as this could potentially reduce fungal load in hay fed to livestock and increase harvest

flexibility.

Adaptation

Established alfalfa plants are very competitive and are well adapted to a wide range of climate and soil conditions. They are medium lived perennial plants that do best in deep loam with high lime content. These plants are drought tolerant and can tolerate some saline conditions, but not high alkaline soils. Alfalfa is very sensitive to soil acidity, and intolerant to flooding (Smoliak et al. 1990).

FUNGAL GROWTH DURING FIELD WILTING AND IN STORED FORAGE

Dry matter (DM) losses and quality decline during hay harvest and storage is primarily due to decomposition fungi. As well as reducing the quality of hay, molding often causes health problems in humans and animals from allergies and mycotoxins (Nash and Easson 1977). Fungal growth in storage often determines the quality. If good quality harvested forage is stored under conditions that permit fungal growth the product will deteriorate.

Growth Requirements

The rate of degradation is dependent on many factors. The requirements for fungal growth include available substrate, a_w, temperature, pH, oxygen levels, light conditions, and time (Moore-Landecker 1991; Cahagnier et al. 1993; Griffin 1994).

Moisture

Like all organisms, fungi require high concentrations of water as a solvent. The water requirements of microorganisms should be defined in terms of the water activity (a_w) in the environment. Water activity is the ratio of the vapour pressure of the substrate to the vapour

pressure of pure water at the same temperature:

$$a_w = p/p_o$$

where p = vapour pressure of solution and p_o = vapour pressure of water (Jay 1992). Water activity is the free water that is available for microbial growth, and at a low a_w, the number of organisms that can survive is reduced. Moisture present in forages and food products consists of both free water and bound water. Bound water is chemically bound to other molecules and is not available for microbial growth. Molds are by far the most numerous and diverse group of microorganisms found in low a_w habitats (Hocking 1991). Storage of dried products with an a_w of 0.70 or lower generally inhibits mold growth, however growth of some *Aspergillus* species has been reported at an a_w as low as 0.62 (Pitt 1981). Magon and Lacey (1984) isolated a number field and storage fungi from freshly harvested and stored wheat. These organisms included *A. versicolor*, *A. amstelodami* and *A. repens* which these authors classified as xerophiles having the ability to grow at an a_w of 0.85 or less. Fresh cut plant material has an a_w of 0.99 which is reduced by drying (Jay 1992). A small change in moisture content (MC) may cause a large change in a_w; for example polished rice at 15% MC has an a_w of 0.71, whereas at 16% MC, the a_w is 0.78 (Scott 1957). Observations by Albert et al. (1989) indicated that alfalfa stem and leaf material differ in a_w at similar moisture contents. Their findings showed that the a_w of stem material at 21.8% MC is higher, 0.85, than for leaf material at 22.8% MC which had an a_w of 0.80.

Nutrition

Fungi absorb soluble simple sugars and amino acids that surround the hyphae. Fungi also secrete enzymes that digest starch, cellulose and proteins into compounds that can be absorbed

through the cell walls. The digestive enzymes are specific and are only secreted when the fungi is in a substrate that can be utilized (Moore-Landecker 1991). For example in forage material, the fungi will utilize the available water-soluble carbohydrates before excreting enzymes that break down starch and cellulose into simple sugars for absorption. Fungal growth depends on the ability of the fungi to digest its substrate, and whether the fungi can obtain all the essential growth factors such as vitamins and minerals from the substrate, or synthesize growth factors from the components within the substrate.

pH

Most fungi grow well when the initial pH is in the range of 4 - 7, however, most show optimal growth in acid conditions at about pH 5 (Pitt 1981). Studies conducted by Pitt and Christian (1968) showed that low pH inhibits most microflora, for example most *Aspergillus* do not grow at pH 3.8 when the a_w is between 0.60 to 0.90. The pH of a medium is often drastically changed by fungal growth, and media that resists pH change may be inhibitory to some fungi (Griffin 1994). Substrates with a high buffering capacity resist pH change. Goering and Van Soest (1972) examined forages during heating, and found that alfalfa with an initial pH of 5.4 did not change over a period of 24 hours at 80°C. Tested alfalfa bales in storage had a pH of 6.5 with no change in pH over a 60 day period (Undi et al. 1996). Griffin (1994) suggests that availability of nutrients in the substrate, metabolic components of the cell wall, and the outer membrane surface are probably affected by the pH. In conditions of high a_w and high pH, bacterial competition reduces the amount of fungal degradation (Pitt 1981). Most vegetables have a higher pH than fruit and, therefore, are more prone to bacterial than fungal spoilage (Jay 1992).

Temperature

Temperature is important in enzyme and chemical activities and, therefore, influences the organism's growth rate. The temperate regions, which represents most of Canada, support the majority of mesophilic fungi which thrive at temperatures between 10 and 40 °C with optimum growth between 15 and 30 °C. Forage wilting temperature is dependant on the surrounding temperature but is usually at optimum for mesophiles and consists mainly of the genera *Alternaria, Cladosporium, Colletotrichum, Fusarium, Phaeoseptoria, Phoma,* and *Ascochyta* (Breton and Zwaenepoel 1990). Psychrophiles grow in cooler environments with optimum growth between 10 and 15° C but most having the ability to grow at lower temperatures (Jay 1992). Thermophiles are found in high temperature environments such as in dung and compost, with optimum growth at temperatures near 40 °C and above (Moore-Landecker 1991). Stored bales have temperatures ranging between 35 and 60 °C depending on the moisture content. The fungi that invade forage at these temperatures are *Absidia, Rhizopus, Aspergillus* and *Humicola* (Breton and Zwaenepoel 1990).

Oxygen

Most fungi are obligate aerobes, requiring oxygen for growth. Some fungi are facultative anaerobes which means they can grow with or without oxygen, such as the water molds that grow in polluted streams and sewage sludge (Moore-Landecker 1991; Griffin 1994). Without exception, conidial fungi which include *Aspergillus species* require oxygen for growth, and most are unaffected by the concentration of oxygen until the oxygen content is below 4.2% (Pitt 1981).

Light

Fungal growth occurs at varying light intensities, however, strong light inhibits most fungal growth. Light affects reproduction, some sporulate in darkness while some sporulate in light only, and some reproduce in both light and dark conditions (Moore-Landecker 1991). Forage windrows when conditioned during sunny weather show reduced mold accumulation because of fast moisture reduction, and the intense light may be another factor contributing to lower molding.

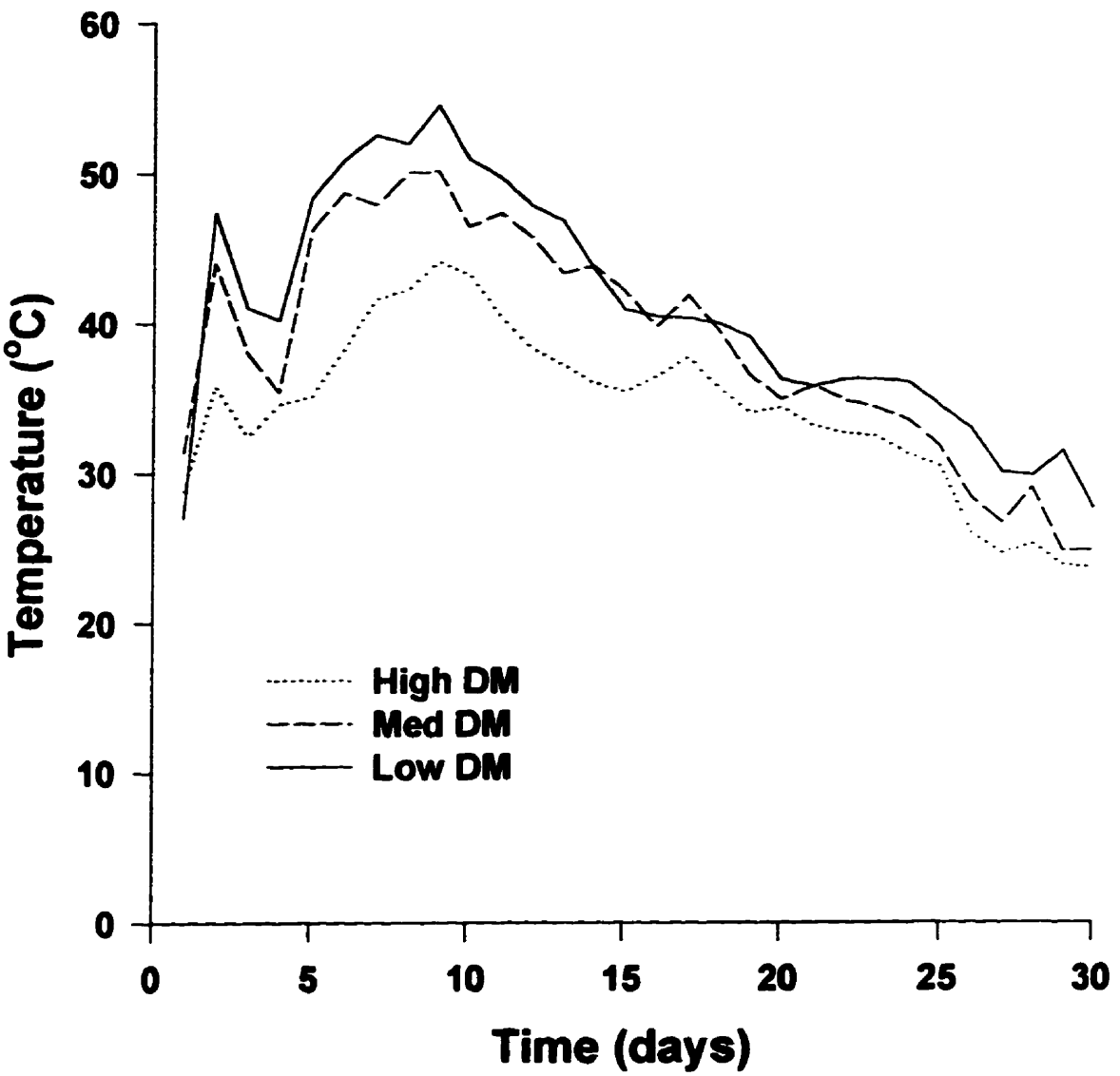
Order of succession

Biodegradation occurs by an orderly sequence of fungi growing in a saprophytic situation. Succession may be related to nutritional factors; simple monomers (monosaccharides and amino acids) are utilized first, followed by disaccharides and simple polymers, and finally complex polymers, for example, lignin, keratin, chitin, etc. are degraded (Garraway and Evans 1984). Succession may be related to temperature, for example organic material in a compost pile or stored hay goes through a number of temperature changes (Figure 1). The invading saprophytes change with each temperature variation (Garraway and Evans 1984). Succession may also be due to a combination of both nutritional and environmental factors.

Fungal spores are common contaminants of both raw and processed agricultural commodities. Spores are ubiquitously found in the soil, water, air and vegetation (Cousin 1995). Most genera found on low moisture substrates such as hay reproduce by asexual spores or conidia which are produced on specialized hyphae called conidiophores (Cahagnier et al. 1993). These molds are classified as Deuteromycotina or Fungi Imperfecti, and consist of mainly *Aspergillus* and *Penicillium* genera (Pitt 1981).

The predominant fungi at baling are field fungi which are usually soil borne and consist mainly of the genera *Alternaria, Cladosporium, Colletotrichum, Fusarium, Phaeoseptoria, Phoma,* and *Ascochyta* (Breton and Zwaenepoel 1991). Low microbial populations exist in hay baled at 84% DM (Gregory et al. 1963). Baling at less than 80% DM can reduce leaf loss and reduce field curing time (Rotz et al. 1988), both of which decrease nutritional losses in the forage. The DM affects the temperature of the stored hay (Figure 1) which in turn affects the type of organisms it supports. Field fungi are eliminated when temperatures approach 50°C, and thermotolerant storage flora invade which include genera; *Absidia, Rhizopus, Aspergillus* and *Humicola* (Breton and Zwaenepoel 1991). The fungal organisms found in bales stored at 64.1-66.2% DM were *Absidia* spp., *Mucor* spp., *Eurotium nidulans* and *Aspergillus fumigatus* in studies conducted by Undi et al. (1996). Cherney et al. (1987) also reported that thermophilic fungi mostly, *Aspergillus* species, are dominant in grass forage stored at 60% DM. As the dry matter increases and the temperatures decrease in storage, xerophilic and thermoresistant microorganisms become the decomposers. These consist of *Paecilomyces variotii, Emericella nidulans* and *Eurotium amestelodami* (Breton and Zwaenepoel 1991; Undi 1995).

Figure 1. Temperature changes during the storage of alfalfa forage baled at Low (64.1-66.2%), Medium (71.0-73.2%), and High (75.4-77.4%) DM contents (Undi et al. 1996).


60
50
40
30
20
10
0
Temperature (°C)
0
5
10
15
20
25
30
Time (days)
High DM
Med DM
Low DM

SCREENING METHODOLOGY

A screening procedure developed to determine post-harvest resistance was recently developed by (Wittenberg et al. accepted). The procedure involves plating fresh alfalfa leaves onto agar, inoculating with a spore suspension, incubating at 25° C, and evaluating the percent leaf area covered by fungal biomass. The evaluations are ranked; the genotypes with high evaluations are considered more susceptible, and low evaluations are considered less susceptible to molding.

The initial work in developing the screening procedure used a spore suspension of *Aspergillus* and *Penicillium* species. Work conducted by Undi et al (1996) indicated that *Aspergillus* species were more prevalent than *Penicillium* species in alfalfa bales at baling and in storage, therefore, only *Aspergillus* species were used in the current studies.

Aspergillus

Aspergillus spores are ubiquitous in nature, and exist wherever decomposing organic matter is present. The health aspects, morphology and growth characteristics of these organisms are described in this section.

There are hundreds of recognized *Aspergillus* species of which 20 species have been identified to cause human infection (Rogers and Kennedy 1991). Any disease caused by any member of the *Aspergillus* genus is classified as aspergillosis. *Aspergillus fumigatus* accounts for 95% of fungal infections in humans (Davies and Sarosi, 1996). Rogers and Kennedy (1991) documented that aspergillosis could be due to: ingestion of food contaminated with mycotoxins, allergy to conidia, or growth of the organism in the body orifices, colonization on debilitated tissues within the respiratory tract, superficial infections of the skin, invasive, inflammatory

infection of the lungs, and systemic fatal disease. *Aspergillus* are opportunistic fungi, and disease due to this organism is increasing because of the increase in immunosuppressed individuals (Waldorf 1989).

The fungus *Aspergillus* was first described by Micheli in 1729 as having structures similar to aspergillums (Davies and Sarosi, 1996). An aspergillum is a perforated globe like devise at the end of a stem used to sprinkle holy water at religious ceremonies. The *Aspergillus* species are classified into groups with similar growth characteristics. There are a number of keys available in the literature that describe the morphology and growth characteristics of a number of *Aspergillus* species, for example Pitt (1981); Hocking (1991); Roger and Kennedy (1991). The five *Aspergillus* species used in the screening procedure are grouped and described in Table 1.

The genus *Aspergillus* is one of the most important fungi in biodegradation as they are adapted to a wide range of a_w and temperatures (Table 2). Most *Aspergillus* organisms are thermotolerant, meaning the spores will survive high temperatures, and still have the ability to germinate when the temperature is suitable. The species belonging to *A. glaucus* group, which includes *A. repens* and *A. glaucus*, are the major decomposers during storage of forage (Kaspersson et al. 1984). This group is succeeded by *A. versicolor, A. flavus, A. nidulans* and *A. fumigatus* (Kaspersson et al. 1984)

Table 1. Characteristics of four *Aspergillus* groups used in the post-harvest fungal resistance screening procedure when grown on Czapek-Dox agar (Roger and Kennedy 1991).

Group and organism	Colony colour	Conidiophore	Conidial head	Conidia
Fumigatus group *A. fumigatus*	white to green to grey green slate grey with age	length ~300um width 5-8 um smooth walled greenish	strongly columnar compact	globose to subglobose elliptical in some isolates echinulate to rarely smooth diameter 2.5-3.0 um
Flavus group *A. flavus*	Yellow to yellowish green to green with age	length 400-850 um (rarely to 2.5 um) uncoloured thick walled coarsely roughened	Radiate, splitting into columns with age	Globose smooth to echinulate diameter 3-6 um most 3.5-4.5 um
Versicolor group *A. versicolor*	Variable, white to yellow orange-yellow tan to yellowish green	length 500-700 um width 5um, merging into funnel shape at vesicle; smooth walled uncoloured or yellowish	Radiate	Globose strongly to delicately echinulate diameter 2-3 um
Glaucus group *A. repens* *A. glaucus*	Green to yellow-green to grey-green orange-yellow areas of cleistothecia with age	length 0.5-1.0 um smooth walled uncoloured broadens toward the vesicle	Radiate to loosely columnar	Ovate to globose spinulose diameter 5-6.5 um

Table 2. Temperature, °C, and A_w optimums and ranges for growth of *Aspergillus* spp. used for Screening Methodology (Wittenberg et al. accepted)

Group and Organism	Temperature °C[y]		a_w	
	Optimum	Range	Optimum	Minimum
Fumigatus group				
A.fumigatus	40-55	12 - 55	0.95[y]	0.82[z]
Flavus group				
A.flavus	35-37	6 - 45	> 0.80[x]	0.78[z]
Versicolor group				
A.versicolor	27	9 - 39	0.98[y]	0.78[z]
Glaucus group				
A.repens	25-27	4 - 40	0.95[y]	0.71[z]
A.glaucus	33-35	10 - 40	0.96[x]	0.62[z] 0.75[x]

[z] Pitt 1981
[y] Pitt and Hocking 1985
[x] Christensen 1987

MOLD INHIBITION

Molding is a problem in forage preservation as environmental conditions and harvesting procedures inoculate the plant material with a wide range of fungi and bacteria. Natural barriers such as physical make-up, chemical inhibitors, and antagonistic microflora are known to protect living plants. A combination of defences may play a role in protecting cut plant material from microbial attack. For example a chemical that inhibits mold, may initially be present but as MC is reduced the activity of this chemical also maybe reduced. Inhibition characteristics are reviewed here as well as their relationship to saprophytic decomposition of harvested plants.

Microbial

Living plants coexist naturally with a variety of microbes most of which have no harmful or beneficial effect to each other, and in some symbiotic relationships one or both organisms benefit. A third type of plant microbe relationship is parasitic and ultimately harmful to the plant. Parasitic organisms can be further broken down into two classifications, biotrophs which depend on the plant host being alive, and necrotrophs which live only on dead tissue (Garraway and Evans 1984). The decomposers of cut plant material, necrotrophs, also known as saprophytes, primarily consist of fungal organisms.

Naturally occurring microorganisms often have a protective effect on the plant by competing against harmful microflora for available substrate; direct antagonism by antibiosis; or by triggering systemic host plant defence responses (Thomashow and Weller 1995). Biological models of systemic acquired resistance documented by Neuenschwander et al. (1996) include; exposure to fungal mycelia, bacterial or viral pathogens which induces resistance to unrelated pathogens in tobacco; cucumber diseases caused by either *Colletotrichum lagerarium*,

or *Cladosporium cucumerinum* which accumulate resistance in cucumbers when exposed to another pathogen, and *Arabidopsis* (a crucifer) which becomes resistant to one fungus when induced by a second fungus. Other examples of resistance has been reported in potatoes, tomatoes, soybeans, red clover, pearl millet, rice and alfalfa. Alfalfa development has incorporated systemic resistance to a number of fungal related diseases as was previously discussed.

Researchers, Janisiewicz and Bors (1995) demonstrated that two antagonists bacterium, *Pseudomonas syringae*, and yeast, *Sporobolomyces roseus*, together were effective in controlling *Penicillium expansum* growth in post harvest wounded apples. These studies demonstrated that *P. syringae* depleted most of the nitrogen while *S. roseus* utilized generous amounts of carbon, therefore depleting the nutrients essential for germination of *P. expansum*. *Pichia guilliermondii*, a post-harvest biocontrol yeast, inhibits *Botrytis cinerea* on citrus and temperate fruits by a combination of nutrient competition and antagonism (Wisniewski et al. 1991). Initially the yeast and the fungus compete for nutrients in the wounded fruit, but once established *P. guilliermondii* attaches to the hyphae of *B. cinerea* and produces citinases and β-glucanases which breakdown the fungus cell walls (Wisniewski et al. 1991). Wisiewski et al. (1991) also suggest that because yeasts produce chitinases and β-glucanases, which are essential in the budding process, they may contribute to controlling growth of fungal decomposers.

Aeromonas and *Serratia* bacterial genera and fungal genera *Gliocladium* and *Trichoderma* produce chitinolytic enzymes (Chernin et al. 1995), and if present on the harvested plant material could control the amount of fungal growth in storage. Chitinolytic enzymes degrade the fungal cell walls, therefore inhibiting fungal growth.

Chemical Inhibitors

Phytoalexins are low molecular weight antimicrobial compounds that are synthesized through secondary metabolism by plants in response to elicitors (Carr and Klessig 1989). Examples of elicitors include fungal degradation enzymes such as cellulases or glucanases, or breakdown products such as cellulose, glucans or fatty acids. Natural elicitors such as heavy metals, wounding, or radiation and UV light also can trigger the production of phytoalexins (Carr and Klessig. 1989). Phytoalexins may be specific to a particular organism; for example, rishitin inhibits *Phytophthora infestans* in potatoes (Tomiyama et al. 1968). Many phytoalexins like chitinolytic enzymes that degrade the fungal cell walls are non-specific.

Induced resistance may be due to natural occurrences such as wounding which could result during strong winds or by insect damage, etc. Damaged areas on wounded plants stimulate a repair mechanism in order to survive. Lignification in wounded wheat leaves has been examined by Pearce and Ride (1981) and Barber et al. (1988). Their studies indicated that chitin, chitosans and ethylene glycol chitin stimulate lignification. Wounding exposes the inner plant tissues to the environmental contaminants that elicit the repair response.

There are many examples of chemical compounds produced by plants that protect the plant against fungal attack. Phytoalexins are produced in rice (*Oryza sativa* L.) when exposed to chitin (Ren and West 1992), and a phospholipid hydroperoxide phytoalexin is produced in soybean seedlings in response to a fungal elicitor (Kendo et al. 1992). Chitanase and β-1,3-glucanase have defence potential against fungi, and many plants produce these two enzymes in response to fungal attack. Harvested immature pea pods exposed to fungal elicitors such as chitosans and glucosamines produce these enzymes (Mauch 1984). Heitz et al. (1994)

documented that tobacco plant bacteria associated with tobacco mosaic virus stimulate the production of antifungal enzymes which have glucanase, chitinase and lysozyme activity. Phytoalexin induction by unrelated organism interaction also occurs in many plants. Smith et al. (1995) documented that a spore suspension of *Helminthosporium sativum*, a nonpathogenic fungus, when applied to the leaf surface of lucerne, 3 major phytoalexins; pterocarpan, sativan, and vestitol, are diffused onto the leaf surface (Smith et al. 1995). These same phytoalexins might be produced in resistant alfalfa genotypes at higher levels which reduces molding in the harvested plants. Yoshida et al. (1993) reported that gramine, an indole protoalkaloid found in barley leaves inhibits spore germination. Ursolic acid from epicuticular waxes of Brazolian Cerrados (*Jacaranda decurrens*) is known to restrict fungi (Varanda et al. 1992).

Physical Inhibitors

The first barrier for microbial attack is the physical and chemical makeup of the outer plant surface (Carr and Klessig 1989). The cuticle, an insoluble polyester of C16 and C18 fatty acid derivatives, and the plant cell wall, which consists of cellulose, polysaccharides and proteins, are passive defences in living plant tissue (Carr and Klessig 1989). These defences protect the plant from desiccation as well as act as a waterproof layer. Attacking microflora secrete degradation enzymes that break down the cell walls and outer plant surfaces (Thomashow and Weller 1995). Enhanced lignification in wounded wheat leaves occurs upon exposure to chitin (Barber et al. 1989), and the subsequent lignin is more difficult to degrade. The plant structural make up is the first barrier to microorganisms, but a_w, drying rates and factors that enhance or impede drying are also important in susceptibility to molding.

Water activity does not relate directly to MC, as indicated in the section on a_w. Stem and

leaf material have very different a_w at similar MC. Water-soluble carbohydrates (WSC) at high concentrations lowers the a_w of a substrate, as water molecules bind to sugars thus the water is unavailable to the microbe (Rockland and Nishi 1980). The water-soluble carbohydrates also can influence the epiphytic microflora by acting as an available energy source which may influence saprophytic fungal activities.

The forage drying rates may vary, and influence the amount of microbial growth. Most materials dry down in two or three phases; a constant rate period with rapid moisture loss, followed by one or two variable rate periods when moisture loss is slow (Potter 1986; Macdonald and Clark 1987). Forage that dries slowly prolongs a damp environment which is suitable for microbial growth, therefore, rapid drying is preferred.

Drying rate of forage depends on climatic conditions; which include temperature, humidity, air velocity, plant factors; such as leaf to stem ratio, stomatal and cuticular resistance, and water retention forces, and management factors like windrow type, forage cutting treatment, and cutting time in relation to weather (Macdonald and Clarke 1987).

High leaf to stem ratios reduce the drying time because leaves have more stomata than stems (Rotz 1995) which allows for quicker moisture loss. The first drying phase is approximately five hours, and in this time frame 20-30 % moisture is lost through the stomata openings (Harris and Tullberg 1980). The stomatal density varies between cultivars, growing conditions and plant age which are factors that affect moisture loss during the first phase of drying (Harris and Tullberg 1980). The time the stomata remain open after cutting may also vary between genotypes. During the second phase of drying, plant metabolism continues until the moisture content falls below 45% (Nash 1978). Water is held more tightly in the plant

material in the third phase of drying (Moser 1995), and moisture loss is very slow.

The cuticle covers the outer plant surface, and acts as a barrier to water loss. Wieghart et al. (1983) indicated that older plant material has a less protective cuticle than younger plants, therefore more mature plants dry quicker. Cuticle thickness and integrity may vary between genotypes allowing rapid water loss in some genotypes and not in others.

Moisture absorption can occur in forages when environmental conditions such as high humidity, dew formation, and rainfall occur (Rotz 1995). Drying time is increased by windrowing as the surface area is reduced, however, shattering losses during baling are reduced when the forage is in windrows (Macdonald and Clark 1987). Weather conditions are important in forage drying, for example sunny conditions can reduce the drying time by a few days (Rotz 1995).

TESTING METHODS

Laboratory testing of forage samples in a more controlled setting than field wilting and storage has a long history which includes studies on the impact of moisture, effectiveness of preservatives, relevance of water activity with fungal growth, and generation of storage temperature curves. Assessments of fungal biomass has shifted from visual estimates to more quantitative methods which measure fungal constituents that can be translated into values of mold accumulation. Various selective media and preparation of inoculum are discussed. Techniques for chemical extractions and physical analysis are also reviewed.

Laboratory forage storage test units

A number of evaluation systems have been used to simplify the complexities of field

experiments, or to deal with limited amounts of forage material when conducting hay harvest and storage trials.

Early laboratory experiments

Festenstein et al. (1965) documented that as early as 1907 laboratory experiments were conducted by Miehe using a wire container filled with hay, and surrounded by cotton insulation to minimize moisture and heat loss. Later, Dewar flasks were used to characterize the moisture and aeration during self-heating process in stored hay (James et al. 1927). Use of Dewar flasks allowed for easy and even mixing of the chopped hay with water which is difficult to achieve in baled hay (Festenstein et al. 1965). These authors found that little heating occurred below 29% water content, and that samples with a moisture content between 29-34% self heated to temperatures between 33 and 55 °C, and samples of greater than 40% moisture heated to 65 °C.

Rotz et al. (1988) used plastic bags containing treated hay samples to analyse microbial inoculants as preservatives. This study was parallelled with a field experiment which had comparable results. A disadvantage to this method is that the plastic bag creates an anaerobic environment which may inhibit some microbial growth normally observed in stored hay.

Two leter glass containers were used in a study conducted by Moore et al. (1985) in which the effect of ammonia on quality changes in orchard grass was analysed. A deflated plastic bag placed over the container allowed for increased volume caused by the addition of NH_3. A closed system was necessary to prevent loss of NH_3.

Humidity chambers consisting of sealed glass jars were used in a study conducted by Albert et al. (1989) to examine different spoilage properties of alfalfa stems and leaves. These experiments used saturated salt solutions to create chambers of different equilibrium relative

humidities.

Baron et al. (1991) compressed forage material, using a mechanical press to achieve suitable density, into polyvinyl chloride tubes (10.2 cm in diameter by 35.6 cm in height) in a study testing propionate preservative. The tube tops remained open to ensure aeration. The tubes were insulated and spaced to prevent heat transfer between samples and to minimize environmental heat loss. This laboratory system resulted in temperatures comparable to small square field bales, but lower temperatures than round bales at similar moisture levels. Similar temperatures should generate similar fungal populations.

Laboratory Mini bales

A baling system designed by Coblentz et al. (1992) allows for laboratory experimentation with mini bales requiring small quantities of forage. These bales are made by packing the forage material into a 10 cm x 10 cm x 36 cm form, and compressing to the required density with a hydraulic press. This system also allows the production of replicate bales of equal density which is a difficult parameter to produce in the field (Coblentz et al.1993). Also this system allows for interaction between hay packages which occurs in conventional stored bales.

According to Coblentz et al. (1994) laboratory mini bales are sensitive to ambient storage conditions, therefore, controlled storage environments may be needed to optimize temperature responses. Baron et al. (1991) indicated that insulation of laboratory hay packages, allowing for aeration and moisture release, are important in experimental design for simulating field conditions. Coblentz et al. (1994) found that mini bales did not maintain the elevated internal temperatures to the extent of conventional bales of the same MC and density. These authors found that by increasing the density of the lab bales by 1.5 to 2 fold, the temperatures were

comparable to conventional bales, however, due to Maillard reaction, damage to proteins was greater in lab scale bales compared to conventional bales at the same moisture content.

Mold determination methods

Mold determination is important for evaluating the amount of contamination in a product and the resultant product quality. There are strict regulations as to the amount of mold that may be present in export commodities, therefore, it is critical to have reliable methods for quantification.

The relative mold index (RMI) is a visual assessment conducted by two appraisers based on colour, amount of mycelial mat and dust present in a sectioned bale (Wittenberg 1991). Bales are scored for colour, dust and mold independently, and each parameter is scored from 0 to 5. A score of 0 represents no mold growth and a score of 5 represents 100% molding. The criteria used by Nash and Eason (1977) for evaluation of several bale sections was scored 0 for no mold, 1 under 25%, 2 for 25 - 50%, 3 for 50 - 75%, 4 for 75 -100%, and 5 for 100% mold growth with brown discoloration. Similarly, Roberts et al. (1987) used ratings from 0 to 5 on cored samples with 0 representing no mold growth, and 5 having mycelial mat throughout the bale. Relative mold index has commonly been used to evaluate forage in the past, but it is a subjective method, and only represents what is visible to the unaided eye, therefore, other methods that are more accurate are preferred.

Plate counts are often used to determine viable microorganisms within a sample. Core samples are stomached or blended, serial diluted, plated onto selective media, and incubated. After a predetermined incubation period the colony forming units (CFU) are counted (Gregory

et al. 1963; Lacey and Dutkiewicz. 1976; Cherney et al. 1987). One disadvantage to this technique is that it is an evaluation of viable reproductive units, and not the fungal biomass present in the sample. Another drawback is that it may not be a true representation of mold growth as some spores may require a longer germination period than allowed in the incubation time. An advantage is that the types of invading mold can be identified.

Near infrared reflectance spectroscopy (NIRS) uses absorption properties of chemical components to analyse a sample. Studies conducted by Roberts et al. (1987) indicated that glucosomine analysis and NIRS for fungal biomass determination have comparable results. This method provides quick results, involves simple sample preparation, and is environmentally friendly because no chemical pretreatment is required. The disadvantages include high instrumentation cost, and low sensitivity to minor components (Shenk et al. 1979, Shenk et al. 1981). Osborne et al. (1993) indicated that the major limitation of NIRS in agricultural testing is the dependence on less precise older chemical methods of analysis.

Ergosterol and chitin are two chemical compounds present in fungi and not in plant material. Analysis for these compounds have been used to measure the mold contamination of agricultural commodities and foods (Cousin 1995). Ergosterol is a component associated with the cell plasma membrane, and is a predominant sterol found in nearly all fungi (Seitz et al. 1977). Chitin is a major constituent of fungal cell walls (Wu and Stahmann 1975).

Ergosterol extraction involves; blending a ground sample with methanol, decanting the supernatant after centrifugation, rewashing with methanol and centrifuging. The two supernatants are combined and refluxed with KOH and ethanol. This mixture is diluted with water and extracted with petroleum ether (Seitz et al. 1977). The extracted compounds are

measured by high performance liquid chromatography, UV spectroscopy, or thin layer chromatography. Ergosterol assay is more sensitive at detecting low levels of fungi than analysis for chitin, and is considered a good method for measuring fungal growth on solid media and soils (Cousin 1995). A quick detection method for ergosterol was developed by Martin et al. (1990). The compound is extracted with cold (4°C) absolute ethanol for 2-3 min. using a mortar and pestle, centrifuged, decanted and rewashed with a second aliquot of ethanol. The extract is then analysed using high performance liquid chromatography. Comparable biomass values were obtained using this method and chitin analysis (Martin et al. 1990). The time required for this analysis is one hour which is much less than for chitin determination which requires approximately eight hours. Ergosterol is easily oxidized, therefore, the levels decrease over long storage periods (Roberts 1995). He also indicated that mycelia and ergosterol accumulate simultaneously for the first period of storage, and that future studies may show that ergosterol accurately represents mold in hay.

Chitin is a polysaccharide of β-(1-4) N-acetyl-D-glucosamine which is a structural component present in fungal cell walls. Chitin is frequently measured to estimate mold contamination of foods, grains and forages. Plant material does not contain chitin, but some chitin is present on bacterial spores, insects and germ plasm, however, the amount is minimal, and actual interference is negligible (Roberts et al. 1987). The glucosamine content varies between species. Schmit et al. (1975) reported that the conidial walls contain about 12% glucosamine, and the mycelial cell walls consist of about 9% in the fungus *Neurospora crassa.*

Chitin must be hydrolysed into units with acid, alkali or enzymes that yield products that can be measured. Acid hydrolysis cleaves the β-(1-4) glycosidic bonds, removes the acetyl

group, and produces glucosamine. Alkali hydrolysis deacetylates chitin to produce chitosan, and enzyme hydrolysis with chitanase yields chitosan (Cousin 1995). Of the three methods, acid hydrolysis is the preferred method because yields are better, and less processing time is required. Plassard et al. (1982) compared glucosamine recovery using acid and alkaline hydrolysis and found yields to be 90% and 50% respectively. The time required for good recovery using acid hydrolysis varies for different fungal organisms. Wittenberg et al. (1989) found that for good glucosamine recovery of forage fungi a hydrolysis time of seven hours at 121°C in 6 N HCl was needed. Glucosamine concentration can be determined colorimetrically, using gas chromatography, high performance liquid chromatography, or an amino acid analyser (Cousin 1995).

Selection of Media

The essential substances required for microbial growth include carbohydrates, proteins, fats, amino acids, vitamins, minerals and water. The ingredient requirements vary between organisms, and by altering those ingredients, the growth for a particular organism can be selected. Environmental change such as pH, a_w, available oxygen, and incubation temperature can act as a selective pressure in culturing microorganisms. Also, inhibitory compounds can be used to select or restrict growth of interfering organisms; for example, antibiotics restrict bacteria, and rose bengal and dichloran both suppress spreading of fungi.

Dichloran 18% glycerol (DG18) is used for enumeration of xerophilic fungi (Pitt and Hocking 1985). Dichloran 18% glycerol agar contains 2 ug l^{-1} dichloran (2,6-dichloro-4-nitroaniline) to inhibit the spreading growth of *Eurotium* colonies (Beuchat and de Daza 1992), and 18% glycerol (v/v) to reduce the a_w to 0.96 (Hocking 1991).

Total bacteria counts can include a large number of organisms, therefore, a rich media with a high a_w and neutral pH of 7 is most suitable. Many types of nutrient agar formulations are available for determination of total bacteria counts (Collins and Lyne 1976). A media known as MRS, named after the individuals, de Man, Rogasa and Sharpe, who formulated and tested it, is commonly used for enumeration of lactic acid bacteria (de Man et al. 1960).

Yeast is enumerated with nutritionally rich Malt Extract Agar (MEA) which is restrictive to bacteria because the pH is near 5 (Pitt and Hockings 1985). This media contains 1.25% malt extract which is a rich carbon source, and yeast extract which contains nitrogen and essential growth factors. Rose bengal chloramphenicol and dichloran rose bengal chloramphenicol medium are used for enumerating both yeast and fungi (Pitt and Hocking 1985). Rose bengal and dichloran restrict the diameter growth which prevents overgrowth of molds (Jarvis 1973), and chloramphenicol inhibits bacteria growth (Pitt and Hocking 1985).

Inoculum Preparation

Microcycle culture technique developed by Smith et al. (1981) is a fast method of culturing spores. This method involves subculturing the fungi, and incubating spores on potato dextrose agar at temperatures 5-10 degrees greater than optimum growth temperature for the initial 24 hours and then reducing to the optimum temperature for the remainder of the incubation period. The initial warm temperatures are used to break dormancy which causes the spore to swell, and germinate into a sporulating structure without the need for vegetative growth. This culturing method allows production of sufficient spores in a shortened time period.

Chemical Extractions

Chemicals commonly used for extracting compounds from plant material include

methanol, ethanol, chloroform, ethyl acetate, cyclohexane, and acetone. Experimentation with a number of extracting solutions is usually required to determine the best solution for extracting the compound of interest.

Yoshida et al. (1993) conducted studies on gramine, a compound that inhibits spore germination, found on barley leaves. Extraction solutions in these studies for removing gramine included; acidic 30% ethanol (0.1 M HCl-EtOH, 7:3), methanol- ammonium hydroxide (100:1), chloroform, and water acidified with 0.1M HCl to pH 4 and pH 6. These researchers reported that gramine extraction with acidified water at both pH levels was less than 0.04% of total gramine. Gramine extraction with water in these experiments was ineffective because a dipping procedure was employed, and the natural wax layer on the leaves prevented gramine dissolution.

More complicated methods for extracting rishitin and phytuberin, two phytoalexins produced in potatoes, are described by Tomiyama et al. (1968); Lyon (1972) and Hildenbrand and Ninnemann (1994). The Lyon (1972) method involves homogenizing with methanol, centrifuging, retaining the residue, extracting the residue with aqueous methanol (60% v/v), extracting three times with chloroform, drying the chloroform fraction, redissolving in acetone, and further purifying with thin layer chromatography. The method described by Tomiyama et al. (1968) homogenized fresh potato slices with methanol chloroform (4:2 v/v), and dried material with a chloroform, methanol, water solution (5:3:2, v/v). The residues of the chloroform fraction were extracted with acetone, dried, and reconstituted with 80% ethanol. This solution was again concentrated and the residue dissolved in hexane. Hildenbrand and Ninnemann (1994) modified the Lyon method for extracting phytoalexins from potatoes. They stirred the tissue with ethanol for one hour, centrifuged, and re-extracted the residue. After the

second centrifugation step the supernatants were combined and reduced *in vacua*. The residue was dissolved in a water ethanol solution (2:3, v/v), extracted three times with dichloromethane, and dried over anhydrous sodium sulphate. The dried extract was reconstituted with 1.5 ml of dichloromethane and analysed by gas chromatography (Hildenbrand and Ninnemann 1994).

Schmit et al. (1975) described the extraction process for three major phytoalexins; pterocarpan, sativan, and vestitol that are diffused onto the lucerne leaf surface. This procedure involves washing the leaf material with 40% ethanol-water solution, subjecting the wash solution to vacuum infiltration, repeating the ethanol-water wash step, shaking on a dark orbital shaker for two hours, rewashing with distilled water, combining all wash solutions; extracting with ethyl acetate (0.5 ml solvent to 1 ml extract solution), drying the solvent portion, and reconstituting with acetonitrile for compound detection using high-performance liquid chromatography.

Disc diffusion assay

The microorganisms are used as an indicator in disc diffusion assays by producing a clear zone of no growth around the disc in the presence of an inhibition compound. Simple assay designs for unknown compounds, such as penicillin, streptomycin, vitamin B_{12}, etc. are described by Heweitt (1977). More recently Madhyastha et al (1994) developed a disc diffusion type assay to detect the mycotoxins food and feed stuffs.

SUMMATION

Selection for resistance to mold diseases has been achieved in living alfalfa plants. The screening procedure developed by Wittenberg et al. (accepted) may have potential as a selection tool for developing cultivars that resist molding after harvest. An effective method for screening

alfalfa for post harvest fungi could be beneficial in developing cultivars with improved resistance to saprophytic organisms during field wilting, and potentially could allow the harvest of hay at a higher moisture content. Baling hay at a higher moisture content would reduce quality decline due to inclement weather and leaf loss. Variation in resistance may be affected by many factors; the natural microflora could influence the concentration and succession of fungal populations and the extent of biodegradation, chemical inhibitors could control invading organisms therefore reducing decomposition, and the physical make-up may restrict microbial entry and may limit the amount of spoilage.

MANUSCRIPT I: Screening procedure verification for post-harvest fungal resistance in alfalfa (*Medicago sativa* L.)

ABSTRACT

Improved alfalfa (*Medicago sativa* L.) cultivars have been selected for forage yield and quality, rangeland and pasture use, multiple disease and pest resistance, however, developing alfalfa cultivars that have resistance to post-harvest fungal invasion is a new area of investigation. Forage quality decline due to decomposition organisms is a problem in field wilting and storage of hay. Cultivars that resist fungal growth after harvest would reduce losses. A leaf screening procedure was developed for the determination of alfalfa genotypes resistant or susceptible to fungal growth after harvest. The objective of this work was to determine if the leaf screening procedure is an effective method for selecting alfalfa genotypes resistant to fungal attack after harvest. The following verification studies were conducted: comparisons of fungal growth when whole plants were cut, chopped and stored under warm humid conditions in the laboratory; and simulated field trials that evaluated mold accumulation during wilting and bale storage. Four genotypes previously identified as having low, variable and high susceptibility to fungal growth after harvest were used. Extent of fungal growth, as measured by glucosamine analysis, for plant material chopped and incubated under conditions conducive to molding, supported plant resistance ratings as determined by the screening procedure. Minimal fungal growth across all genotypes in the simulated field wilting study made comparative evaluation impossible, however, laboratory-scale bales produced from genotypes having resistance to fungal attack had lower glucosamine concentration, 2.47 mg g^{-1} DM, after 9 days of storage than those produced from genotypes identified as susceptible, 3.82 mg g^{-1}DM. These studies suggest that monitoring the colonization of *Aspergillus* species on fresh leaf material is a valid procedure to screen alfalfa genotypes for susceptibility to molding during harvest and storage.

Keywords: alfalfa, mold, glucosamine, fungal resistance, harvest, storage

Abbreviations: d, day; DM, dry matter; ha, hectare; WSC, water soluble carbohydrates

INTRODUCTION

Alfalfa breeding has been an active area of research for over 75 years, and has resulted in improving cultivars for forage yield, rangeland and pasture use, forage quality, and multiple disease and pest resistance. During the mid-1970s, resistance to Phytophthora root rot caused by the fungus *Phytophthora megasperma* Drechs f.sp. *medicaginis*, and anthracnose caused by *Colletotrichum trifolii* Bain, were first incorporated into new cultivars (Barnes et al. 1988). Resistance to Fusarium wilt caused by *Fusarium oxysporium* Schl. f. sp. *medicaginis* Weiner, and Verticillium wilt caused by *Verticillium albo-atrum* Reike and Berth, were the main focus of alfalfa development in the 1980s (Barnes et al. 1988). Page et al. (1992) reported that 100 cultivars were available with variable levels of resistance to Verticillium wilt in the early 1990s. Development of alfalfa with fungal resistance is evident in the living plant, however, developing alfalfa cultivars that have resistance to post-harvest fungal invasion is a new area of investigation.

Alfalfa is grown for seed, pasture, the dehydration industry, hay, silage and green manure. Twenty-one thousand ha of alfalfa were grown for pedigreed seed in 1996 (Canadian Seed Growers' Association 1996), and according to the 1991 census 5,753,511 ha of tame hay, most of which consisted of alfalfa, was grown in Canada (Statistics Canada 1992).

Poor field wilting conditions or high moisture storage supports increased numbers of post-harvest decomposition organisms (Undi 1995) that reduce hay quality by lowering nutritive value, digestibility, and acceptability (Nash and Easson 1977; Kaspersson et al. 1984). The wilting organisms are primarily field fungi which are usually soil borne and consist mainly of

the genera *Alternaria, Cladosporium, Colletotrichum, Fusarium, Phaeoseptoria, Phoma,* and *Ascochyta* (Breton and Zwaenepoel 1991). These field organisms are eliminated when temperatures approach 50°C, and thermotolerant storage fungi invade, which include *Absidia, Rhizopus, Aspergillus* and *Humicola* (Breton and Zwaenepoel 1991). Microbial studies conducted by Undi et al. (1996) on stored baled alfalfa indicated that by day 10 the total fungi counts were 5.0×10^4 colony forming units g^{-1} in low DM alfalfa bale storage. These post-harvest decomposition organisms not only reduce forage quality, but result in health problems for both the livestock and individuals who handle hay (Gregory et al. 1963; Lacey 1975; Nash and Easson 1977).

An effective method for screening alfalfa for post harvest fungi would be beneficial in developing cultivars with improved resistance to saprophytic fungal organisms during field wilting, and potentially could allow harvest of hay at a higher moisture content. Baling hay at a higher moisture content reduces quality decline due to inclement weather and leaf loss.

A leaf screening procedure to determine post-harvest fungal resistance in alfalfa was recently developed (Wittenberg et al. accepted). The procedure involves plating fresh alfalfa leaves onto agar, inoculating with a spore suspension consisting of 5 *Aspergillus* species, incubating at 25 °C, and evaluating the percent fungal biomass coverage on the leaves.

The objective of this research was to compare the fungal resistance ratings observed with the screening procedure with the actual fungal growth that occurs on the same genotypes when subjected to wilting and storage conditions conducive to molding. Three studies were conducted with 4 genotypes, which ranged in sensitivity to post-harvest molding: a modified leaf screening procedure which tested individual *Aspergillus* species as well as a combined inoculum; whole

plant incubation which measured the actual mold accumulation at different stages of incubation; and field wilting and bale storage which determined mold accumulation over time. Results from each of these studies was compared to post-harvest fungal susceptibility as determined by the screening procedure.

METHODS AND MATERIALS

Plant populations

Four alfalfa (*Medicago sativa* L.) genotypes selected from a population of 40 genotypes from the cultivars arrow, algonquin, cimmaron and rambler were used for these experiments. The four genotypes rated as most resistant (R-1), most susceptible (S-4), or intermediate in susceptibility (V-2 and V-3) to fungal colonization when exposed to an inoculum consisting of *Aspergillus* spore suspension in a screening procedure outlined by Wittenberg et al. (accepted). Two sets of plants were used: Set 1 consisted of cloned genotypes which were established in 1993 in the greenhouse and planted in the field in five blocks with five plants per genotype in each block, in a randomized block design. Set 2 consisted of cloned plant material established in the greenhouse and transplanted into four field blocks in the spring of 1995. Each field block consisted of 24 clones per genotype planted in three rows with a 75 cm row spacing and a 37.5 cm spacing between plants within the row. Clones were maintained in the field for the duration of the experiments.

Inoculum

Work conducted by Undi et al. (1996) indicated that *Aspergillus* species were more prevalent than *Penicillium* species in stored alfalfa, therefore, for these studies only *Aspergillus* species were used. Freeze dried cultures of *Aspergillus flavus* DAOM 214728, *Eurotium*

amstelodamii DAOM 215938 (also referred to as *Aspergillus glaucus* in this text) *Aspergillus fumigatus* DAOM 196947, and *Aspergillus versicolor* DAOM 213352 were obtained from the Canadian Collection of Fungus Cultures, Agriculture and Agri-Food Canada, Ottawa, ON and *Eurotium repens* var *columnaris* 36963 (also referred to as *Aspergillus repens* in this text) was obtained from American Type Culture Collection, Rockville, Maryland. The organisms were subcultured on 10-20 ml of Potato Dextrose Agar (PDA) in 50 or 100 ml screw cap flasks, and incubated at warm temperatures for 24 hours to break dormancy (Smith et al. 1981) and an additional 2 - 4 days at a lower temperature. *Aspergillus glaucus*, *A. repens* and *A. versicolor* were initially incubated at 36°C for 24 hours, then the temperature was reduced to 25°C; and *A. flavus* and *A. fumigatus* were incubated at 41°C to break dormancy, after which temperatures were reduced to 25°C.

The conidia of each species was harvested by adding 10 ml of 0.1% Tween 80 (Mallinckrodt Specialty Chemicals Paris, NY.) solution, and 10-30 sterilized glass beads to each flask which was then agitated 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, and the resulting pellet re-suspended in 0.1% Tween 80 solution to a concentration of approximately 1 x 10^6 spores ml^{-1}, determined using a haemocytometer. The plates were inoculated with a spore suspension (10^6 spores ml^{-1}) of the five individual species of *Aspergillus*; *A. flavus*, *A. glaucus*, *A. repens*, *A. versicolor*, and *A. fumigatus*, as well as a combined inoculum at a rate of 1 - 3 x 10^5 spores per plate. The combined inoculum consisted of equal aliquots of each of five *Aspergillus* species and represented the inoculum used as per the screening procedure protocol.

Screening Procedure using individual and combined inoculum

Dichloran 18% glycerol agar (DG18) plates were poured in advance using 18 - 20 ml media plate^{-1}. This media contains 2 ug l^{-1} dichloran (2,6-dichloro-4-nitroaniline) to inhibit the spreading growth of *Eurotium* or *Aspegillus* colonies (Beuchat and de Daza 1992), and 18% glycerol (v/v) to reduce the a_w to 0.96 which inhibits bacteria growth (Hocking 1991). This media is commonly used for enumeration of xerophilic fungi (Pitt and Hocking 1985) which have the ability to grow in low moisture products such as hay.

Three leaf screening trials were conducted using leaves collected from Set 1 plants. Trial 1 was conducted on July 13, 1995 using plant material that was at 50% bloom except for S-4 which was in the bud stage. Trial 2 started on July 11, 1996 and used plant material in the early bud stage for all genotypes except for S-4 which was in the vegetative phase. Trial 3 began on August 30, 1996 with plant material in the bud stage. The plants were individually harvested for all three trials 9 to 10 cm above the soil surface using scissors, placed in sterile bags, and transported to the laboratory. A sanitation step, which involved washing the scissors, and the operator's gloves with 95% ethanol, was performed after each plant was cut to avoid cross-contamination. Fully developed leaves, without insect bites, tears or blemishes, were removed from the harvested plant and placed onto a sterile paper towel. Four to 6 leaves from each plant were placed onto prepared agar plates such that the entire leaf surface made contact with the agar. Plates were prepared in duplicate, and sprayed with 1 - 3 x 10^5 spores per plate using a nalgene aerosol bottle. The plates were recapped, and incubated at 25 °C. The evaluation was conducted when 70% of all the plates showed colonization on 20% of the leaf area. The rating system for alfalfa leaf spot disease (James 1971) was used as a guide to determine the percent

leaf area with fungal coverage.

Whole plant incubation

Three whole plant incubation trials were initiated on the same day as the leaf screening trials with the same plant material. Four g of leaf and stem material was collected from each clone, chopped and placed into eight empty petri plates, and incubated at 25 °C to allow growth of epiphytic microflora. Two plates for each clone were removed from incubation and frozen (-20 °C) on days 0, 5, 8, and 11. Plates and contents were freeze dried for DM, WSC and glucosamine determinations. The procedure steps were the same for all three whole plant incubation trials except a sampling change in trial 2; duplicate plates for each clone were removed from incubation and frozen (-20 °C) on days 0, 6, 8, and 11.

Field wilting and bale storage

First cut plants from set 2 were used for trial 4. All genotypes were in late bud stage except S-4 which was in early bud stage. Ninety-six plants from 4 different blocks representing each genotype were cut on June 17, with a cordless grass shear (Makita, Electric Works Ltd. Japan). The combined plant material from the four blocks was placed into one windrow for each genotype. The windrows were arranged so that the height, width and density were the same for all genotypes. Genotypes with greater volume of material had longer windrows. Four forage samples (100 - 200 g) per windrow were taken one hour post cutting, and daily at 9:00 and 16:00 hours until the moisture content was approximately 45%. Samples were collected in sterile sample bags, and frozen (-20°C) for dry matter and glucosamine analysis. Dried forage was used to produce 12 laboratory-scale bales per genotype that were at 55 % DM. A laboratory-scale baling system designed by Coblentz et al. (1993) was used with some bale

production modifications. The forage was packed into the bale chamber by folding the material into 10 cm lengths, compressed with a hydraulic press to a density of 437 kg m^{-3}, and then tying the compressing material with twine. Genotypes V-3 and S-4 were baled 4 days after cutting and stored at 4°C until all the bales were ready for incubation. Bales from V-2 and R-1 were made 6 d post cutting due to a slower wilting rate. Bales were randomly placed and then tied together into 2 stacks; stack one had 4 bales each of R-1, V-2 and S-4, V-3 bales were missing due to shortage of plant material; and stack two had four bales of each genotype. Three sides of each stack were insulated with additional laboratory-scale bales, and the remaining three sides with styrofoam to minimize heat and moisture loss. The insulator bales and styrofoam were secured around the stack with twine. A Trendicator (Model 400A; Doric Scientific Div. Emerson Electric Co. San Diego, CA) was used to monitor bale temperatures during bale storage using thermocouple wires which had been inserted into one bale genotype^{-1} stack^{-1}. This was to ensure that incubation temperatures corresponded to normal hay stack temperature changes (Undi 1995). The stacks were placed in an incubator so that the temperature could be controlled if needed. Four bales representing each genotype were core sampled using a Penn State core sampler, and the samples were frozen (-20°C) on day 0, 9, and 24 of incubation for dry matter and glucosamine analysis.

Trial 5 began on July 22 using second cut plants, at 10% bloom except for genotype R-4 which was in the bud stage. The same conditions and procedures were used as in trial 4 with bales for genotypes V-3 and S-4 constructed on day 4, and V-2 and R-1 prepared on day 5. Due to the shortage of material, stack one consisted of 2 R-1 bales, 3 S-4 bales, 4 V-2 bales and 0 bales for V-3. Bales were removed from the stacks for sampling on d 0, 9 and 24.

Chemical Analysis

Forage samples were weighed before and after freeze drying to determine the DM. The whole plant incubation samples were ground with a Moulinex coffee and spice mill Model 980 (Moulinex Canada Ltd, Toronto ON). The dried field wilted and stored bale samples were ground with a Tecator Cyclotec Model 1093 (Fisher Scientific Ltd. Toronto, ON) using a 1 mm screen. The glucosamine analysis were conducted according to the protocol reported by Wittenberg, et al (1989). A method, using 3,5-dinitrosalacylic acid (Aldrich Chemical Co Milwaukee WS) developed by Solominski et al (1993), was used to determine water soluble carbohydrate content on the dried sample.

Statistical Analysis

Data was analysed using General Linear Models (GLM) (SAS Institute Inc. 1985). The leaf screening procedure data from each of the three trials (1, 2 and 3) was analysed as a 4 by 6 factorial with 4 genotypes and 6 inoculums as the factors of interest. The effect of genotype, inoculum and their interaction were tested with inoculum by clone within genotype as the error term. The combined data from trials 1, 2, and 3 was analysed as a completely randomised block design with each trial representing a block. The three way interaction of inoculum, genotype and trial was used as the error term to test all other factors.

The glucosamine values for whole plant incubation trials 2 and 3 were each analysed for time 0, 5, 8 and 11 days independently. Dry matter at each sample time was used as a covariate, and genotype was tested using 0.7967 clone (geno) and 0.2033 error as the error terms. Initial dry matter and water soluble carbohydrates were analysed as a completely randomised design with genotype, and observations taken at time zero of incubation. Dry

matter loss was calculated for each genotype at each time period in the whole plant incubation studies by the following equation:

[(initial DM (g) - DM (g) at each sampling time)/initial DM(g)] x 100.

The dry matter loss for each time was analysed as a complete random design.

Data from each time of wilting and bale storage was collected by time with 4 samples per time in trials 4 and 5 and analysed as a completely randomised design with genotype as the main effect. Least squares means for factors of interest were compared using an experiment-wise Type 1 error rate of 0.05.

RESULTS AND DISCUSSION

Screening Procedure using individual and combined inoculum

The fungal coverage ranged from 10.05 - 42.89% in trial 1 with inoculums *A. repens* and *A. versicolor* colonizing more leaf material than *A. flavus* and *A. fumigatus* (Table 3). The range for colonization of leaves in trial 2 was from 10.05 - 71.73% for all inoculants, and *A. flavus* and *A. repens* had values of 71.73% and 71.23%, respectively. The highest fungal coverage was observed with *A. repens* (72.52%) and *A. glaucus* (79.47%) in trial 3, and the lowest coverage was with *A. fumigatus* (10.21%). *Aspergillus repens* consistently had high fungal coverage while *A. fumigatus* had low colonization for all trials. Interaction comparisons indicate that *A. repens* had the highest fungal coverage for the 3 trials combined.

There was no inoculum by genotype interaction when the combined data for trials 1, 2, and 3 was analysed indicating that the general ranking of genotypes across inoculant types was similar. However, data from individual trials indicates that the ability to see significant

differences between plant genotypes varied when different inoculant types were used Figure 2. The resistant genotype, R-1, had lower leaf area colonized by fungi in all three trials for inoculants *A. repens*, and *A. versicolor*. Lower fungal coverage on the leaves was observed on the resistant genotype for all inoculums except for *A. fumigatus* in trials 2 and 3.

The resistant genotype had lower fungal coverage than the remaining genotypes comparing the inoculum by genotype interaction with the exception of inoculant *A. fumigatus* (Table 3). These results show that a spread of 15 - 26 % units between resistant and susceptible genotypes was observed for these inoculants. A large range in coverage is preferred as it increases the separation between resistant and susceptible.

These findings indicated that modifying the screening procedure to use either *A.repens* or *A. versicolor* inoculums rather than using a combination of the original five *Aspergillus* species may improve the ability to identify alfalfa genotypes with post-harvest resistance to fungal attack. Incubation was at 25°C which is most suitable for *A. repens* and *A. versicolor* which grow optimally between 25-27°C and at 27°C, respectively (Pitt and Hocking, 1985). If the incubation temperature had been 35°C the ratings for *A. flavus* and *A. glaucus* may have shown greater difference between resistant and susceptible genotypes because these organisms grow best between 35-37°C and 33-35°C, respectively (Pitt and Hocking 1985). The a_w in these trials was not a factor which influenced fungal growth because the *Aspergillus* species used in this procedure grow best at an a_w greater than 0.80, and the a_w of the media and the plant material was 0.96 and 0.99, respectively. The results in the 3 trials demonstrated that *A. fumigatus* was not effective at selecting susceptibility differences. This organism grows best at temperatures between 40 and 55°C, therefore, growth is poor at 25°C, the procedure incubation

temperature. Furthermore, *A. fumigatus* produces minute conidia (2-3.5 um) which become airborne and are easily inhaled. This organism is the causative agent for the majority of allergic and invasive fungal diseases (Roger and Kennedy 1991).

Whole plant incubation

The concentration of glucosamine on day 0 for trial 1 ranged from 1.26 to 1.44 mg g^{-1} (Table 4), which were lower than reported values of 1.50 to 1.60 mg g^{-1} in sampled alfalfa at 30% bloom at the end of wilting (Undi 1995). The glucosamine concentration in this trial was lower because it related to the initial stages of wilting whereas the reported value was for the end of wilting 3 to 4 days after cutting. The plants in trial 1 were at 50 % bloom except for S-4 which was in early bud stage. The glucosamine accumulation on day five ranged from 2.17 for the resistant genotype to 3.05 mg g^{-1} in the susceptible genotype, and on day 11 the concentration had increased to 9.79 and 12.95 mg g^{-1} for resistant and susceptible, respectively. Undi (1995) reported glucosamine accumulation ranging from 2.20 to 3.00 mg g^{-1} on day 14 of bale storage. The high values obtained in this trial indicated that this was a good test to determine if one genotype was more susceptible to molding. No differences were observed for initial DM in this trial (Table 5).

The initial glucosamine ranged from 1.46 to 1.64 mg g^{-1} in trial 2 (Table 4) which were similar to concentrations reported in the previous paragraph at the end of wilting (Undi 1995). Glucosamine accumulation was lower ($P<0.05$) for the resistant genotype than the susceptible genotype on day 5, 8 and 11 of incubation. The susceptible genotype, S-4, in this trial was in the vegetative growth stage which may be a better substrate for fungal growth, therefore, it was not surprising that more fungal accumulation was measured for this genotype. Dry matter

losses, which accounts for the weight of forage and fungal biomass, were significantly higher for the susceptible genotype on day 6 in trial 2 (Table 5). No differences in dry matter loss was observed among these genotypes on days 8 and 11.

The plants were all in bud stage for trial 3 which eliminated the maturity bias that could have influenced results in trial 1 and 2. The initial glucosamine concentrations were in the same range as trial 2, and the same trend in glucosamine accumulation was observed with significant differences measured on days 5, 8 and 11 (Table 4). Differences were not observed for initial DM% and for DM% lost between resistant and susceptible genotypes at each incubation time (Table 5).

Glucosamine concentration increased more rapidly for the susceptible genotype than the resistant genotype in all plate incubation trials (Table 4). The susceptible genotype had significantly more ($P<0.05$) glucosamine accumulation on day 5 than the resistant genotype for all trials with the greatest difference between susceptible and resistant observed in trial 3. All plant material was at the bud stage for this trial which indicated that physiological stage differences between genotypes was not a factor that contributed to glucosamine differences observed in the first two trials.

Day 5 in the whole plant incubation trial would most closely correspond to field wilting conditions that are conducive for molding. Therefore, during field wilting the susceptible genotype should support more molding than the resistant genotype under the same conditions.

The concentration of water-soluble carbohydrate was significantly different ($P<0.05$) between genotype V-3 (87.91 mg g^{-1} DM) and S-4 (106.87 mg g^{-1} DM), but there was no difference between the susceptible and resistant genotypes (data not shown). No differences

between resistant and susceptible genotypes for WSC concentration at cutting, indicated that this was not a factor that contributed to molding in the harvested alfalfa plants in this trial. Low DM and medium DM alfalfa bales after one day of storage have been reported at 65 mg g^{-1} and 56 mg g^{-1}, respectively (Undi 1995) which are lower than the WSC concentrations observed in this trial. The lower concentration of WSC reported by Undi (1995) was observed because WSC are readily metabolized in harvested plants until respiration stops (Moser 1995). Losses had occurred during wilting and one day of bale storage, whereas, in this trial the samples were frozen at time zero to minimize WSC losses.

Field wilting and bale storage

Field wilting was more rapid ($P<0.05$) for genotypes V-3 and S-4 than for the other 2 genotypes in trial 4 (Table 6). Harris and Tullberg (1980) documented that leaves dry quicker than stems. This could be one of the reasons for different drying rates as it was observed that genotypes V-3 and S-4 had slender stems and more leaves than V-2 and R-1. Other factors that affect water loss include plant structure, growth state, stomatal concentration and cuticle makeup which are also reviewed by Harris and Tullberg (1980). The initial DM was lowest for genotype V-2 for unknown reasons.

During wilting in trial 4 the mean daily temperatures ranged from 13 to 23°C and the mean relative humidity was 56 to 87% with 4.57 mm precipitation on day 2. These conditions were suitable for mold growth, however, the mold accumulation from cutting to the time of baling did not increase (Table 7). The temperatures within the windrows were not determined, however, the windrow temperatures would be expected to be higher than the surrounding temperatures due to natural plant respiration. Plant respiration continues in the cut plant until

the moisture content reaches 30-40% (Rees 1982), therefore heat would have been generated throughout the wilting phase for this trial. The majority of wilting fungi are mesophiles (Sharma 1989) which thrive at temperatures between 10 and 40°C with optimum growth between 15 and 30°C (Jay 1991).

Wilting fungi are soilborne organisms and are dispersed by the wind, rain, and harvesting equipment. Heavy equipment disturbs the soil and stirs up dust containing fungal organisms. For these trials hand cutting with shears reduced the amount of airborne spores during harvest which may have contributed to the low glucosamine accumulation across all genotypes during wilting. Another reason for low molding may be the gentle method of handling compared to conventional forage cutting. Traditional cutting methods damage the stems and surfaces of the plants which exposes inner tissues and allows release of plant fluids. The damaged areas and the plant exudates may improve the conditions for fungal growth.

Statistical differences in DM between the resistant and susceptible genotypes were not observed in Trial 5 on day 4 during wilting (Table 6). Two days were needed to construct the laboratory-scale bales, therefore V-3 and S-4 were baled on day 4 because these genotypes had a higher DM % of 52.03 and 50.85, respectively. Bales for genotypes R-1 and V-2 were constructed on day 5. The mean temperatures in this trial ranged from 17 to 21°C and the daily means for relative humidity were between 62 and 87% with 2.13 mm of precipitation on day 2 of wilting. The conditions were poor for good drying, but conducive for mold growth, however, glucosamine accumulation during field wilting was low and differences could not be determined (Table 7).

The conditions for both trials were similar except the precipitation was 4.57 mm in trial

4, and 2.13 mm in trial 5. The precipitation was on day 2 for both trials but the amount in trial 4 may have had an effect on DM differences. The growing period for the plants used in trial 4 started with cool spring temperatures in which little growth occurred, which was then followed by very warm temperatures where the plants grew rapidly developing very tall (60-70 cm) and thick stems. Trial 4 used first-cut plant material which normally in alfalfa has lower leaf to stem ratio than later cuttings (Rotz, 1995). Trial 5 used second-cut plant material that had shorter and thinner stems. Plants in trial 4 were in late bud stage except for S-4 which was in early bud stage, and for trial 5 genotype S-4 was at late bud stage while the remaining plants were at 10% bloom. The physical condition of the plants in trial 4 may account for the differences observed in DM% which were not observed in trial 5.

Bales stored in trial 4 showed differences in DM on day 9 with S-4 and V-2 being lower ($P<0.05$) than R-1, but by day 24 there was no difference in DM amongst the genotypes (Table 6). Differences in DM% were not observed in trial 5 during bale storage. Studies conducted by Undi (1995) indicate that in low DM (65.3%) and medium DM (72.4%) moisture loss in stored alfalfa bales in the first 14 days was more than 80%. The moisture loss from day 0 to day 24 in bale storage was 67.8% for the susceptible genotype and 77.8% for the resistant genotype in trial 4, and in trial 5 losses were 76.0 % for the susceptible and 79.6% for the resistant genotype. The bale stacks were stored in an incubator, but the temperature was not controlled. The incubator humidity was not monitored, but condensate observed on the ceiling of the incubator, indicated that the humidity was high, which accounted for the reduced moisture loss in storage. Since the trial was designed to have conditions suitable for molding, the high humidity was desirable.

Differences in glucosamine concentration were not observed between the resistant and susceptible genotypes during bale storage in trial 4 (Table 7). The susceptible genotypes had more ($P=0.0232$) glucosamine that the variable genotype V-2 on day 9. Glucosamine concentrations ranged from 4.21 to 5.16 mg g^{-1} across all genotypes by day 24 of storage. These concentrations were higher than the concentration (3.0 mg g^{-1}) detected by Undi (1995) in stored low DM (65.3 %) alfalfa bales on day 21. The higher glucosamine concentrations in this trial at day 24 indicated that the conditions were suitable for molding.

The glucosamine differences were not detected in stored bales representing the four genotypes in trial 5 (Table 7), however, combined results of trial 4 and 5 indicated that the glucosamine accumulation on day 9 was greater ($P<0.05$) for the susceptible genotype than for the resistant genotype (Table 8).

The temperature in hay storage normally increases in the first few days due to plant respiration, which is followed by a temperature drop, and then a second temperature increase which corresponds to the growth phase of the saprophytic fungi (Gregory et al. 1963; Breton and Zwaenepoel 1991). The temperature curve in trial 4 showed the three corresponding peaks which are normally observed in stored bales (Figure 3). A third peak occurred near day 20 in trial 4. Breton and Zwaenepoel (1991) documented that the number of temperature peaks that occur in stored hay correspond to the number of successive microflora groups that decompose the stored bales. The number of successive groups depends on the amount of available moisture in the stored bales. Studies with mini bales conducted by Coblentz et al. (1996), observed that the second increase in temperature normally begins near day 5 and the temperatures remain high for approximately 15 days. The temperature drop in trial 4 may have been due to changes in

the microflora; a second group succeeding the death of the first group of saprophytes. The temperatures in trial 5 fluctuated at the beginning, and increased by day 10 to temperatures that correspond to normal stack temperatures (Figure 3).

Mold develops best when the a_w is between 0.80 and 0.85 (Jay 1992), which relates to moisture content from 18 to 25% (Albert et al. 1989), and above and below this a_w range conditions are less conducive to molding. The susceptible genotype wilted rapidly in the first phase of drying, therefore had the optimum a_w for molding before the resistant genotype which dried slower in this first phase. This may be the reason the mold accumulation on day 9 was greater for the susceptible genotype, but by day 24 the glucosamine differences were not evident between genotypes.

CONCLUSIONS

Aspergillus repens and *A. versicolor* each provided different resistance and susceptibility ratings ($P<0.1$), therefore were effective for separating resistant and susceptible alfalfa genotypes. *Aspergillus fumigatus* should be removed from the screening procedure for safety reasons, and because it was not an effective selection fungi.

Alfalfa genotypes S-4 and V-3, which are susceptible to post-harvest fungal attack, had a more rapid rate of moisture loss than the resistant genotype R-1, during the field wilting trial. This suggests that rapid drying may be associated with increased susceptibility to molding after harvest. Low glucosamine accumulation during field wilting may have been related to the harvest procedure used in these studies. When the bale storage trials were analysed separately the glucosamine concentrations were not different between genotypes, however, when the results

were combined, the glucosamine concentrations were higher ($P<0.05$) in the susceptible genotype as compared to the resistant genotype on day 9 (Table 8). Glucosamine accumulation in bale storage and in the whole plant incubation trials support the rating results of the screening procedure. This indicates that this procedure has potential as a selection tool for developing cultivars that are resistant to post-harvest fungal growth.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Canada /Manitoba Agreement on Agriculture Sustainability. Appreciation is extended to Angela Oswald, Heather Kienzle, Markian and Chris Babij for their assistance in harvesting, baling and sampling, and to Janice Haines and Peter Mills for conducting the chemical analysis.

Figure 2. Leaf area colonized, %, when alfalfa genotypes ranging in post-harvest susceptibility were incubated with five *Aspergillus* species, either individually or in a combined inoculum. The study was replicated three time with n = 8 for each trial. Standard errors were 6.92, 9.71 and 7.18 for trials 1, 2, and 3, respectively and are represented with SE bars.

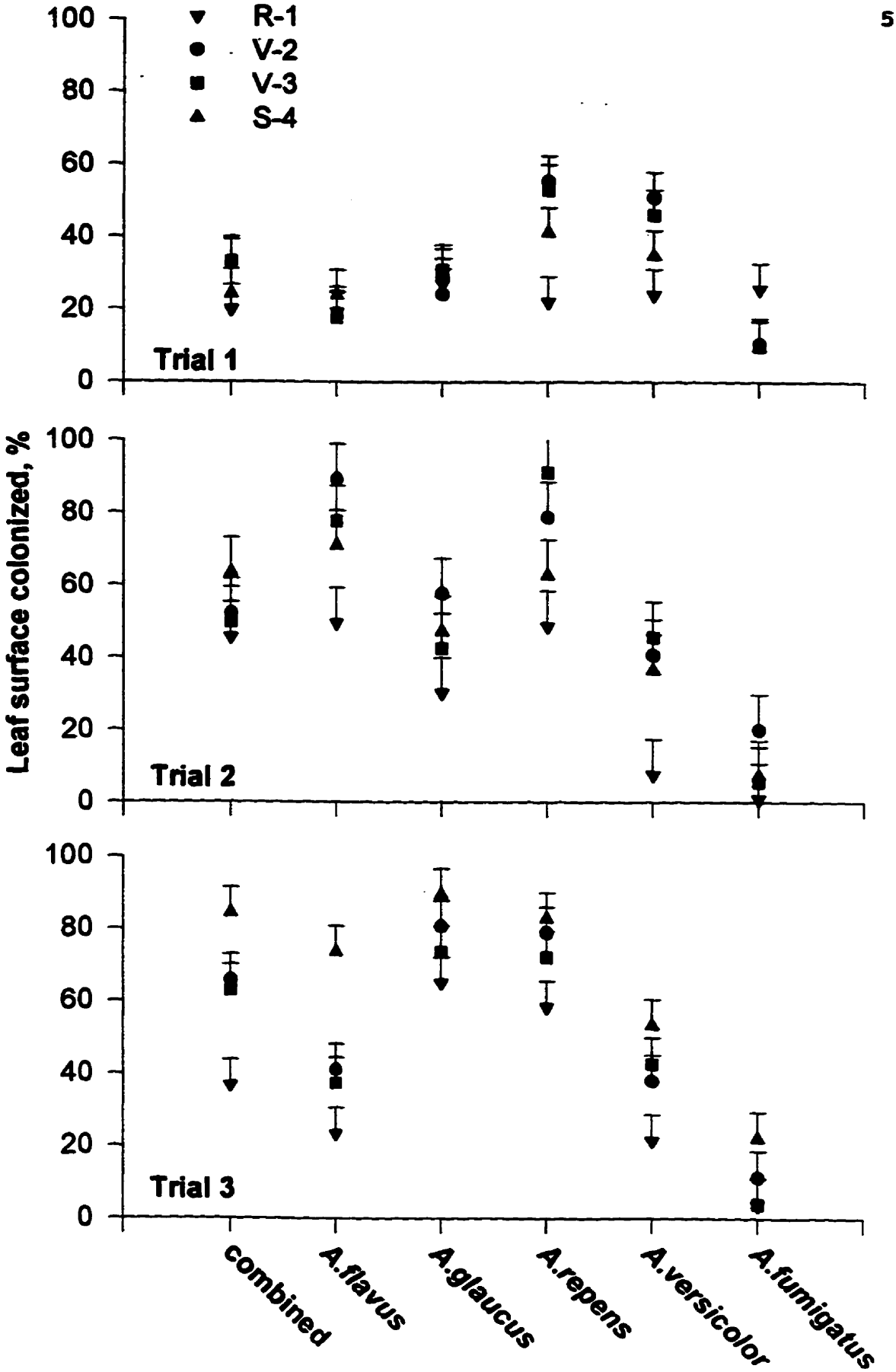

R-1
V-2
V-3
S-4
Leaf surface colonized, %
100
80
60
40
20
0
Trial 1
Trial 2
Trial 3
combined
A.flavus
A.glaucus
A.repens
A.versicolor
A.fumigatus

Figure 3. Daily temperatures of resistant (R-1), variable (V-2 and V-3), and susceptible (S-4) alfalfa genotypes in simulated bale storage study with a reference curve representing conventional bale storage for alfalfa baled at 64.1-66.2% DM (Undi, 1995).

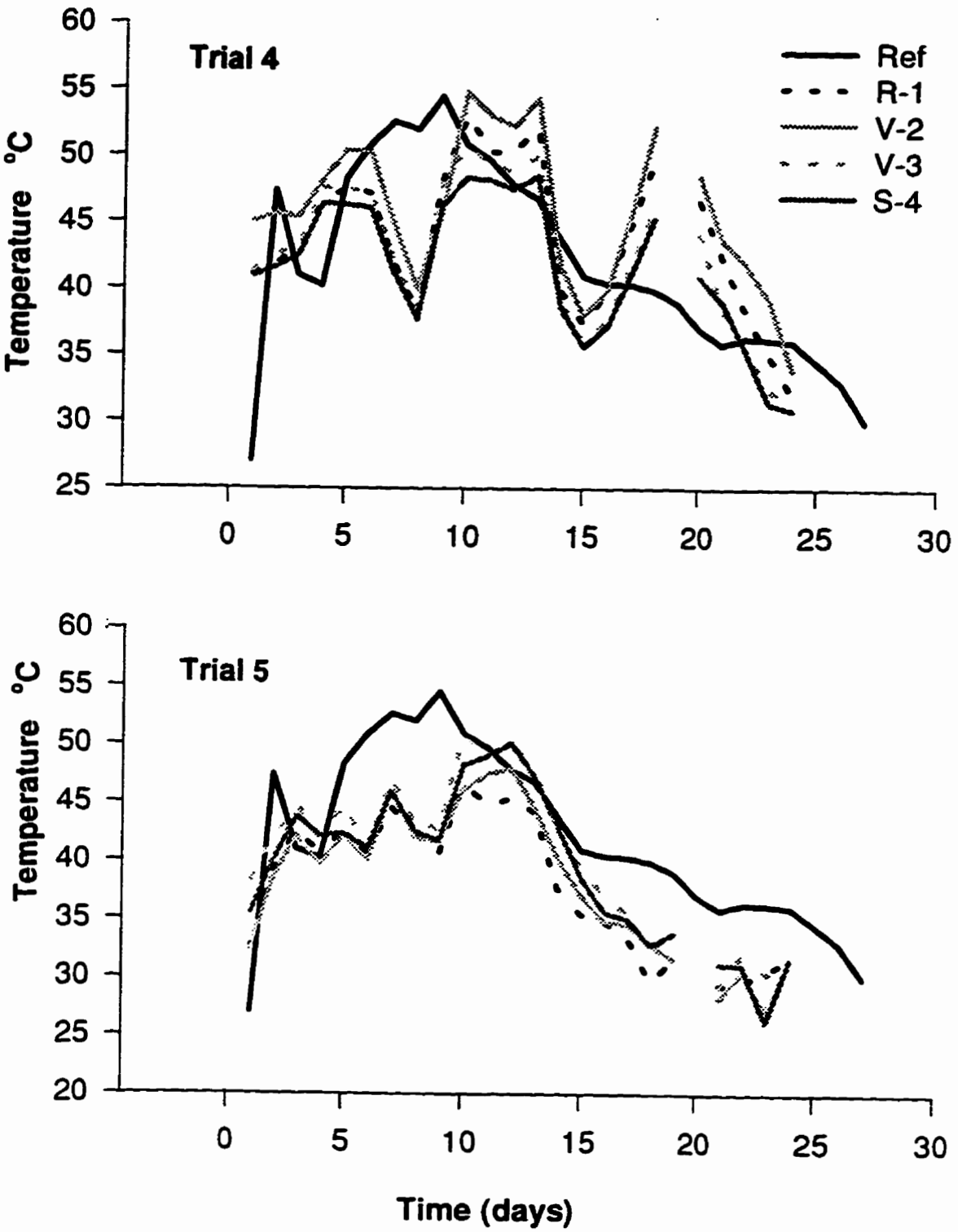

Trial 4
Ref
R-1
V-2
V-3
S-4
Temperature °C
60
55
50
45
40
35
30
25
0
5
10
15
20
25
30
Trial 5
Temperature °C
60
55
50
45
40
35
30
25
20
0
5
10
15
20
25
30
Time (days)

Table 3. The effect of interaction comparisons for the trait % leaf area colonized by fungal biomass in trials 1, 2 and 3. Least squared means were compared for interactions of inoculum by trial, genotype by trial, and inoculum by genotype.

Inoculum by trial	Trial 1	Trial 2	Trial 3	Combined[z]
A. repens	42.89[a]	71.23[a]	72.52[ab]	62.21[a]
A. glaucus	32.98[ab]	44.44[b]	79.47[a]	52.30[b]
combined	27.50[ab]	52.75[b]	62.36[b]	47.54[c]
A. flavus	21.51[b]	71.73[a]	43.80[bc]	45.68[c]
A. versicolor	40.12[a]	32.58[bc]	40.12[c]	37.19[d]
A. fumigatus	10.05[c]	10.05[d]	10.21[d]	9.53[e]
SE	3.23	3.23	3.23	1.87
Genotype by trial	Trial 1	Trial 2	Trial 3	Combined[z]
R-1	19.72[b]	30.40[b]	34.76[c]	28.29[b]
V-2	38.16[a]	57.02[a]	52.23[b]	49.14[a]
V-3	31.54[a]	51.92[a]	50.28[b]	44.58[a]
S-4	27.28[a]	48.02[a]	67.55[a]	47.62[a]
SE	2.64	2.64	2.64	1.52
Inoculum by genotype	R-1	V-2	V-3	C-4
A. repens	43.05[b]	71.49[a]	72.06[a]	62.25[a]
A. glaucus	40.87[b]	60.74[a]	52.09[a]	55.49[a]
combined	34.04[b]	50.04[a]	48.06[a]	57.29[a]
A. flavus	30.71[b]	51.77[a]	44.07[a]	56.16[a]
A. versicolor	17.68[b]	44.69[a]	44.84[a]	41.54[a]
A. fumigatus	3.40	15.36	6.39	12.96
SE	3.73	3.73	3.73	3.73

[z] Combined means for 3 trials

[a-c] different letters in the same column indicates difference for inoculum by trial and for genotype by trial interactions using least significant difference (LSD) with experiment wise error rate of 0.05; different letters in the same row indicates difference for inoculum by genotype interactions.

Table 4. Mean glucosamine concentration, mg g^{-1} DM, of harvested alfalfa plant material that was chopped and placed in petri plates for incubation over an 11 day period, n = 4.

	Genotype				
	R-1	V-2	V-3	S-4	SE
Trial 1[z]					
day 0	1.26	1.44	1.35	1.34	0.2
day 5	2.34[b]	2.32[b]	3.08[ab]	3.28[a]	0.2
day 8	7.10	6.33	6.53	8.17	0.9
day 11	10.56[b]	10.54[b]	11.22[ab]	13.97[a]	0.5
Trial 2					
day 0	1.64	1.59	1.46	1.56	0.2
day 6	6.07[b]	6.23[b]	7.24[ab]	8.52[a]	0.5
day 8	9.21[b]	8.91[b]	9.73[b]	12.12[a]	0.7
day 11	11.04[b]	11.45[ab]	13.10[ab]	14.61[a]	1.0
Trial 3					
day 0	1.52	1.67	1.44	1.69	0.1
day 5	3.08[b]	4.31[ab]	4.45[ab]	6.57[a]	0.5
day 8	8.36[b]	10.30[ab]	10.42[ab]	13.48[a]	0.8
day 11	10.84[b]	11.82[b]	12.29[b]	15.90[a]	0.9

[z] Glucosamine values in this set were calculated using an averaged lab dry wt.
[a b] different letters in the same row indicate a difference using least significant difference (LSD) with experiment wise error rate of 0.05.

Table 5. Initial dry matter content, %, of freshly harvested alfalfa plant material used in trials 1, 2 and 3, and the DM lost, % of original DM, during incubation for trials 2 and 3, n = 4.

	Genotype				
	R-1	V-2	V-3	S-4	SE
Trial 1					
Initial DM	25.09	24.21	24.26	23.67	1.3
Trial 2					
Initial DM	22.78	23.12	23.46	26.47	0.9
DM lost by d 6	12.46^{b}	16.75^{ab}	15.13^{ab}	20.88^{a}	1.6
DM lost by d 8	17.00	29.42	21.32	27.65	2.9
DM lost by d 11	18.81^{b}	31.78^{a}	27.59^{ab}	30.68^{ab}	2.7
Trial 3					
Initial DM	23.31	20.75	22.34	21.20	0.6
DM lost by d 5	11.14^{ab}	10.82^{ab}	8.91^{b}	21.25^{a}	2.5
DM lost by d 8	20.70	25.42	22.47	28.80	2.0
DM lost by d 11	29.34	23.18	27.15	35.33	2.0

^{a,b} different letters in the same row indicate a difference using least significant difference (LSD) with experiment wise error rate of 0.05.

Table 6. Effect of alfalfa genotypes on forage DM, %, at the time of cutting, after field wilting, and during stack storage of lab-scale bales in trials 4 and 5, n = 4.

	Genotype				
	R-1	V-2	V-3	S-4	SE
Trial 4					
at cutting	22.61[a]	19.64[b]	22.71[a]	21.79[a]	0.4
wilting d 4[z]	39.86[a]	38.52[a]	50.08[b]	49.25[b]	1.6
stack storage d 0	58.45[a]	57.02[a]	52.62[b]	49.92[c]	0.4[y]
stack storage d 9	71.17[a]	66.56[b]	ND[x]	67.65[b]	0.8
stack storage d 24	75.11	73.11	73.04	73.61	1.8[y]
Trial 5					
at cutting	22.83[a]	19.77[b]	22.17[a]	21.62[a]	0.3
wilting d 4[z]	45.39[ab]	44.16[b]	52.03[a]	50.85[ab]	1.7
stack storage d 0	62.11	60.32	59.58	58.37	1.1[y]
stack storage d 9	68.71	63.41	66.31	64.81	1.3
stack storage d 24	78.05	77.65	78.44	76.40	1.2[y]

[z] V-2 and S-4 were baled on d 4, and stored at 4 °C until V-3 and R-1 were baled.
[y] SE averaged from set of means when observation count varied.
[x] ND, not determined.
[a b] different letters in the same row indicate a significant difference using least significant difference with experiment-wise error rate of 0.05.

Table 7. Effect of alfalfa genotype on glucosamine concentration, mg g^{-1} DM, at the time of cutting, after field wilting, and during stack storage of laboratory-scale bales in trials 4 and 5, n = 4.

	Genotype				
	R-1	V-2	V-3	S-4	SE
Trial 4					
at cutting	0.86	1.18	0.98	1.23	0.1
wilting d 4	1.38	1.11	1.47	1.29	0.1[z]
stack storage d 0	1.20	1.36	1.31	1.14	0.1[z]
stack storage d 9	2.89[ab]	2.80[b]	ND[y]	4.54[a]	0.4
stack storage d 24	4.28	4.41	5.16	4.21	0.3
Trial 5					
at cutting	1.47	1.42	1.42	1.44	0.2
wilting d 4	1.61	1.48	1.68	1.40	0.2
stack storage d 0	1.53	1.38	1.29	1.70	0.1
stack storage d 9	2.05	3.20	2.91	3.11	0.4
stack storage d 24	3.58	4.27	4.21	4.58	0.3[z]

[z] SE averaged from set of means when observation count varied.
[y] ND, not determined.
[a b] different letters in the same row indicate a significant difference using least significant difference with experiment-wise error rate of 0.05.

Table 8. Effect of alfalfa genotypes on forage DM, %, and glucosamine concentrations, mg g^{-1} DM, at the time of cutting, after field wilting, and during stack storage of laboratory-scale bales, n = 4. Combined results of trial 4 and 5.

	Genotype				
Trial 4 and 5	R-1	V-2	V-3	S-4	SE
Forage DM %					
at cutting	22.72[a]	19.71[b]	22.44[ac]	21.71[c]	0.2
wilting d 4[z]	42.62[a]	41.34[a]	51.05[b]	50.05[b]	1.2
stack storage, d 0	60.28[a]	58.67[a]	56.10[b]	54.15[b]	0.6[y]
stack storage, d 9	69.94[a]	64.99[b]	ND[x]	66.23[b]	0.8
stack storage, d 24	76.92	75.38	67.28	74.71	4.8[y]
Forage glucosamine mg g^{-1}					
at cutting	1.16	1.30	1.20	1.34	0.1
wilting d 4	1.32	1.30	1.58	1.35	0.1
stack storage, d 0	1.37	1.37	1.30	1.41	0.1[y]
stack storage, d 9	2.47[b]	3.00[ab]	ND	3.82[a]	0.3
stack storage, d 24	3.92	4.34	4.09	4.40	0.4

[z] V-2 and S-4 were baled on day 4, and stored at 4 °C until V-3 and R-1 were baled.
[y] SE averaged from set of means were observation count varied.
[x] ND, not determined.
[a b] different letters in the same row indicate a significant difference using least significant difference with experiment-wise error rate of 0.05.

MANUSCRIPT II: Physical, chemical and microbial factors in alfalfa (*Medicago sativa* L.) related to post-harvest spoilage

ABSTRACT

Decomposition fungi often are responsible for dry matter losses and quality decline during the harvest and storage of hay. Differing levels of susceptibility to post-harvest fungal growth has been observed in alfalfa (*Medicago sativa* L.) genotypes. Studies were conducted with alfalfa genotypes ranging in post-harvest susceptibility to colonization by *Aspergillus* species in order to investigate chemical, microbial and physical factors that may contribute to inhibition of mold growth after harvest. The results of the chemical extraction studies were inconclusive, however, darkened zones observed in the disc diffusion assay may indicate the presence of an inhibitory compound for all genotypes. The susceptible alfalfa genotype supported the growth of higher ($P<0.05$) populations of lactic acid bacteria than the variable and resistant genotypes in two trials, and in one trial differences were observed between the susceptible and resistant genotypes for total bacteria, yeast, or mold counts. No difference between genotypes was observed for water activity and water soluble carbohydrate concentration of fresh plant material, however, drying rates were lower ($P<0.05$) for the more resistant genotypes. Plant characteristic resulting in rapid wilting and high epiphytic lactic acid bacteria populations may be associated with susceptibility to post-harvest mold growth.

Key words: alfalfa (*Medicago sativa* L), mold inhibition, extraction, drying rates, *Aspergillus*, epiphytic bacteria

Abbreviations: A_w, water activity; CFU, colony forming units; DM, dry matter; LAB, lactic acid bacteria; TB, total bacteria; WSC, water soluble carbohydrate

INTRODUCTION

Two main concerns in hay production are dry matter losses and quality decline. Baling at high moisture content (28-35%) decreases losses due to shattering and rain damage (Baron and Mathison 1990), however, increased losses in dry matter and crude protein occurs in storage (Rees 1982; Collins et al. 1987; Wittenberg and Moshtaghi-Nia 1990; Undi 1995). Poor field wilting conditions or high moisture storage supports increased numbers of decomposition organisms (Undi 1995) that reduce hay quality by lowering nutritive value, digestibility, and acceptability (Kaspersson et al. 1984; Undi 1995). The wilting organisms are primarily field fungi which are usually soil borne and consist mainly of genera *Alternaria, Cladosporium, Colletotrichum, Fusarium, Phaeoseptoria, Phoma*, and *Ascochyta* (Breton and Zwaenepoel 1990). These organisms are eliminated in storage when temperatures approach 50°C, and thermotolerant storage flora invades. The storage decomposition genera include *Absidia, Rhizopus, Aspergillus* and *Humicola* (Breton and Zwaenepoel 1990), and these organisms can cause health problems for both the livestock and individuals who handle hay (Gregory et al. 1963; Lacey 1975; Nash and Easson 1977).

Studies conducted by Wittenberg et al (accepted) demonstrated that alfalfa (*Medicago sativa* L) genotypes showed variation in susceptibility to post-harvest fungal attack. Potential mechanisms that reduce molding after harvest include; chemical inhibitors; epiphytic microflora; substrate constituents; and physical characteristics such as drying rate and a_w.

The first barrier for microbial attack is the physical and chemical makeup of the outer plant surface (Carr and Klessig 1989). The plant cell wall, which consists of cellulose,

polysaccharides and proteins is another block against fungal growth. Attacking microflora secrete degradation enzymes that break down the plant cell walls and outer plant surfaces (Thomashow and Weller 1995), however, many living plants respond by producing compounds that inhibit microbial growth (Carr and Klessig 1989). Some observations of restricted fungal invasion have been made in harvested plant material. Harvested pea pods, exposed to fungal cell wall constituents such as glucosamines and chitosans, produce two antifungal enzymes, glucanase and chitanase (Mauch 1984), thought to protect the seed pod from fungal attack.

Microbial organisms can protect plants against harmful microflora by competition, antagonism or by triggering a systemic host plant defence response (Thomashow and Weller 1995). Competitive organisms could deplete the substrate of growth factors required by pathogenic or damaging microflora. Janisiewicz and Bors (1995) demonstrated that two antagonists bacterium *Pseudomonas syringae*, and yeast *Sporobolomyces roseus* when colonizing together, were effective in controlling *Penicillium expansum* growth in post harvest wounded apples. The *P. syringae* depletes most of the nitrogen while *S. roseus* utilizes generous amounts of carbon, which exhausts the nutrients essential for germination of *P. expansum* (Janisiewicz and Bors 1995). Also, *Aeromonas* and *Serratia* bacterial genera and the fungal genera *Gliocladium* and *Trichoderma* produce chitinolytic enzymes (Cherin et al. 1995) and, if present on the harvested plant material, may control the amount of fungal growth on plant material during wilting and storage. Induced resistance may be due to natural occurrences such as wounding; pathogen attack causing a plant response such as the production of chitanase; or an unrelated organism interaction. For example, Heitz et al. (1994) documented that tobacco plant bacteria associated with tobacco mosaic virus stimulate the production of antifungal enzymes

which have glucanase, chitinase and lysozyme activity.

Temperature, humidity, air velocity, type and thickness of the material affect the rate a material dries. Most materials dry down in two or three phases; a constant rate period with rapid moisture loss, followed by one or two variable rate periods when moisture loss is slow (Potter 1986; Macdonald and Clark 1987). Forage that dries slowly prolongs a damp environment which is suitable for microbial growth, therefore, rapid drying is preferred. The available water for microbial growth (a_w) at less than 0.70 restricts mold growth (Pitt 1981; Jay 1991;). Water-soluble carbohydrates (WSC) at high concentrations will lower the a_w of a substrate because water molecules bind to sugars thus making the water unavailable to microbes (Rockland and Nishi 1980). Water-soluble carbohydrates also can influence the epiphytic microflora by acting as an available energy source, thus influencing saprophytic fungal activities.

The objective of this work was to investigate possible mechanisms that could account for variation in susceptibility to fungal attack in post-harvest alfalfa (*Medicago sativa* L) forage. Two populations of alfalfa, each having genotypes that range in susceptibility to post-harvest *Aspergillus* growth, were used to compare the presence of chemical inhibitors, epiphytic microflora populations, and whole plant a_w, WSC, and wilting rates.

MATERIALS AND METHODS

Plant populations

Two sets of alfalfa plant material were used for the following series of trials. Set A consisted of four genotypes, R-1A, a resistant genotype on which leaf colonization occurred more slowly than on the susceptible genotype, S-4A, and two variable genotypes, V-2A and V-

3A, that had a range of intermediate responses during the screening procedure. These genotypes were selected during the development of the screening procedure for selecting alfalfa genotypes that showed reduced fungal growth after harvest (Wittenberg et al. accepted). Six plants of set A (three clones of R-1A and three clones of S-4A) were grown in the greenhouse, and a second set of cloned material was transplanted in the field in 1993. The greenhouse temperature ranged from 18 - 28°C, and natural lighting was supplemented with six Phylips Agro 430 watt lamps. Insects were controlled by alternate use of three chemicals; Thiodan, an indosulfan (Chipman Inc. ON.) Trumpet, a bendiocarb, (Plant Products Co ON). and Decis, a detamethrin, (Hoechst, PQ). Set B consisted of six genotypes, three resistant identified as R-1B, R-2B and R-3B, and three susceptible identified as S-1B, S-2B and S-3B, selected from an initial population of 1144 genotypes representing 22 alfalfa cultivars. Cloned material from these six genotypes were transplanted into the field in the spring of 1996, and used for microbial, WSC, and drying rate trials.

Chemical extraction trials

Plant material in late bud to early bloom was cut, placed in sterile bags, and taken to the laboratory for immediate processing. Approximately 10 g of fresh plant material was crushed with an equal amount w/v of extraction solution for 30 min using a stomacher (Seward Medical Laboratory Blender Stomacher 400, CanLab Scientific, Mississauga, ON). The resulting solution was applied dropwise using a micropipette onto blank filter paper discs (6.35 mm in diameter, BBL Microbiology Systems, Becton Dickinson Co, Cockeyville, MD) to concentrations of either 50 and 100 ul per disc by applying 10 ul doses, and allowing the disc to dry thoroughly between applications. A total of four discs, two at each concentration, were

made for each plant harvested. The discs were evenly placed onto dichloran 18% glycerol (DG18),(Pitt and Hocking 1985; Beuchat and De Daza 1992) agar plates. Each agar plate contained four discs representing one resistant plant, and four discs representing one susceptible plant. Preinoculated and plain agar plates were used. The preinoculated agar plates were sprayed with an *Aspergillus* spore suspension (10^6 spores ml^{-1}) consisting of *A. flavus, A. glaucus, A. repens, A. versicolor,* and *A. fumigatus* at rate of 1 - 3 x 10^5 spores per plate two days before the experiment. The plain agar plates were inoculated in the same manner after the discs were plated. The agar plates were incubated at 25°C, and visual evaluations were conducted daily to determine if an inhibition zone surrounded the plated discs.

Extraction trial 6 used two clones of both resistant and susceptible genotypes R-1A and S-4A. Three extraction solutions; methanol and water (80:20 v/v); ethanol and water (80:20 v/v); and chloroform were used. Preinoculated and plain agar plates were used for trial 6.

Extraction trial 7 used four clones from all genotypes in set B; due to shortage of material, two clones per genotype were extracted with the first extraction solution and another two clones per genotype with chloroform. Discs contained 100 ul extract. Two extraction solutions were employed; the first being methanol and water acidified with phosphoric acid to pH 2.2 (80:20 v/v), and the second being chloroform. Trial 7 tested individual *Aspergillus* species *A. flavus, A. glaucus, A. repens, A. versicolor,* and *A. fumigatus* as inoculums as well as a combined inoculum consisting of the above five *Aspergillus* species, with all plates being inoculated after the discs with 100 ul extract were plated.

Genotypes from set B plus R-1A and S-4A were used in extraction trial 8. Fourteen hours prior to harvest the alfalfa plants were bruised by brushing the plants in two directions 90°

to each other with a floor brush, and sprayed with an *Aspergillus* spore suspension (10^6 spore ml^{-1}) consisting of *A. flavus, A. glaucus, A. repens, A. versicolor,* and *A. fumigatus*. Plants were then harvested and processed using either methanol and acidified water to pH 4 (80:20 v/v), or chloroform as the extraction solutions.

Plants from R-1A and S-4A, three clones per genotype were combined and samples extracted with acidified water (w/v) for trial 9. The water was acidified with phosphoric acid to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5.

Epiphytic microflora studies

Alfalfa plants were cut individually using scissors and placed in sterile bags for transporting to the laboratory. A sanitation step which involved washing scissors, and the operator's gloves with 95% ethanol was performed after each plant cutting to avoid cross-contamination. Ten g of fresh plant material was washed in 90 ml of 0.1% Tween80 (CAS No 9005-65-6 Mallinckrodt, Mississauga, ON) in sterile distilled water, at medium setting for two minutes using a stomacher. The wash solutions were serially diluted and plated using the spread plate technique on four media types: nutrient agar; media formulated by de Man, Rogosa, and Sharpe (1960) known as MRS media (BBL MRS 95617 Becton Dickinson Systems, Cockeysville, MD); malt extract agar (Pitt and Hockings 1985); and Rose Bengal with chloramphenicol media (Jarvis 1973; Pitt and Hocking, 1985) to assess for total bacteria (TB), lactic acid bacteria (LAB), yeast, and mold counts respectively. All plates were incubated at 25°C. Total bacteria, LAB, and yeast were counted on day two, and mold counts were completed on day seven of incubation.

Two trials were conducted with plant material maintained under field conditions. Set A

were first cut plants harvested at early bud stage for trial 10, and trial 11 used plants from set B plants plus R-1A and S-4A from set A. Trial 11 were second cut plants at early bud stage with the exception of S-4A which was in vegetative phase.

Wilting studies

Two drying rate trials were conducted; trial 12 used plants in set A and trial 13 was with set B plants. Third cut plants were harvested at early bloom for both trials. Four clones representing each genotype were cut and two samples taken. One 50 g sample of freshly cut plant material was weighed and placed in a pre-weighed brown paper bag (13 x 21 x 16 cm) with the top opened. The bags were placed on a greenhouse bench in a randomized block such that there were 4 blocks each containing one clone per genotype. Bags within each block were re-arranged at each weighing to avoid potential environmental bias. Plant material and bags were weighed at 7:00 and 17:00 daily. A second sample of fresh material was stored (-20°C) until freeze dried for dry matter (DM) and water soluble carbohydrate (WSC) determination of forage. A method, using 3,5-dinitrosalacylic acid (Aldrich Chemical Co Milwaukee WS) developed by Solominski et al (1993), was used to determine water soluble carbohydrate content on the dried sample.

A_w studies

Two clones per genotype for plants in set B were harvested and the leaves were separated from the stems for initial a_w determination. Stem material was cut with a knife into 1 - 3 mm pieces and then further crushed using a mortar and pestle. Leaf material was cut into small pieces and crushed with a mortar and pestle. Once a uniform consistency was achieved, the samples were bagged to avoid moisture loss, cooled to 4°C, and analysed using a water

activity meter (Decagon Model CX-1, Decagon Devices Inc., Pullman Washington, USA)

Statistical analysis

Numerical differences were not observed for results from the chemical extraction, therefore, statistical analyses were not performed. Data were analysed using General Linear Models (GLM) (SAS Institute Inc. 1985).

Epiphytic microbial counts were converted to natural logarithms, and analysed as a completely randomized design with each genotype as the factor of interest. Blocking effects were ignored because the plants were in close proximity to each other in the field. Contrasts were performed to help define differences.

Water soluble carbohydrates at time zero of incubation were analysed as a completely randomized design with genotype as the main effect and clone within genotype as the error term. Least squares means for factors of interest using preplanned comparisons and holding experiment-wise Type 1 error rate to 0.05.

Wilting rates, was measured as the regression of the water remaining (g 100 g^{-1} fresh plant material) on drying time (k value) for each genotype using the following equation:

$$Y = Ae^{kt},$$

where Y = water remaining g % of fresh sample, t = time of observation, e is the base natural logarithm, and A and k are parameters to be estimated. For analysis the above equation was converted by taking the natural log of both sides giving a new equation:

$$\log Y = a + kt$$

where a = intercept and k = slope. The k-values for the four genotypes were compared using a t-test.

RESULTS AND DISCUSSION

Chemical Extraction studies

Disc diffusion assays use microorganisms as an indicator of presence or absence of a compound by evaluating the zone around each disc for altered microbial growth or inhibition. Simple assay designs for unknown compounds such as penicillin, streptomycin, vitamin B_{12}, etc., are described by Hewitt (1977). More recently Madhyastha et al. (1994) developed a disc diffusion type assay to detect the mycotoxins in food and feedstuffs.

A clear zone indicates fungal inhibition in these trials, and darkened zones represent an altered fungal growth. Vegetative growth appears white due to clear mycelia, and reproductive growth produces coloured conidia. An environmental stress, such as the presence of a chemical can cause this change in growth (Smith et al. 1981; Dahlberg and Etten 1982; Garraway and Evans 1984).

Distinct clear zones indicating fungal inhibition, surrounded four discs, two with 50 ul and two with 100 ul extract on one agar plate for the resistant genotype (R-1A) when extraction was with chloroform, and the plate inoculated after disc placement (Table 9). These clear zones were present until day five. Plated discs containing extract from another clone of the same genotype did not respond in the same manner. The remaining discs in trial 6 for both genotypes consistently were surrounded by dark zones for all three extraction methodologies when discs were placed on agar plates which were subsequently inoculated and incubated. To avoid effects of extraction solutions on the assay the discs were thoroughly dried. Preliminary trials were conducted to determine the time required for disc drying, and the effects of the extraction chemicals on the assay when placed on the plates with and without drying. No difference was

observed in fungal growth between discs placed before and after drying which indicates the chemicals evaporated before germination occurred. Therefore, the dark zones of fungal growth around discs appear to be due to a compound that was extracted from the plant material.

All discs in trial 7 were surrounded by darkened zones by day 3 of incubation with the exception of one disc representing a R-1B plant which was extracted with methanol and water (80:20 v/v) and inoculated with *A. versicolor*. This disc had a yellow zone (0.5 to 1.0 cm) that was present until day 5. Conidia of *A. versicolor* typically varies in colour and are often green, yellow, orange or brown. Why all the discs were surrounded by green growth except for one is unknown. The development of clear zones for discs containing the chloroform extract from R-1A was not observed. Discs with R-1A extract prepared with a methanol:H_2O solution in trial 8 had darkened zones surrounding them on day 3, and the remaining extracts from both resistant and susceptible genotypes had dark zones on day 4. Resistant and susceptible plant extracts prepared with the chloroform had darker zones on day 3. Clear zones around discs containing R-1A extract prepared with chloroform as observed in trial 6 was not repeated in this trial.

In trial 9, discs prepared with acidified water at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 were surrounded by 0.5 cm yellow zones on day three. Plates were completely overgrown by day four, and the colour was uniform over all plates. Yoshida et al (1993) extracted gramine, a phytoalexin found in *Hordeum* and *Phalaris*, with acidic 30% ethanol (0.1M HCl-EtOH, 7:3), methanol-ammonium hydroxide (100:1), chloroform and water adjusted to pH 4 and pH 6 with 0.1 M HCl. These researchers reported that gramine extraction with acidified water was less than 0.04% of total gramine for both pH levels. Gramine extraction with water in these experiments was ineffective because a dipping procedure was employed, and the natural wax

layer on the leaves prevented gramine dissolution. Acidification in trial 9 was to facilitate dissolving the possible phytoalexins that may be present in the plant material. The early sporulation on all plates in this trial suggest that water may have extracted a compound that stimulated a phase change as most fungal species are not affected by pH over a broad range of 3 to 8, and optimum being around pH 5 (Pitt and Hocking 1985).

Extraction solutions used for these trials were ineffective for extracting sufficient compounds inhibiting fungal growth to form clear zones around the discs. Extractions with chloroform resulted in darkened zones for both resistant and susceptible genotypes on day 2 which may indicate the presence of a phytoalexin at a low concentration. Clear zones for one clone of the resistant genotype extracted with chloroform in trial 8 also suggest the presence of a phytoalexin, however, in subsequent trials this response was not repeated. Differences were not observed when different *Aspergillus* species were tested as inoculums.

Epiphytic Microflora studies

Epiphytic microflora, the microorganisms naturally present on the forage crops, is influenced by location, climate, plant maturity and the type of crop. Lin et al. (1992b) reported standing corn supported more LAB than alfalfa. Lactic acid bacteria numbers tend to increase with maturity, and can range from 10^1 to 10^6 colony forming units (cfu) g^{-1} (Lin et al. 1992a). Lin et al. (1992b) reported a combination of yeast and mold counts of 10^6 cfu g^{-1} on standing alfalfa plants. Undi et al. (1996) indicated microbial populations of 2.2×10^5 cfu TB g^{-1} DM for Low DM alfalfa, and 9.4×10^4 cfu TB g^{-1} DM for Medium DM alfalfa with total fungal counts for these samples at 1×10^3 cfu g^{-1} DM at the end of field wilting. Microbial trials 10 and 11 indicate that differences in epiphytic microflora also occurs between alfalfa genotypes.

No differences ($P>0.05$) amongst set A genotypes were observed for epiphytic mold and yeast in trial 10 (Table 10). Differences between the two variable genotypes were observed for total bacteria counts in freshly harvested plant material, however, there was no difference between the resistant (R-1A) and susceptible (S-4A) genotypes. Lactic acid bacteria (LAB) populations were greater ($P<0.05$) for S-4A than the remaining genotypes. The results for TB, yeast and mold may have affected by evaluations on the variable genotypes V-2A and V-3A.

A comparison of epiphytic microflora on four resistant and four susceptible genotypes in trial 11 showed that genotype, S-4A, supporting higher ($P<0.05$) populations of TB, LAB, yeast, and mold than R-1A, a resistant genotype (Table 11). In general the resistant genotypes had lower ($P<0.05$) TB, and yeast populations than the susceptible genotypes (Table 11), and with no differences ($P>0.05$) for LAB and mold. Plants from Set A were more physiologically mature than those of Set B and this may account for some of the differences observed between genotypes categorized as either resistant or susceptible.

Comparisons for all resistant genotypes against all susceptible genotypes indicated that TB, yeast and mold populations were greater ($P>0.05$) for the susceptible genotype. A 3 fold difference for TB and 2 fold difference for yeast and mold populations were observed for susceptible genotypes. The TB population on the susceptible genotypes was 2 fold greater than the resistant genotypes for set B genotypes.

Lactic acid bacteria are generally associated with preservation, and they are important organisms in silage fermentation. The antimicrobial effects of lactic acid bacteria have been reported in wine fermentation (de Saad and de Nadra, 1992; Pardo et al. 1989), and sauerkraut and pickle production (Jay 1991). Conversely, preservative hay inoculums containing lactic acid

bacteria have not been reported as effective antifungal agents (Rotz et al 1988; Wittenberg and Moshtaghi-Nia 1990).

Genotypes R-1A and S-4A were evaluated in both microbial trials, and the results indicate that microflora was consistently higher ($P<0.05$) for the susceptible genotype than the resistant genotype for LAB. The trend for higher mold and TB was also evident for the susceptible genotype. The genotype S-4A belongs to a creeping type cultivar, whereas, R-1A grows more upright. Plants that grow closer to the soil would more easily become inoculated with microflora than upright plants. Another suggestion is that the susceptible genotype may be a more favourable substrate for epiphytic microorganisms as well as for post-harvest fungi. The high epiphytic population on the susceptible genotypes may also prepare the harvested plant material for the succeeding saprophytic fungi. The more mature plants, R-1A and S-4A in trial 11, supported more LAB than the less mature genotypes from set B which corresponds with experiments conducted by Lin et al. (1992a). The results in trial 11 for TB ranged between 10^6 to 10^7 cfu g^{-1} which corresponded to results obtained by Undi et al. (1996) for TB ranging from 10^4 to 10^6 cfu g^{-1}. Lactic acid bacteria was between 10^3 to 10^5 in trial 11 which were higher than for trial 10, but according to Lin et al. (1992a) LAB can range between 10^2 to 10^4 cfu g^{-1}. Mold on alfalfa at the end of wilting has been reported at 10^3 (Undi et al. 1996). Values for mold in trials 10 and 11 were between 10^3 and 10^6 cfu g^{-1}. Yeast counts were between 10^3 and 10^5 cfu g^{-1} for trial 10 and for trial 11 from 10^4 and 10^6 cfu g^{-1}.

Wilting studies

There was no difference in the initial MC % for genotypes in trial 12, however, V-2A had a higher ($P<0.05$) initial MC % than the remaining genotypes in trial 13. All three

resistant genotypes dried slower ($P<0.05$) than the susceptible genotypes S-1B and S-3B, and two resistant genotypes R-3B and R-2B, dried slower that S-2B in trial 12 (Table 12). The resistant and variable genotypes dried slower ($P<0.05$) than the susceptible genotype in trial 13 (Table 13). Results of both drying trials demonstrate that a 12.5 to 21.7% slower drying rate was associated with resistant genotypes. The presence of an inhibitory compound which at higher moisture would be active longer may be the reason for reduced mold growth in the resistant genotype, however, the presence of an inhibitory compound was not established in the extraction studies. The epiphytic microbial profile may be more stable on the plants that have a slower drying rate and this population may have an inhibitory effect on colonization by saprophytic organisms on freshly cut plant material.

A_w and WSC determination

Determination of WSC (Table 12) indicated there was no difference ($P>0.05$) between genotypes. Moser (1995) documented that WSC concentrations vary throughout the day having the lowest concentrations at 600 h and the highest amount at 1800 h, and can increase 4% throughout the day. Water soluble carbohydrates are readily metabolized in harvested plants until respiration stops (Moser, 1995). The WSC concentrations recorded by (Undi 1995) for alfalfa bales following one d of storage for Low DM and Medium DM alfalfa were 65 mg g^{-1} and 56 mg g^{-1} respectively. The alfalfa cut at mid day for WSC determination, and stored at -20° C to minimize the WSC loss due to respiration had higher values than those recorded by Undi (1995),(Tables 12 and 13).

Water activity is a better indicator for potential spoilage than forage moisture content because it represents the free water in the substrate whereas moisture content accounts for both

free and bound water. Mold develops best when the a_w is between 0.80 and 0.85 (Jay 1992), which relates to a moisture content from 18 to 25% in alfalfa (Albert et al. 1989). Water activity greater than 0.85 and less than 0.80 is less conducive to molding. Albert et al. (1989) also demonstrated that at any moisture content, alfalfa leaves have a lower a_w than stems. The ratio of stem to leaf material in stored alfalfa could have an effect on the rate of spoilage. No difference in standing plant a_w levels were observed for resistant versus susceptible genotypes or between leaf and stem material (Table 12). Analysis at different stages during wilting may have resulted in differences. The material must be uniform throughout when samples are tested for a_w using the water activity meter Decagon Model CX-1. Sampling at different stages in wilting would be difficult because as the material drys it is more difficult to compact the material into a sample cup without air pockets. An uneven sample and air pockets would reduce the accuracy of testing.

CONCLUSIONS

The rate at which the plant material dries may be an important factor in susceptibility to post-harvest fungal growth. The physical plant structure that allows for quick moisture loss may also be conducive for mold growth.

Microbial studies showed higher lactic acid bacteria populations on the susceptible S-4A genotype in both trials. Higher TB populations may prepare the plant material for invading fungi. Epiphytic microflora differences between the susceptible and resistant genotypes suggest that the susceptible genotypes may be more favourable for both epiphytic and saprophytic organisms.

Fresh cut plant material did not vary in a_w between resistant and susceptible genotypes,

or between leaves and stem material, however, as the plant material dries, variation in a_w may be an important factor between resistant and susceptible genotypes. Initial water-soluble carbohydrate concentration were not related to alfalfa susceptibility to post-harvest fungal growth in this experiment.

Inconsistent results in the chemical studies made conclusive comparisons impossible. The extraction procedures may have been inadequate for removing sufficient amounts of inhibition compounds to create a response. The extractions indicate that some compounds may have been removed from the plant material that stressed the fungus into conidiation forming the darkened zones.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Canada /Manitoba Agreement on Agriculture Sustainability. Appreciation is extended to Janice Haines and Peter Mills for chemical analysis, and Angela Oswald for microbial work.

Table 9. Effect of plant extracts, extracted from alfalfa genotypes classified as susceptible and resistant to post-harvest fungal attack when extracted with methanol:H_2O, ethanol:H_2O, and chloroform as extraction solutions, on fungal growth using a disc diffusion assay.

	Genotypes			
	R-1A		S-4A	
	Clone 1	Clone 2	Clone 1	Clone 2
Methanol:H_2O				
d 2	DDii	iiii	DDii	iiii
d 3	DDDi	DDii	DDii	DDii
d 4	DDDi	DDDD	DDii	DDDD
d 5	DDDD	CCCC	DDii	CCCC
Ethanol:H_2O				
d 2	DDii	iiii	DDii	iiii
d 3	DDii	DDii	DDDD	iiii
d 4	DDDD	DDDD	DDDD	DDDD
d 5	DDDD	CCCC	DDDD	CCCC
Chloroform				
d 2	DDii	IIII	DDii	DDDD
d 3	DDii	IIII	DDii	DDDD
d 4	DDDD	IIii	DDDD	DDDD
d 5	DDDD	DDDD	DDDD	DDDD

C indicates the plates were evenly covered with fungal growth
D dark zone surrounding the disc
i no dark or inhibition zones surrounding the disc
I inhibition zone surrounding the disc
Two clones of each resistant and susceptible were used in this trial.

Table 10. Total counts of epiphytic microflora, expressed as the natural logarithms of the number of colony forming units g^{-1} fresh plant material, for alfalfa genotypes ranging in susceptibility to post-harvest fungal growth trial 10, n = 4.

	TB[z]	LAB[y]	Yeast[x]	Mold
Genotype				
R-1A	5.31[ab]	3.24[b]	3.57	4.40
V-2A	4.75[b]	3.00[b]	3.50	3.87
V-3A	6.08[a]	2.81[b]	3.83	4.38
S-4A	5.97[ab]	4.13[a]	5.14	4.91
SE	0.28	0.19	0.40	0.30

[z] Total bacteria

[y] Lactic acid bacteria

[x] F test shows significance for this parameter, however, lsmeans could not separate the means.

[a b] Means in the same column with different letters are different, using Least Square Difference (LSD) with an experiment-wise error rate of 0.05.

Table 11. Total counts of epiphytic microflora expressed in natural logarithms of colony forming units g^{-1} fresh plant material, for alfalfa genotypes ranging in susceptibility to post-harvest fungal growth trial 11, n = 4. Contrasts between all resistant and all susceptible genotypes, and between resistant and susceptible genotypes in set B.

	TB[z]	LAB[y]	Yeast	Mold
Resistant Genotypes				
R-1A	6.00[b]	4.62[b]	4.49[b]	5.24[b]
R-1B	6.59[ab]	4.13[bc]	4.22[b]	3.97[c]
R-2B	6.64[ab]	3.74[c]	4.53[b]	3.69[c]
R-3B	6.31[b]	3.83[c]	4.70[b]	3.77[c]
Susceptible Genotypes				
S-4A	7.08[a]	5.53[a]	5.71[a]	6.08[a]
S-1B	6.72[ab]	3.72[c]	4.58[b]	4.03[c]
S-2B	6.87[ab]	3.83[c]	4.62[b]	3.87[c]
S-3B	6.97[a]	3.45[c]	4.31[b]	3.98[c]
SE	0.16	0.17	0.18	0.12
Resistant vs susceptible contrasts	----------	----------	Pr> F	----------
All genotypes	0.01	0.66	0.02	0.01
Set B genotypes	0.01	0.10	0.89	0.15

[z] Total bacteria
[y] Lactic acid bacteria
[a-c] Means in the same column with different letters are different, using Least Square Difference (LSD) with an experiment-wise error rate of 0.05.

Table 12. Comparison of initial moisture content (MC) %, n =4, drying slopes[z], n =4, water activity, A_w, n = 2, and water-soluble carbohydrate (WSC) concentration n = 4 on set B genotypes.

	initial MC[y] %	slope	WSC mg/g	stem a_w	leaf a_w
Resistant Genotypes					
R-1B	76.68	-0.77[bc]	99.74	0.989	0.986
R-2B	76.30	-0.67[cd]	103.58	0.990	0.989
R-3B	77.78	-0.66[d]	104.75	0.989	0.987
Susceptible Genotypes					
S-1B	76.39	-0.89[a]	94.07	0.991	0.991
S-2B	77.71	-0.87[ab]	110.87	0.989	0.987
S-3B	75.61	-0.88[a]	105.69	0.989	0.987
SE	0.85	0.04	5.48	0.0009	0.0009

[a,b] different letters indicate difference in slope using a t-test $\alpha = 0.05$.

[z] slope = -log (g H_2O in sample at time t /g initial H_2O in sample) /d

[y] MC = dry wt / initial wt x 100

Table 13. Comparison of initial moisture content MC %, drying slopes[z], and water soluble carbohydrate (WSC) concentration on set A genotypes, n = 4.

Genotype	Initial MC[y]	Slope[z]	WSC mg g^{-1}
R-1A	72.86[b]	-0.72[b]	128.16
V-2A	75.56[a]	-0.74[b]	114.48
V-3A	72.73[b]	-0.75[b]	106.88
S-4A	73.71[b]	-0.92[a]	130.43
SE	0.31	0.03	7.31

[a, b] Initial MC % with different letters are different, using Least Square Difference with an experiment-wise error rate of).05, and different letters indicate difference in slope using a t-test $\alpha = 0.05$.

[z] slope = -log (g H_2O in sample at time t /g initial H_2O in sample) /d

[y] MC = dry wt / initial wt x 100

MANUSCRIPT III: Alfalfa (*Medicago sativa* L.) genotype selection for post-harvest fungal resistance from 22 cultivars

ABSTRACT

Resistance to a number of fungal diseases are routinely incorporated into newly developed cultivars. Alfalfa development for post-harvest resistance is a new area of research. A screening procedure was developed for screening alfalfa genotypes that resist fungal growth post-harvest. The procedure involves plating fresh harvested leaves, inoculating the plated leaves with an *Aspergillus* spore suspension, incubating and evaluating the fungal coverage on the leaves. This procedure was used to select two populations; a resistant population that exhibited reduced fungal growth, and a susceptible population that supported fungal growth on fresh leaf material after harvest. The initial population consisted of 1144 genotypes representing 22 cultivars. The two populations obtained from the initial screening consisted of 87 genotypes categorized as resistant and 88 genotypes as susceptible. The first screening showed that cultivar, Rambler, was more susceptible ($P<0.05$) to fungal growth after harvest than all other cultivars tested with the exception of Class and Rushmore. The cultivar Arrow was rated most resistant ($P<0.05$) to fungal growth and was significantly more resistant than Rambler, Class, Rushmore, Algonquin, GH 787, Apollo Supreme and Pickseed 8920 MF. The 175 selected genotypes were screened a second time, and the 12 selected as resistant and 10 as susceptible were cloned. Selected cloned genotypes were subjected to the leaf screening procedure a third time to obtain a final working population of three resistant and three susceptible genotypes. The mean leaf area colonized in the third screening ranged from 18.6 to 27.8% for the resistant genotypes, and from 46.4 to 66.3% for the susceptible genotypes.

Keywords: alfalfa, plant breeding, selection, procedure, post-harvest fungi

Abbreviations: cm, centimeter; l, liter; ml, milliliter

Introduction

During the mid-1970s the focus for alfalfa development was in disease resistance. Arrow was one of the first registered alfalfa cultivars with resistance to Verticillium wilt (Pickseed 1997) which is caused by the fungus *Verticillium albo-atrum*. Cultivar development today routinely incorporates resistance to a number of microbial diseases (Barnes et al. 1988). Most new cultivars have resistance to at least four fungal diseases; Verticillium wilt, Fusarium wilt, Anthracnose and Phytophthora root rot (Table 14). Older cultivars such as Algonquin, Allouette, Beaver and Rambler have resistance to bacterial wilt, but are susceptible to most fungal diseases (Pickseed 1997). Disease resistance to parasitic organisms in the living plant may also reduce the saprophytic microflora in harvested plants. If the resistance in the living plant is due to an inhibitory compound the presence of this chemical may decrease molding after harvest.

A technique developed by Wittenberg et al. (accepted), for selecting alfalfa plants that resist post-harvest fungi, has been recently verified in this laboratory (Manuscript I). This procedure should be beneficial in developing cultivars of plants that resist saprophytic organisms during field wilting, and potentially could allow harvest of hay at a higher moisture content reducing detrimental exposure to environmental elements. Also, baling hay at a higher moisture content reduces leaf loss in harvesting, and results in higher quality.

The objective of this work was to select two populations of alfalfa: a resistant population that shows reduced post-harvest fungal invasion, and a susceptible population that supports post-harvest fungal growth. These populations were selected for future study to determine trait heritability and potential plant characteristics or mechanisms associated with increased resistance

to post-harvest fungal growth (Manuscript II).

MATERIALS AND METHODS

Screening Procedure

The screening procedure recently developed by Wittenberg et al. (accepted) for selecting alfalfa plants that resist post-harvest fungal growth was used to select two populations for further study. The screening procedure was conducted by the following steps: plants were individually harvested 9 to 10 cm above the soil using scissors, placed in sterile bags, and transported to the laboratory. Four to six fully developed leaves, without insect bites, tears or blemishes, were removed from the freshly harvested plant and placed onto prepared agar plates such that the entire leaf surface make contact with the agar. Plates were prepared in duplicate, and sprayed with 1 - 3 x 10^5 of spores per plate using a nalgene aerosol bottle. Due to availability of *Aspergillus* spp. the inoculum varied slightly between selection cycles. The plates were recapped, and incubated at 25°C. The evaluation was conducted when 70% of all the plates showed colonization on 20% of the leaf area. The rating system developed for alfalfa leaf spot disease (James 1971) was used as a guide to determine the leaf area with fungal coverage. The ratings were ranked; the genotypes with high percentage ratings were considered more susceptible, and low ratings were considered less susceptible to molding.

Plant material

Seed representing 22 registered cultivars was obtained from various companies (Brett Young Seeds, Winnipeg, MB; Pickseed, Lindsey, ON; W.L. Research, Evansville, WI; ABI, Ames, IA; Forage Genetics, West Salem, WI and Great Plains Research, Apex, NC). The seed

was initially planted in the field in the spring of 1994. Fifty-two established genotypes from each cultivar were transplanted into 2 liter milk cartons and maintained in the greenhouse for screening. The greenhouse temperature ranged from 18 - 28°C, and natural lighting was supplemented with six (Phylips Agro) 430 watt lamps. Insects were controlled by alternating the use of three chemicals; Thiodan or indosulfan, (Chipman Inc. ON.), Trumpet or bendiocarb, (Plant Products Co. ON.) and Decis or detamethrin, (Hoechst, PQ). Six control genotypes which had been identified during the screening procedure development were transplanted into milk cartons and maintained under the same conditions. The control genotypes consisted of two genotypes ranked as resistant, two as susceptible and two that ranked intermediate. Four clones of each control genotype were included with each screening.

The 175 genotypes selected after the first screening were maintained in the greenhouse and re-screened on June 2, 1995. Twenty-two selected genotypes from the second screening were cloned on June 27, 1995. Twelve established clones of each selected genotype were transplanted into clay pots. These plants were maintained in the greenhouse for the third screening which reduced the final population to three resistant and three susceptible genotypes. Twelve clones for each of the six genotypes in the final population were maintained under the previously described greenhouse conditions. Screening occurred when the majority of the plants were in a late bud stage of growth.

First screening

The first screening began on March 1, 1995 which using the original plant material (Table 15). The plants were screened in 4 blocks (one block per week) with each block consisting of 286 experimental genotypes representing 22 cultivars for a total of 1144 genotypes.

The inoculum for the first screening consisted of a mixture of 3 *Aspergillus spp.* at a concentration of 1 - 10^6 spores ml^{-1}. Freeze dried cultures of *Aspergillus flavus* DAOM 214728, *Eurotium amstelodamii* DAOM 215938 also referred to as *A. glaucus, and A. fumigatus* DAOM 196947 were obtained from Agriculture Canada, and the method for inoculum preparation described in Manuscript II was used.

The plated control plants were evaluated daily for mold growth, and when 70% of these plates had 20% fungal biomass coverage on the leaves the entire block was evaluated. The plates were evaluated on day 5 for the 4 blocks. Two individuals evaluated the plates; one evaluator rated the first plate per genotype and a second evaluator rated the duplicate plate.

The genotypes were ranked from the one with the least percent fungal coverage on the leaf surface to the one with the highest percent leaf area coverage, based on mean values of the duplicate plates. The genotypes were selected as resistant if the duplicate mean for leaf area covered was less than the lowest mean value in the control plants in each block. If this did not yield 20 plants, then the 20 lowest rated plants were selected from each block. Plants selected for the susceptible population were chosen similarly as the 20 plants in each block having the high duplicate means for fungal leaf area coverage, and greater than the means for the control plants. Other limitations for the resistant genotypes were; no more than 10% of the plants from cultivars OAC Minto, Beaver, and Algonquin were to be selected, and both resistant and susceptible genotypes should not include more than 20% of genotypes from one cultivar. No other limitations were imposed for the susceptible genotypes.

The first screening ratings were analysed as a completely randomized block design using General Linear Models (SAS Institute Inc. 1985) to determine if there were significant differences

in susceptibility between cultivars. Cultivar was tested against genotype within cultivar. The block consisted of 286 genotype representing 22 cultivars which were screened in one week. The block by cultivar interaction was also tested. Least square means were compared using an experiment-wise error rate of 0. 05.

Second Screening

Selected genotypes from the first screening, which consisted of 87 resistant and 88 susceptible genotypes, were re-screened on June 2 in one complete block. Six control genotypes, described in the first screening, were tested with the 175 selected genotypes. The second screening inoculum consisted of equal aliquots of *A. fumigatus*, *A. flavus*, *A. versicolor*, and *A. glaucus* at 1 x 10^5 spores ml^{-1} and *A. repens* at 1 x 10^4 spores ml^{-1}. *Aspergillus versicolor* DAOM 213352 was obtained from Agriculture and Agri-Food Canada, Ottawa, Canada and *Eurotium repens* var columnaris 36963 also referred to as *A. repens*, from American Type Culture Collection, Maryland.

Plates were scored by two evaluators, but due to the inconsistent ratings by one evaluator only evaluations by the other evaluator were considered for the selection. The evaluations were ranked and divided into two groups; a resistance group of genotypes with scores less than 10%, and a susceptible group with scores greater than 90%. A ranking system which included the mean scores of the first screening as well as the second screening were used for this selection. Differences were calculated between the first and second screening means for each genotype. The resistant genotypes with differences less than 10 percentage units were selected for the resistant population. The susceptible population consisted of genotypes selected from the high ranked genotypes with differences less than 10 percentage units between the first

and second screening. Twelve genotypes were classified as resistant and ten as susceptible from this screening.

Third screening

The third screening conducted on Nov. 2, 1995 tested the selected 22 genotypes from the second screening. Four clones for each genotype were tested using an inoculum that consisted of equal aliquots of all five *Aspergillus* species at 1 x 10^6 spores ml^{-1}.

One individual evaluated all the plates; to reduce bias the duplicate plates were divided into two sets; the entire first set was rated, then the second set was rated. The means from each genotype were calculated using the ratings for the four clones tested in duplicate. The three genotypes selected as resistant were selected on the basis of lowest leaf area coverage in the third screening and rated as resistant in the second screening. Similarly, the three susceptible genotypes were selected on the basis of the highest leaf area coverage in the third screening, and that were classified as susceptible in the second screening.

RESULTS AND DISCUSSION

The range for ratings in the first screening were from 0 % - 100 % fungal coverage on the leaf material for block. Variation for skewness was observed across the 4 blocks and calculated values for skewness were 0.24, 1.05 and 0.55 for blocks 1, 2 and 4 respectively and -0.18 for block 3. Blocks 1 and 3 were closest to normal distribution and block 2 deviated the most since the ratings for fungal leaf coverage in this block were low. The overall means for ratings in blocks 1, 2, 3 and 4 were 47, 29, 55 and 34 ($\pm$ 4.55), respectively. The first screening was very large and required the assistance of a number of individuals which in turn increased the chance of error. Incorrect leaf placement and length of time to plate leaves, and

length of time required to evaluate the plates may also have contributed to a high degree of variation between duplicates in this study.

The overall mean for fungal coverage on the leaves in the first screening was 41 $\pm$ 16 % of leaf area. Cultivars did vary ($P<0.05$) in susceptibility to post-harvest fungal attack (Table 15). Rambler was more susceptible ($P<.05$) to fungal growth after harvest than all other cultivars tested with the exception of Class and Rushmore. Rambler does not have resistance to fungal diseases that affect the growing plants, therefore, it may have lower resistance to post-harvest fungal growth; conversely both Class and Rushmore, which also had low resistance to post-harvest fungal growth, have resistance to Vertcillium wilt, Fusarium wilt, Anthracnose and Phytophthora root rot (Table 14). The cultivar Arrow was rated most resistant ($P<0.05$) to fungal growth and was significantly more resistant than Rambler, Class, Rushmore, Algonquin, GH 787, Apollo Sup. and Pickseed 8920 MF.

There was a cultivar by block interaction ($P<0.05$). Several cultivars changed ranking from one block to the next, probably due to differences in genotypes used from block to block. Both resistant and susceptible genotypes had been selected from most cultivars after the first screening indicating great variability within cultivars (Table 15).

Eighty-seven plants categorized as resistant and 88 categorized as susceptible were chosen in the first screening from the initial population. No genotypes from cultivars Rambler and Pickseed 8920MF were selected for resistance, and cultivars DK133 and Greenfield had no genotypes that rated susceptible.

The overall mean for the second screening was 59 $\pm$ 18% leaf area covered by fungal growth and differences ($P<0.05$) were observed for genotypes. Some genotypes that were

selected resistant in the first screening were rated as susceptible in the second screening. Also, some genotypes selected as susceptible in the first screening rated resistant in the second screening. Reasons for the reversal of ratings could be related to improper plant identification, change in inoculum used, incorrect leaf placement and length of time required to evaluate plates. Twelve resistant and 10 susceptible genotypes, were selected.

The third screening overall mean was 37 ± 15% leaf area covered by fungal biomass, and differences were observed between genotypes and clones within genotypes. Five of the twelve genotypes identified as resistant following the second screening, had fungal coverage greater than 40% indicating susceptibility, and three of the ten genotypes previously identified as susceptible had less than 25% coverage. The resistant genotypes selected were Dominator 27 (R-1B), Arrow 43 (R-2B), and Proof 40 (R-3B), the susceptible plants were Rambler 9 (S-1B), Cimmaron VR 10 (S-2B), and GH 787 1 (S-3B).

Variable ratings were observed between clones which accounts for deviation between the third screening mean for each selected genotype and the means from the first and second screenings (Table 16). Four clones were tested in duplicate in the third screening giving a higher mean for the resistant genotypes and a lower mean for the susceptible genotypes. The variation among clones could be due to environmental differences within the greenhouse. Other factors that may have contributed to variation are inoculum change, and leaf placement error.

There are some concerns using this screening procedure for selecting genotypes that are classified as resistant or susceptible. Three screenings were conducted, and results indicate the relative rating for a genotype can be reversed. If the selection procedure is based on one screening then the evaluation may not accurately reflect the type of post-harvest susceptibility

of that genotype, therefore, more than one screening may be required.

Evaluation of fungal growth may vary from one evaluator to another, or from morning to afternoon analysis. It is difficult to keep the ratings consistent in large screenings, for example, the first screening was very large, and 620 plates were evaluated in one day. Smaller screenings are recommended.

Placement of leaves onto agar is a tedious task, and leaves are frequently placed with little agar contact. The leaf shape may also affect the flat contact of the leaf on the agar surface. Leaves that are not in contact with the agar are usually less supportive of mold growth.

Leaf placement on media that supports mold growth must be questioned. Is the fungal growth on the edge of the leaf influenced by the growth on the agar or is the leaf more supportive of fungal growth?

The screening procedure has potential as a selection tool, however, some procedure modifications may be required. For example, the use of leaf plugs (equal size pieces of leaf material cut with a round form) could reduce the problem of leaf contact on the agar surface. Also the plugs could allow diffusion of any inhibitor compounds that may be present in the leaf tissue which may enhance selection.

CONCLUSIONS

Three susceptible genotypes and three resistant genotypes were selected using the screening procedure developed by Wittenberg et al. (accepted) from an initial population of 1144 plants. Three screenings were conducted: the first screening reduced the population to 175 genotypes, the second screening selected 12 resistant and 10 susceptible genotypes, and the third screening reduced the number to the final population with three resistant and three susceptible

genotypes. The major concern was the dramatic changes in ranking of some genotypes from one screening to the next.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Canada /Manitoba Agreement on Agriculture Sustainability. Appreciation is extended to Falicitas Katepa-Mupondwa, Janice Haines, and the many fellow students who helped plate leaves during each screening.

Table 14. Fungal disease resistance ratings for alfalfa cultivars tested for post-harvest fungal resistance[Z].

Cultivar	Bacteria Wilt	Verticillium Wilt	Fusarium Wilt	Anthracnose Race 1	Phytophthora Root Rot
Algonquin[Y]	MR	S	-	-	S
Allegro[X]	HR	R	HR	HR	HR
Alouette[Y]	R	S	-	-	S
Apollo Supreme	HR	R	HR	HR	R
Arrow	HR	R	HR	MR	HR
Beaver[Y]	R	S	-	-	S
Cimmaron VR	HR	R	HR	HR	R
Class	HR	R	R	HR	HR
DK133	HR	R	HR	HR	HR
Dominator[Z]	HR	R	HR	HR	HR
GH787[X]	HR	R	R	HR	HR
Green Field	HR	R	HR	HR	HR
OAC Minto[W]	R	S	-	-	-
Pickseed 8920MF[Y]	HR	R	-	-	R
Proof	HR	R	HR	HR	HR
Rambler[Y]	MR	S	-	-	S
Rushmore	HR	R	HR	HR	HR
Sterling	HR	R	HR	HR	HR
Ultraleaf 87	HR	R	HR	HR	HR
Venture	HR	R	R	HR	R
WL322					
WL322HQ	HR	R	HR	MR	R

[Z] Note: Information obtained from (Certified Alfalfa Seed Council 1997) unless indicated otherwise.
Resistance Ratings: % resistant plants; 0-5, S; 15-30, MR; 30-50, R; <50, HR.
[Y] Pickseed Canada (1997) [Z] Undersander et al. (1995) [W] Agriculture Canada, 1983
- No resistance rating means that the cultivar has not been included in a disease evaluation for the listed disease.
Disease scientific names: Bacteria wilt; *Corynebacterium insidiosum*, Verticillium Wilt; *Verticillium albo-atrum*, Fusarium wilt; *Fusarium oxysporum f.sp. medicaginis*, Anthracnose root rot; *Colletotrichum trifolii*, Phytophthora root rot; *Phytophthora megasperma f. sp. medicaginis*.

Table 15. Alfalfa cultivars tested for post-harvest fungal resistance, and the selected genotypes representing resistant and susceptible genotypes for first (SE=3.10), second and third screenings.

Cultivar	First Screening Mean	No resistant genotypes selected at each screening			No susceptible genotypes selected at each screening		
		1	2	3	1	2	3
Arrow	27[a]	13	2	1	1		
OAC Minto	33[abc]	5			4		
Green Field	33[abc]	7					
Dominator	34[abc]	8	2	1	1		
Alouette	35[abc]	7			2		
Ultraleaf 87	36[abc]	4			3		
WL322HQ	37[abc]	5	2		4	1	
Beaver	38[abc]	1			3		
Venture	39[bcde]	5			1		
DK133	40[bcde]	4	1				
Allegro	42[bcde]	4			7	1	
Cimmaron VR	42[bcde]	8	1		8	1	1
Sterling	43[bcde]	1			1		
WL322	43[bcde]	1			2		
Proof	43[bcde]	5	3	1	4		
8920MF	44[bcde]				3		
Apollo Supreme	47[bcd]	2			3		
GH787	49[bcd]	1			4	1	1
Algonquin	49[bcd]	2			7	2	
Rushmore	53[abc]	2	1		8	1	
Class	55[ab]	2			8	1	
Rambler	66[a]				14	2	1

[a-e] different letters indicate difference using least significant difference with experiment wise error rate of 0.05.

Table 16. Selected genotypes after three screenings and their mean rating (% fungal coverage) for each screening.

		Screening					
		1		2		3	
Genotype	Lab ID	n	Leaf area colonized	n	Leaf area colonized	n	Leaf area colonized
Resistant							
Dominator 27	R-1B	2	7	1	9	8	24
Arrow 43	R-2B	2	6	1	8	8	28
Proof 40	R-3B	2	8	1	10	8	19
Susceptible							
Rambler 9	S-1B	2	91	1	90	8	46
Cimmaron VR 10	S-2B	2	98	1	98	8	66
GH 787 1	S-3C	2	99	1	100	8	58

GENERAL DISCUSSION

The screening procedure was tested three times in the verification trials (Manuscript I), and it was used three times to select a resistant and susceptible population for future studies (Manuscript III). The screening procedure has potential as a selection tool, but some modifications are required. Results from trials 1, 2 and 3 show that the resistant genotype consistently had lower fungal coverage for inoculants *A. repens* and *A. versicolor*. Therefore, it is recommended that the inoculum should include these species individually or combined.

The whole plant incubation studies indicate that the susceptible genotype had more glucosamine accumulation than the resistant genotypes on days 5, 8 and 11. The incubation period was 11 days in duration which may not have been long enough. Differences may have been observed had the incubation period been extended to 24 or 30 days. The optimum growth conditions may have been reached at an earlier stage during incubation for the susceptible genotype allowing the fungal biomass to accumulate.

Increases in glucosamine were not observed in field wilting trials 4 and 5. Poor molding may be due to low contamination during hand harvesting. The moisture content at baling controls the species and number of organisms that invade the forage during storage. The bales for these studies were stored at a high MC, therefore, the moisture level at the time of the third peak may have been adequate for a succeeding group of organisms. The storage conditions were suitable for molding with the accumulation of glucosamines by day 24 being 4.21 - 5.16 mg g^{-1}. This is more than the reported results of 3.1 mg glucosamine g^{-1} on day 21 of conventional square bales in storage at 65.3% DM (Undi et al. 1996). Differences were not evident between the resistant and susceptible genotypes in wilting and storage trials until the data from trials 4

and 5 were combined. The combined data indicate that the differences were present at day 9 but not on day 24. As discussed previously, the susceptible genotype may support optimum fungal growth before that of the resistant genotype. As a result, it accumulates a higher concentration by day 9, but shows no differences on day 24.

These findings indicate that the susceptible genotype had more mold on day 9 in stored bales, and on days 5, 8 and 11 for whole plant incubation, but there is no indication that these differences would be observed after long term storage under conditions conducive to molding. On the other hand, drying is most rapid in the first 14 days of storage (Unci et al. 1995), therefore, an alfalfa cultivar that shows reduced molding in the first phase of storage would be beneficial for preservation of hay.

Chemical analysis results conducted were inconclusive. The testing method may have been inadequate for detecting inhibition compounds. The data collected showed some changes in fungal growth on the disc diffusion assays, but conclusions could not be made. Chemical extractions with a concentrating step may have produced different results.

Microbial studies showed that the susceptible genotype supported higher counts for lactic acid bacteria in both trials. This may indicate that the susceptible genotype was a better substrate for both epiphytic and saprophytic organisms, or this genotype was more susceptible to contamination since it has a more prostrate growth habit than the resistant genotype.

The susceptible genotype dried more rapidly than the resistant genotype which suggests that optimum a_w for fungal growth was reached for the susceptible genotype before the resistant genotype. A combination of rapid drying, and potential high contamination on the susceptible genotype are reasons for arguing that this genotype may not be different in susceptibility.

The screening procedure was used to select resistant and susceptible populations from an initial population of 1144 genotypes from 22 cultivars. The first screening trial indicated rating differences among cultivars, genotypes within cultivar and blocks.

FUTURE CONSIDERATIONS

Aspergillus fumigates should not be used in the inoculum because this organism produces minute spores (2-3 um) which are readily inhaled. *Aspergillus fumigates* is the causative agent for approximately 95% of fungal infections in humans (Davies and Saros 1996). Furthermore, this organism grows best at 40°C, therefore, it grows very slowly at the incubation temperature of the screening procedure, which was verified by the results in the screening procedure with different inoculums. The use of one *Aspergillus* spp. would simplify preparation and reduce the chance of contamination. *Aspergillus repens* and *A. versicolor* gave a large spread between the genotypes and therefore would both be good organisms to use. They also grow best at 26°C which is similar to the screening procedure incubation temperature of 25°C.

Large screenings are not recommended as it is difficult to keep the ratings consistent, for example, in the first screening, 620 plates were evaluated in one day. Another problem with the evaluation is that differences often occur from one evaluator to another. It is difficult to train people to evaluate objectively throughout the day as fatigue and other influences are factors that affect the rating results. There should be a time limit for this evaluation.

Placement of leaves onto agar is a tedious task, and leaves are frequently placed with little agar contact. The leaf shape may also affect the contact of the leaf on the agar surface. Observations indicate that leaf surfaces that do not directly contact the agar do not support mold growth and therefore may be classified as resistant in the evaluation. The use of leaf plugs could reduce the problem of leaf contact on the agar surface. Also, the plugs could allow diffusion of any inhibitor compounds that may be present in the leaf tissue which may enhance

selection.

Leaf placement on media that supports mold growth must be questioned. Is the fungal growth on the edge of the leaf influenced by the agar or is it because the leaf is more supportive of fungal growth?

The screening procedure does not include a sterilization step, and heavily contaminated plant material may be at a disadvantage due to larger populations of decomposition organisms. A sterilization step is important because the objective is to test all plants in an equal manner. Sterilized plants have zero viable spores on the surface, and when they are inoculated with 1 x 10^5 spores, they are tested equally.

There are some concerns using this screening procedure for selecting genotypes that are classified as resistant or susceptible. Three screenings were conducted, and some genotypes selected as resistant in one screening were rated as susceptible in the next screening, or selected genotypes as susceptible were rated resistant in the subsequent screening. If the selection procedure is based on one screening, then the evaluation may not accurately reflect the type of post-harvest susceptibility of that plant, and therefore, more than one screening may be required.

This procedure has potential as a selection tool for developing cultivars that are resistant to post-harvest fungal growth. However, some modifications to the screening procedure are required.

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APPENDIX

Appendix IA: Effect of inoculant type and plant genotype on harvested alfalfa plants (Manuscript I).

Trial 1
Dependent Variable: % coverage

Source	df	MS	Pr>F
Inoculant	5	4797.5	0.0001
plant genotype	3	2866.8	0.0002
Inoculant*Geno	15	293.4	0.7101
Inoculant*Clone(Geno)	72	383.3	0.0001
Error	96	157.0	

Trial 2
Dependent Variable: % coverage

Source	df	MS	Pr>F
Inoculant	5	18828.3	0.0001
plant genotype	3	6417.2	0.0001
Inoculant*Geno	15	618.5	0.6581
Inoculant*Clone(Geno)	72	759.2	0.0015
Error	96	38140.8	

Trial 3
Dependent Variable: % coverage

Source	df	MS	Pr>F
Inoculant	5	20297.1	0.0001
plant genotype	3	84.82.8	0.0001
Inoculant*Geno	15.	338.6	0.6519
Inoculant*Clone(Geno)	72	412.7	0.4552
Error	96	38329.3	

Inoculant*Clone(Geno) error term used to test inoculant, genotype, and inoculant by genotype.

Appendix IB: Effect of % fungal coverage on harvested alfalfa plants (trials combined).

Source	df	MS	Pr>F
Inoculant	5	31153.1	0.0001
Plant genotype	3	13188.5	0.0001
Inoculant*Genotype	15	592.4	0.1139
Trial	2	26165.1	0.0001
Inoculant*Trial	10	6555.4	0.0001
Genotype*Trial	6	62326.5	0.0001
Inoculant*Genotype*Trial	30	333.7	0.7339
Error	503	203509.3	

Inoculant*Genotype*Trial error term used to test above factors.

Appendix IC: Effect of plant genotype on glucosamine concentration for whole plant incubation of freshly harvested alfalfa plants (Manuscript I).

Trial 1

Dependent Variable :GLU

Source	df	MS	Pr>F
Time d 0			
Geno	3	0.3871	0.9552
Clone (Geno)	12	0.7626	0.4990
DM	1	0.4176	0.4729
Error	16	0.2527	
Time d 5			
Geno	3	0.4090	0.0194
Clone (Geno)	12	0.4595	0.1291
DM	1	0.0145	0.8119
Error	16	0.5051	
Time d 8			
Geno	3	4.7342	0.3641
Clone (Geno)	12	5.1679	0.0876
DM	1	1.7091	0.4174
Error	16	1.8498	
Time d 11			
Geno	3	2.0314	0.0015
Clone (Geno)	12	3.0587	0.0274
DM	1	21.2765	0.0004
Error	16	2.7538	

Appendix IC: Continued.
Trial 2
Dependent Variable: GLU

Source	df	MS	Pr>F
Time d 0			
Geno	3	0.1414	0.7901
Clone (Geno)	12	0.1308	0.7170
DM	1	0.1077	0.4546
Error	15	0.1828	
Time d 6			
Geno	3	1.7372	0.0257
Clone (Geno)	12	1.6396	0.8287
DM	1	3.3154	0.2971
Error	15	2.8407	
Time d 8			
Geno	3	3.5469	0.0264
Clone (Geno)	12	3.5511	0.4840
DM	1	16.3651	0.0475
Error	15	3.5122	
Time d 11			
Geno	3	5.5735	0.0330
Clone (Geno)	12	63.6736	0.6756
DM	1	0.0398	0.9406
Error	15	6.9312	

Appendix IC: Continued.
Trial 3
Dependent Variable: GLU

Source	df	MS	Pr>F
Time d 0			
Geno	3	0.0705	0.6851
Clone (Geno)	12	0.0805	0.8301
DM	1	0.1279	0.3542
Error	15	0.1399	
Time d 5			
Geno	3	5.5817	0.1599
Clone (Geno)	12	0.9262	0.9705
DM	1	0.0092	0.9552
Error	15	2.8155	
Time d 8			
Geno	3	13.7058	0.0403
Clone (Geno)	12	1.9493	0.7979
DM	1	0.0174	0.9433
Error	9	3.2463	
Time d 11			
Geno	3	1.3628	0.7676
Clone (Geno)	12	3.2795	0.5522
DM	1	20.5332	0.0299
Error	15	3.5698	

Appendix ID: Effect of plant genotypes on dry matter content of freshly harvested alfalfa plants (Manuscript I).

Trial 1
Dependent Variable: DM

Source	df	MS	Pr>F
Time d 0			
Geno	3	1.3695	0.8906
Error	12	5.1076	

Trial 2
Dependent Variable: DM

Source	df	MS	Pr>F
Time d 0			
Geno	3	34.4873	0.0589
Error	12	3.5133	

Trial 3
Dependent Variable: DM

Source	df	MS	Pr>F
Time d 0			
Geno	3	5.3047	0.0471
Error	12	1.4850	

Appendix IE: Effect of plant genotype on DM loss during incubation of whole plant material in petri plates (Manuscript I).
Trial 2
Dependent Variable: DM % lost

Source	df	MS	Pr>F
Time d 6			
Geno	3	49.7102	0.0174
Error	12	9.8727	
Time d 8			
Geno	3	131.7861	0.0347
Error	12	31.9850	
Time d 11			
Geno	3	138.1093	1.1207
Error	12	29.0200	

Appendix IE: Continued.
Trial 3
Dependent Variable: DM % lost

Source	df	MS	Pr>F
Time d 6			
Geno	3	124.0019	0.0165
Error	12	24.2313	
Time d 8			
Geno	3	50.4084	0.0601
Error	12	15.5237	
Time d 11			
Geno	3	103.0330	0.0046
Error	12	13.9581	

Appendix IE: Effect of genotype on forage glucosamine concentration at sampling times during field wilting and bale storage (Manuscript I).
Trial 4
Dependent Variable: GLU

Source	df	MS	Pr>F
At cutting			
Geno	3	0.1182	0.1049
Error	12	0.0464	
Field wilting d 4			
Geno	3	0.0928	0.1092
Error	11	0.0364	
Stack storage d 0			
Geno	3	0.0402	0.5662
Error	11	0.0571	
Stack storage d 9			
Geno	2	0.8437	0.0232
Error	9	0.6529	
Stack storage d 24			
Geno	3	0.7807	0.2247
Error	12	0.4657	

Appendix IE: Continued.
Trial 5
Dependent Variable: GLU

Source	df	MS	Pr>F
At cutting			
Geno	3	0.0021	0.9954
Error	12	0.0972	
Field wilting d 4			
Geno	3	0.0064	0.5463
Error	12	0.0867	
Stack storage d 0			
Geno	3	0.3465	0.1731
Error	10	0.0568	
Stack storage d 9			
Geno	2	0.8348	0.1709
Error	8	0.3867	
Stack storage d 24			
Geno	3	0.7085	0.0907
Error	11	0.2544	

Appendix IF: Effect of dry matter at sampling times during field wilting and bale storage (Manuscript I).

Trial 4
Dependent Variable: DM

Source	df	MS	Pr>F
At cutting			
Geno	3	8.1225	0.0002
Error	12	0.5232	
Field wilting d 2			
Geno	3	7.9091	0.0706
Error	12	2.6027	
Field wilting d 3			
Geno	3	30.9273	0.0009
Error	12	2.8147	
Field wilting d 4			
Geno	3	147.9946	0.0003
Error	12	10.1928	
Stack storage d 0			
Geno	3	60.7427	0.0001
Error	11	0.7319	
Stack storage d 9			
Geno	2	23.1968	0.0086
Error	9	2.7492	
Stack storage d 24			
Geno	3	6.4216	0.6559
Error	11	11.5858	

Appendix IF: Continued.

Trial 5
Dependent Variable: DM

Source	df	MS	Pr>F
At cutting			
Geno	3	6.8941	0.0001
Error	12	0.3247	
Field wilting d 2			
Geno	3	14.9004	0.0042
Error	12	1.9710	
Field wilting d 3			
Geno	3	36.82	0.0015
Error	12	3.7741	
Field wilting d 4			
Geno	3	61.1986	0.0125
Error	12	10.9817	
Stack storage d 0			
Geno	3	8.3016	0.2022
Error	10	4.4917	
Stack storage d 9			
Geno	3	15.4032	0.0944
Error	8	5.1140	
Stack storage d 24			
Geno	3	2.8923	0.6425
Error	11	5.0190	

Appendix IG: Effect of genotype on forage glucosamine concentration at sampling times during field wilting and bale storage combined data from trials 4 and 5 (Manuscript I).
Trial 4 and 5
Dependent Variable: GLU

Source	df	MS	Pr>F
At cutting			
Geno	3	0.0523	0.5468
Trial	1	1.359	0.0006
Geno*Trial	3	0.080	0.4356
Error	24	0.0722	
Field wilting d 4			
Geno	3	0.1345	0.3583
Trial	1	0.8183	0.0151
Geno*Trial	3	0.0836	0.5616
Error	24	0.1194	
Stack storage d 0			
Geno	3	0.0162	0.8358
Trial	1	0.3512	0.0215
Geno*Trial	3	0.1396	0.0916
Error	21	0.0569	
Stack storage d 9			
Geno	3	2.1426	0.0240
Trial	1	1.9980	0.0684
Geno*Trial	2	1.5094	0.0849
Error	17	0.5276	

Appendix IG: Continued
Stack storage d 24

Geno	3	0.3681	0.7888
Trial	1	0.0291	0.8692
Geno*Trial	3	0.4244	0.7510
Error	22	1.0485	

Appendix IH: Effect of genotype on DM at sampling times during field wilting and bale storage combined data from trials 4 and 5 (Manuscript I).
Trial 4 and 5
Dependent Variable: DM

Source	df	MS	Pr>F
At cutting			
Geno	3	14.7781	0.0001
Trial	1	0.0597	0.7106
Geno*Trial	3	0.2384	0.6451
Error	24	0.4240	
Field wilting d 2			
Geno	3	12.8731	0.0046
Trial	1	28.7159	0.0017
Geno*Trial	3	9.9365	0.0140
Error	24	2.2869	
Field wilting d 3			
Geno	3	63.7048	0.0001
Trial	1	8.9544	0.1123
Geno*Trial	3	4.0475	0.3210
Error	24	3.2944	
Field wilting d 4			
Geno	3	199.4683	0.0001
Trial	1	108.1716	0.0039
Geno*Trial	3	9.7250	0.4468
Error	24	10.5872	

Appendix IH: Continued

Stack storage d 0			
Geno	3	54.7436	0.0001
Trial	1	222.5625	0.0001
Geno*Trial	3	12.0323	0.0109
Error	21	2.5223	
Stack storage d 9			
Geno	3	30.6758	0.0016
Trial	1	40.9042	0.0047
Geno*Trial	2	0.2039	0.9487
Error	17	3.8620	
Stack storage d 24			
Geno	3	131.5000	0.5064
Trial	1	486.6008	0.0991
Geno*Trial	3	159.0293	0.4250
Error	22	164.0796	

Appendix IIA: Effect of genotype on epiphytic populations of freshly harvested alfalfa plants (Manuscript II).

Genotypes from trial 10

Dependant Variable: TB

Source	df	MS	Pr>F
Geno	3	1.55	0.0181
Error	12	0.3112	
Dependent Variable: LAB			
Geno	3	1.37	0.0014
Error	12	0.1385	
Dependent Variable: Yeast			
Geno	3	2.35	0.0460
Error	12	0.6535	
Dependent Variable: Mold			
Geno	3	0.72	0.1610
Error	12.	0.3517	

Appendix IIA: Continued.

Genotypes from trial 11

Dependant Variable: TB

Source	df	MS	Pr>F
Geno	7	0.85	0.0003
Error	51	0.1778	
Dependent Variable: LAB			
Geno	7	3.49	0.0001
Error	54	0.2141	
Dependent Variable: Yeast			
Geno	7	1.49	0.0001
Error	46	0.2053	
Dependent Variable: Mold			
Geno	7	5.89	0.0001
Error	56	0.1234	

Appendix IIA : Continued

Contrasts

Dependent Variable TB

Source	df	MS	Pr>F
All genotypes res vs sus	1	3.96	0.0001
Set B genotypes res vs sus	1	1.27	0.0101
Error	51	0.18	0.0001
Dependent Variable LAB			
All genotypes res vs sus	1	0.04	0.6555
Set B genotypes res vs sus	1	0.61	0.0975
Error	54	0.21	
Dependent Variable Yeast			
All genotypes res vs sus	1	1.26	0.0171
Set B genotypes res vs sus	1	0.01	0.8890
Error	56	0.21	
Dependent Variable Mold			
All genotypes res vs sus	1	1.66	0.0005
Set B genotypes res vs sus	1	0.27	0.1455
Error	56	0.12	

Appendix IIB: Effects of drying rate on freshly harvested alfalfa plants (Manuscript II).

Genotypes from set A

Dependent Variable: Log of water remaining

Source	df	MS	PR>F
Geno	3	0.01	0.8050
Day	1	97.52	0.0001
Day*Geno	3	0.34	0.0001
Error	72	0.03	

Genotypes from set B

Source	df	MS	PR>F
Geno	5	0.04	0.0775
Day	1	150.50	0.0001
Day*Geno	5	0.46	0.0001
Error	108	0.08	

Appendix IIC: Effect of water soluble carbohydrates (WSC) on freshly harvested alfalfa plants (Manuscript II).

Dependent Variable:
WSC

Source	df	MS	Pr>F
Geno	3	504.18	0.12
Error	12	213.53	

Appendix IID. Results of trial 7 disc diffusion assay using 4 discs per genotype extracted with methanol:H_2O.

	Genotypes					
Inoculum	R-1B	R-2B	R-3B	S-1B	S-2B	S-3B
A. flavus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. glaucus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. repens						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. versicolor						
d 3	DDDD	DDDY	DDDD	DDDD	DDDD	DDDD
d 4	DDDD	DDDY	DDDD	DDDD	DDDD	DDDD
d 5	DDDD	DDDY	DDDD	DDDD	DDDD	DDDD
A. fumigatus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
combined						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD

D dark zone surrounding the disc
i no dark or inhibition zones surrounding the disc
I inhibition zone surrounding the disc
Y yellow zone surrounding the disc

Four discs with 100 ul extract for clones 1 and 2 per genotype were tested in this trial.

Appendix IIE. Results of trial 7 disc diffusion assay using 4 discs per genotype extracted with Chloroform.

	Genotypes					
Inoculum	R-1B	R-2B	R-3B	S-1B	S-2B	S-3B
A. flavus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. glaucus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. repens						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. versicolor						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
A. fumigatus						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
combined						
d 3	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD

D dark zone surrounding the disc
i no dark or inhibition zones surrounding the disc
I inhibition zone surrounding the disc
Four discs with 100 ul extract for clones 3 and 4 for each genotype were tested.

Appendix IIF. Results of Trial 9 disc diffusion assay using 4 discs per genotype extracted with methanol:acidified water (80:40 v/v) or chloroform.

	Genotypes							
	R-1B	R-2B	R-3B	R-1A	S-1B	S-2B	S-3B	S-4A
Methanol:H_2O								
d 3	iiii	iiii	iiii	DDDD	iiii	iiii	iiii	iiii
d 4	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD	DDDD
Chloroform								
d 3				DDDD				DDii
d 4				DDDD				DDDD

D dark zone surrounding the disc
i no dark or inhibition zones surrounding the disc
I inhibition zone surrounding the disc
Two discs per clone with 100 ul extract and 2 clones per genotype were tested in this trial.
Set B plants were not extracted with chloroform due to shortage of material

Appendix IIG. Results of Trial 9 disc diffusion assay genotypes extracted with acidified water.

pH/day	Genotypes	
	R-1A	S-4A
pH 3.0		
d 3	DD	DD
pH 3.5		
d 3	DD	DD
pH 4.0		
d 3	DD	DD
pH 4.5		
d 3	DD	DD
pH 5.0		
d 3	DD	DD
pH 5.5		
d 3	DD	DD
pH 6.0		
d 3	ii	ii
d 4	DD	DD
pH 6.5		
d 3	ii	ii
d 4	DD	DD

D dark zone surrounding the disc
i no dark or inhibition zones surrounding the disc
I inhibition zone surrounding the disc
Two discs with 100 ul extract per genotype at each pH were tested.

Appendix IIIA: Cultivar comparison for leaf area colonized in Screening I (Manuscript III).
Dependent Variable: Rating

Source	df	MS	PR>F
CV	21	3937.8	0.0001
Geno(CV)	264	522.0	0.0005
Rep	3	37522.7	0.0001
Rep(CV)	63	1432.5	0.0001
Error	787	377.8	

Tests of Hypotheses using the Anova MS for geno(CV) as the error term.

Appendix IIIB: Genotype comparison for leaf area colonized in Screening III (Manuscript III).
Dependent Variable: Rating

Source	df	MS	PR>F
Geno	20	1356.5	0.0001
Clone(geno)	63	841.8	0.0001
Error	92	221.7	

Tests of Hypotheses using the Anova MS for clone(geno) as the error term.