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(57) Abstract

Methods for identification of tannin-expressing alfalfa plants, alfalfa plants having measurable endogenous tannin levels and methods of breeding for alfalfa cultivars having measurable endogenous tannin levels are disclosed. Methods for feeding ruminants with forage from alfalfa (*Medicago sativa* L.) plants having measurable endogenous tannin levels, which provides bloat safety, bypass proteins, increased pest resistance and improved ruminant health and nutrition are also disclosed. The methods involve the use of alfalfa plants having measurable endogenous tannin levels as a major source of feed.

ALFALFA PLANTS HAVING MEASURABLE ENDOGENOUS TANNIN LEVELS FOR USE AS ALFALFA FORAGE FOR IMPROVED RUMINANT HEALTH AND NUTRITION AND METHODS OF IDENTIFYING AND BREEDING TANNIN-EXPRESSING ALFALFA PLANTS

FIELD OF THE INVENTION

The present invention relates to alfalfa plants and methods of alfalfa breeding, animal feeding and animal nutrition. In particular, the invention relates to alfalfa plants having measurable endogenous tannin levels as measured by a radial diffusion assay, developing such alfalfa plants, with the tannin levels providing bloat safety, protein protection, and improved ruminant health and nutrition, and providing pest control in the alfalfa plants.

BACKGROUND

Alfalfa Grazing, Bloat and Current Management Practices

Alfalfa (*Medicago sativa* L.), of all currently grown field crop species, produces the largest amount of crude protein per acre and is the most important forage legume grown in the United States. It has a wide range of adaptation from temperate to tropical regions. Its importance in world agriculture can be attributed to a number of morphological and physiological characteristics that contribute to its high yield of nutritious forage, rapid recovery after cutting, longevity, and tolerance to environmental stress. Because alfalfa fixes atmospheric nitrogen, it limits the need for chemical nitrogen application and adds a beneficial nitrogen carryover effect in crop rotation.

Fresh alfalfa forage consumption has usually been associated with bloat in ruminants. Although bloat is rarely encountered when feeding alfalfa in the form of hay, silage, pellets or haylage (Howarth, 1988), bloat is a serious digestive disorder of beef cattle and dairy cows that graze forage legumes such as alfalfa and white clover. The disorder is caused by the formation of stable foam in the rumen which prevents gas escape, and often results in animal death.

A number of reviews relating to bloat have been published, with several of them reported by Bush and Burton (1994). Digestion of forage by the microflora in the rumen releases large quantities of CO_2 and methane. Bloat is the distention of the ruminal cavity that occurs when these gases are retained by the development of a persistent foam within the rumen. Distention of the rumen exerts pressure on the circulatory and pulmonary systems causing severe stress and sometimes death of the animal due to asphyxiation. Bloat can be chronic and nonlethal and it can also be sudden and

deadly. Without wishing to be bound by any particular theory of mechanism of action the presence of soluble proteins, namely the major plant enzyme ribulose bisphospate carboxylase, is at this time the best simplified explanation of the bloat-causing potential of alfalfa herbage (Howarth, 1988). Concentration of soluble protein in the rumen fluid is higher in animals that subsequently bloat than in those that do not bloat (Hall et al., 1988).

Alfalfa grazing, when well conducted, has been shown to be one of the most cost-effective farm practices (Lacefield et al., 1996). Alfalfa is well suited for hay production, but the propensity of this legume to cause bloat has restricted its use for grazing. Grazing alfalfa has not been practiced to any great extent in the U.S. (Lacefield et al., 1996). Even with this low rate of usage, mortality of cattle from bloat in Canada and the United States may be as high as 1% to 1.5% of all alfalfa-grazed cattle. The value of these losses is estimated at $125 million (Goplen, 1989; Howarth, 1988). What makes bloat frightening for most producers is that, its appearance, albeit infrequent, is also quite unpredictable and the losses it causes can be severe. In New Zealand, where grazing is a common practice, 80% to 90% of the producers treat for bloat at some time in the year (Clark, 1996). Currently, bloat preventative management techniques, discussed more fully below, are available but have not proven to adequately address the bloat problem.

Ironically, grazing of alfalfa has been shown to be an economically viable option for dairy farms, specifically in Michigan (Rotz, 1996). Depending on the grazing strategy used, the overall result was an increase in the annual return to management or farm profitability of $100 to $240/dairy cow, even when the added cost of the use of bloat control additives is considered (Rotz, 1996). Bloat-free alfalfa would also likely expand the use of this more nutritious forage species for the benefit of the grower.

Requirements for establishing an alfalfa stand for grazing are similar to requirements for establishing a stand for hay production. A thick, healthy and productive stand has the greatest potential for animal performance and production per acre.

Recommendations for grazing a stand of alfalfa approximate hay harvest. Rotational grazing has been shown to be the best agronomic practice, as opposed to continuous grazing, for yield quality and stand persistence. General recommendations are to graze an area (paddock) for one week or less and allow 4 to 6 weeks for plants to recover before grazing again. Plants should not be grazed for more than 10 to 12 days. Dividing the field into smaller paddocks is necessary for rotational grazing. There should be a minimum of 5 individual paddocks to allow rotating the animals into a new

paddock each week with a 4-week recovery. Planning needs to be made for gates, lanes, access to water, ease of cattle movement and field slopes.

Although pure stands can be grazed successfully, alfalfa-grass mixtures are most often employed to minimize the risk of bloat. To prevent bloat associated with grazing alfalfa varieties having non-increased endogenous tannin levels, the following management practices are commonly suggested: 1. Grow grass and alfalfa in mixtures; 2. Feed bloat preventing compounds; 3. Pre-feed hay or other feed before turning animals into pastures, especially when plants are wet from dew; 4 Limit access to legume pasture until animals are adapted to the new feed; 5. Do not graze immature or frost damaged alfalfa or alfalfa-grass; 6. Provide salt and minerals; 7 Observe cattle closely when turning in for the first time; 8. Observe cattle closely during cool, cloudy, rainy weather for signs of bloat.

There currently are a number of effective products on the market to control bloat. However, with the exception of some bolus-delivery systems (e.g. Monensin) which can be relatively expensive, the grazier must ensure that sufficient amounts of the products are ingested daily or twice daily by every animal in the herd (Clark, 1996). Manual drenching or voluntary intake (from treated block, grain topdressing or through in-line delivery system) can be used. The voluntary intake approach introduces a significant risk because of individual animal variability in timing and amount of food gathering.

In Argentina, grazing is the pasture management method of choice. There, the growers prevent bloat by a constant monitoring of the animals; by growing alfalfa in mixture with grass; and by grazing alfalfa when it is in bloom. It is well recognized that the optimal stage for alfalfa harvest is at the mid-bud to first flower stage (Reinhart, 1990;Sheaffer et al., 1988). This combines high quality forage with high plant and animal productivity. Therefore, the practice of late alfalfa harvest (when in bloom) with grass mixtures has the major disadvantage of not allowing the growers to make full use of alfalfa potential as a high quality forage.

Tannins in Plants

Tannins as used herein means phenolic compounds that precipitate proteins. They can form complexes with starch and cellulose but they have a greater affinity for protein than for starch. Their molecular weight ranges from 500 to >20,000. Tannins are soluble in water with the exception for some high molecular weight structures.

Tannins are usually divided into two groups:

1 - Hydrolyzable tannins (HT);

2 - Condensed tannins (CT) often called proanthocyanidins

HT are molecules with a polyol (generally D-glucose) as central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups. Classes of HT include gallotannins (gallic acid), ellagitannins (ellagic acid), taragallotannins (gallic and quinic acid) and caffetannins(caffeic and quinic acid). The most common source of gallotannins is tannic acid. HT are usually present in low amounts in plants.

CT are more widely distributed in plants than HT. These are the type of tannin found in temperate legumes such as sainfoin and birdsfoot trefoil (Reed, 1995). This is also the type of tannin (Epicatechin) found in the palisade layer of the alfalfa seedcoat (Koupai-Abyazani et al., 1993). They are oligomers or polymers of flavonoid units linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. They are called condensed tannins due to their condensed chemical structure. The term proanthocyanidin is derived from the acid catalyzed oxidation reaction that produces the red anthocyanidin upon heating CT in acidic alcohol solutions. A more complete description of the chemical aspect of tannins is available in Hagerman (1992).

Tannins can be located in different parts of plants. Chiquette et al. (1988) reports that birdsfoot trefoil has tannin vesicles located just under the epidermal layer, frequently adjacent to the stomata. In *Lespedeza cuneata* tannins are especially evident in the vacuoles of paraveinal mesophyll cells, a layer of cells in contact with the lower layer of palisade parenchyma. These cells are thought to facilitate lateral transport within the leaf (Mosjidis et al.,1989). Tannins have also been found in the hypodermis (below suberized epidermis) of root tissues and between the outer integument and aleurone layer of seeds.

Plant tannins are well known for their bloat preventative characteristics. They prevent bloat by working on two important aspects of stable foam formation: reduction of soluble protein and reduction of gas formation. By forming complexes with proteins, condensed tannins precipitate the proteins that produce foam and reduce their availability to the proteolytic bacteria. This reduced bacterial activity in turn limits the production of gas in the rumen (Wood and Plumb, 1995).

Several methods exist for tannin determination. Most of them are discussed in Hagerman and Butler (1989), Lowry et al. (1996), or Reed (1995). No single method will give satisfactory results for quantitative analysis, in relationship to nutritional effects, because the chemical properties that are involved in the reactivity of polyphenols in colorimetric or precipitation assays may differ from the properties that underlie their nutritional or toxic effect. (Reed, 1995).

Colorimetric procedures share the analytical problem of the lack of suitable standards. The most commonly used tannin standards are tannic acid, catechin and quebracho. However, there are differences in chromophore reaction with these standards from those of the compounds from the plant extracts. Therefore, this can lead to large under- and overestimates of the content of polyphenols and is probably the single largest cause for the great deviation in tannin concentrations that are reported in the literature (Reed, 1995). The method using the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA-HCl) protocol (Li et al., 1996) appears to be one of the most reliable and sensitive.

Even though tannins have been reported as having differing molecular weights and chemical composition, no significant differences were detected between the biological effects of different tannins. Methods based on protein precipitation are often more realistic for estimating the content of tannins in plants because these methods are more closely related to biological effects.

However, these methods also have their disadvantages, mostly related to sample preparation. The radial diffusion (RD) method developed by Hagerman (1987) is a simple and specific method that overcomes most of these problems and still maintains good sensitivity. It has also been shown to have a good correlation (R^2= 0.63) with *in vitro* gas production of rumen fluid (Wood and Plumb, 1995). Nevertheless, it has been recommended by Perez-Maldonado et al. (1995) that a plant protein (fraction 1, associated with the ribulose bisphosphate carboxylase) be used as a protein standard instead of the common bovine serum albumin (BSA). The recommendation for doing so comes from the fact that in their experiment, BSA had a constant affinity of 2.6 g protein per gram of tannic acid, *Desmodium intortum* and *Lotus pedunculatus* tannins. However, with a purified plant protein, they had affinities of 3.5, 2.1 and 0.8 protein per gram of the respective tannin source. Some care is therefore needed in trying to predict ruminal behavior based on the tannin binding assay with BSA. As an alternative, a calibration could be built to predict the binding power of the alfalfa tannin with purified plant protein. BSA based RD readings could, therefore, be adjusted to represent the expected reaction in the rumen.

Handling of tissue samples and extraction of tannins are critical parts of any tannin assay. Extraction and analysis of fresh tissue is preferred in order to minimize chances for tannin polymerization and the formation of tannin complexes reducing extractability (Hagerman, 1988; Terrill et al 1989), but immediate analysis is not possible in all cases (when dealing with large numbers of samples or when assaying samples from remote locations). Lyophilization (freeze drying) is thought to be the

gentlest method of preservation and may be equivalent to fresh material although reduced tannin extractability has been noted in some cases (Hagerman, 1988) Several scientists have reported decreased tannin extraction from heating and drying (Hagerman, 1988; Terrill et al.,1989; Telek, 1989) and there are reports of oxidation affecting tannins during storage and extraction.

Three solvents are commonly used to extract tannin from plant samples: hot aqueous methanol, aqueous acetone and acidic methanol (Hagerman, 1988). It appears that aqueous acetone (usually 70%) is preferred by most (Hagerman, 1988, Li et al 1996).

Tannins in Forage Species and Bloat Safety

In forage species, tannin content has been associated with bloat safety All tannin-containing legumes such as birdsfoot trefoil(*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia* Scop.), sericea lespedeza (*Lespedeza cuneata*), and crownvetch (*Coronilla varia*) are considered bloat safe. Grasses are also considered bloat safe, but there are reports that rapidly growing perennial ryegrass can cause bloat (Clark, 1996) Other forage species, such as alfalfa or white clover, with very little or no tannin, can cause bloat if the conditions are favorable. While white clover is not considered to be a bloat safe legume it does contain small amounts of tannins, 0.2 to 0.7 % according to Stockdale and Dellow (1995); or even less (0.06%) according to Li et al., (1996).

Nevertheless, higher amounts of tannins in the flowers could explain why less incidences of bloat are observed when flowering white clover is fed to cows as the sole feed compared with clover with few or no flowers (Stockdale, 1994). By looking at many forage species, Li et al. (1996) extrapolated that 0.2-0.5% dry matter (DM) would be sufficient to prevent bloat from alfalfa and recommended 0.5% as a breeding objective. This represents a condensed tannins (CT) to soluble protein ratio of about 1:20.

Tannin level in forages is mostly genetically determined but can vary during the growing season. For example, in low tannin varieties (2-5% total tannins) of sericea lespedeza, concentration of tannins can vary from one to two percent during the growing season (Windham et al., 1988). Barry (1985), reporting some of his previous work, mentioned the increase of tannin concentration when birdsfoot trefoil is grown in acid, low-fertility soil (70-80g/kg DM) versus fertile soil (20-30g/kg DM). Lege et al. (1993) with cotton, recommends consideration of the tissue sampled when analyzing tannin after finding that younger tissue had higher condensed tannin concentration

Extensive searches of germplasm of alfalfa and closely related species (*M falcata* and *Trigonella* spp.) have failed to find notable amounts of tannins, with the exception of the seed coat (Reed, 1995). Li et al.(1996) recently reported the observation of tannins in trichomes of alfalfa but even their sensitive technique did not allow the quantification of any measurable amount of phenolics. In fact, it is significant to note that in most studies, alfalfa is actually used as the null standard for tannins (Robbins et al., 1987). Alfalfa tannin levels of 0.85g/kg were reported by Kraiem et al. (1990). However, the colorimetric method they used may have suffered from precision problems, as discussed in a later section. The 0.37% and 0.13% DM amounts they also report for sainfoin and trefoil are not in the range commonly admitted for these species. Minor amounts of tannins in alfalfa have been reported by Niezen et al. (1995) and Wang et al., (1996a) using an acid-butanol analytic method, but they also stated that alfalfa does not contain tannins, or that only traces are present.

The lack of tannins in alfalfa has caused several researchers to consider genetic engineering as the sole way to obtain alfalfa varieties having measurable tannin levels (Broderick 1995; Bush and Burton,1994; Dalrymple et al., 1984; Daminani et al., 1993;Gruber et al., 1994). Vogel and Sleper (1994) also stated that breeders lack definite selection criteria for bloat, and until the causes of bloat are clearly delineated, forage breeders will not be able to successfully address this problem. Attempts have been made to develop interspecific hybrids between alfalfa and sainfoin, or between alfalfa and birdsfoot trefoil, using protoplast fusion, but stable hybrids "were not obtained" (Li et al., 1993).

This lack of genetic variability for tannins in alfalfa has motivated researchers to find other ways to obtain bloat safety in alfalfa. Cheng et al., (1995) have attempted to develop bloat-safe cultivars of alfalfa by reducing the initial rate of digestion of the forage in the rumen. They refer to a low initial rate of digestion (LIRD varieties), measured on the basis of the extent of DM disappearance after 4 hours of incubation in the rumen. Although they have shown that some reduction in the risk of bloat can be achieved, the new cultivar cannot be considered completely bloat-safe, and the inherent forage quality of the crop is reduced. Rumbaugh (1969) has measured genetic differences for foaming properties in alfalfa but the range of variability for alfalfa is not likely to reach the bloat safety level without significant reduction of the feed value of the crop.

A low to moderate content of tannins (up to 4 or 5%) in alfalfa would be a desirable trait to enhance the efficiency of protein utilization in ruminants (Waghorn et al., 1987; West et al., 1993; Waghorn and Shelton, 1995). The protective role of

condensed tannins in the rumen is now universally accepted as a viable proposition (D'Mello, 1992). Numerous feeding studies have shown that excessive ruminal protein degradation may be the most limiting nutritional factor in higher-quality temperate legume forage (Broderick, 1995). When protein degradation is very rapid, ruminal microbes cannot utilize all of the amino acids and ammonia released. More protein is degraded than is synthesized and the consequent loss of ammonia from the rumen is referred to as "ammonia overflow". Improving protein utilization using various formulas or protectants have been the object of patents (Meyer, 1988; McAskie, 1989, Lyon et al., 1981; Burroughs and Trenkle, 1980).

Small genetic variability has been found in alfalfa for protein degradability in silage or in the rumen (Broderick, 1995). These differences can be attributed to structural or protein constituents and are not independent of other forage quality factors such as protein content or digestibility. However, the chemical reaction between condensed tannins and proteins offers a new dimension to improve the protein utilization in the grazing ruminants, which include beef cattle, dairy cattle and sheep. Condensed tannins, by complexing with proteins, reduce their degradation and hence lower the proportion of non-protein nitrogen such as ammonia (Waghorn et al.,1987). The tannin-protein complex, stable at the rumen pH (5 to 7), would by-pass or escape microbial digestion in the rumen, with protein becoming available for digestion in the low pH of the stomach (2.5 to 3.5) or higher pH (8) of the lower intestinal tract.

Table 1 summarizes the different effects of tannins across different plants and ruminants species. Low to moderate tannin levels (2-3%) such as in the Waghorn et al., (1987) study, increased the flux of essential amino acids through the abomasum (real stomach) in sheep. It would also enhance utilization and concentration of cystine (a limiting amino acid in legumes and a major component of wool protein) for biosynthetic reactions (Wang et al., 1994; Lee et al., 1995). Increased concentration of growth hormones in the plasma was also observed as an effect of additional tannins but the cause and consequences are not well understood (Barry et al.,1986a). A recent study conducted in New Zealand (Waghorn and Shelton, 1995) has shown that a grass and lotus mixed diet containing as little as 1.8% CT was able to substantially reduce apparent digestibility of nitrogen, reduce ammonia concentration, and maintain the nutritive value of the forage. Wang et al. (1996a) concluded that the principal effect of tannins in growing lambs on birdsfoot trefoil versus alfalfa as a feed was to increase wool growth. However, the greater rate of carcass gain of lambs grazing trefoil was thought to be caused by factors other than CT. The same group (Wang et al.,1996b)

has observed a slower decline in milk production and higher milk protein than ewes fed birdsfoot trefoil supplemented with polyethylene glycol (PEG), a tannin binding agent.

When looking at different legume species Broderick (1995) reports that a quadratic regression explains the relationship between the tannin concentration and the protein degradation rate. The minimal degradation rate was estimated to be at 27g of tannic acid equivalent/kg of DM. However, these data were collected across species of very different forage quality and it is not known what the relation could be in a single, high forage quality background such as alfalfa. Because the effect of tannins is affected by the level of protein in the forage, other scientists (Chiquette et al., 1989, Tanner et al., 1994) prefer to talk about free tannins or the tannin:soluble protein ratio. Free tannins, defined as the total water-soluble condensed tannins that have exceeded the binding capacity of plant proteins, could be detrimental to the ruminant if they precipitate proteins of the gut wall and extracellular carbohydrate degrading enzymes secreted by rumen bacteria. Because alfalfa and birdsfoot trefoil contain similar amounts of crude protein it is expected that the optimal tannin concentration needed in grazing should approach that of birdsfoot trefoil. In *Lotus* spp. it is estimated that the ideal tannin concentration should be in the 20-40g/kg DM range (Barry, 1985; Barry et al., 1986b).

TABLE 1: Nutritional Effects of Tannins on Ruminants

Levels	Tannins (%DM)	Plant Type	Treatments	Animal Fed	Effects	Reference
Low	0 2, 0 7	*Trifolium repens*	Maize silage	cow	lower rumen amonia concentration	Stockdale and Dellow, 1995
	1.26 to 2 00	Sorghum	*Silage*, Fresh	sheep	TN reduced by ensiling. No diff. in DM intake.	Montgomery et al., 1983
					no diff. for digestibility	
					high fiber-bond proteins in bird-resistant types	
	1.8	*L pedunculatus*	with ryegrass	sheep	reduce apparent digestibility of N	Waghorn and Shelton, 1995
					no effect on liveweight gain or wool growth	
	1 or +/- 5	*Lotus corniculatus*	Low or high TN strains	nylon bag	lower TN improved digestibility	Chiquette et al., 1988
	0,2.2.5 0	Ryegrass & *Lotus sp*	High, low no TN	sheep	high TN increase cystein blood concentration	Lee et al., 1995
					best wool and body weight gain on low TN	
	2 2	*Lotus corniculatus*	w/o PEG	sheep	similar DM intake, lower apparent N digestion	Waghorn et al., 1987
					lower rumen [NH3], greater flus of essential AA	
Mod.	2.7	*Lotus corniculatus*	w/o PEG	sheep	increase utilization of cystine reduce apparent digestion of N	Wang et al., 1994
	0,3.6,	*Medicago sativa*	0,3.6% TN	Sheep, Deer	protein digestibility reduction	Hagerman et al., 1992
	1 7,3 9, 5 2.6 2	Peanut skins		cow	decrease rumminal NH3, higher milk yields at moderate [TN]	West et al., 1993
					higher fat but lower protein as TN increase	
	3 4	*Lotus corniculatus*	w/o PEG	sheep	increase wool growth	Wang et al., 1996a
	4.4	*Lotus corniculatus*	w/o PEG	sheep	increase milk production and milk protein	Wang et al., 1996b
	9.5, 4 5 1 4	*Lotus corniculatus*	w/o PEG	sheep	TN increase [plasma growth hormone]	Barry et al., 1986a
	5	*Cenatonia siliqua*	w/o PEG	Sheep	indication of trypsin and amylase inhibition	Silanikove et al., 1994
	?	Sorghum	Bird-resistant or non-	Beef	TN decrease small intestinal a a digestibility	Streeter et al., 1993
	?	Forages, trees shrub	various formulas	Deer	TN reduces cell soluble digestibility	Robbins et al., 1987
	1.8, 4, 7 1	various	w/o PEG	Goats	depression of protein digestion	Silanikove et al., 1996
	5	*Hedisarum coronanum*	nematodes, drenched	sheep	improved parasitized lambs (wool) performance	Niezen et al., 1995
	5.0-5 5	*Lotus corniculatus*	w/o PEG	sheep	increase net absorption of methionine	McNabb et al., 1993
High	4.6, 10 6	*Lotus peduncalatus*	w/o PEG	sheep	reduced intake	Barry and Duncan, 1984
	7.6-9 0	*Lotus peduncalatus*	w/o PEG	sheep	low live weight gain on high-TN	Barry, 1985
	7 or 18	*Lespedeza cuneata*	Low or high TN strains	Sheep	HT decreased intake and digestibility	Terrill et al., 1989

If low to moderate tannin levels show beneficial effects in the ruminant, a level of tannins that is either too low or too high would not be desirable. With respect to high levels of tannin, the astringent reaction in the mouth of the animal would first reduce the palatability of the feedstuff and consequently, feed intake, a key component of ruminant growth rate and milk production. West et al., (1993), using peanut skins in a protein

rich ration concluded that peanut skins should be limited to no more than 16% of the ration. This corresponded to tannin levels of 5.18%. Toxicity or negative effects from high levels of tannins for the ruminant may result from their affinity for essential amino acids (especially methionine and lysine) or from damage to the mucosa and gastrointestinal tissues. Feeding high tannin containing rations to goats, Silanikove et al. (1996) have shown that intake and digestibility can be improved by adding polyethylene glycol (PEG), a tannin binding agent to the ration. Similarly, *Lotus pedunculatus* cultivars containing 20g/kg DM tannins was considered beneficial to the animal but high levels (76-90g/kg DM) had reduced live weight gain in sheep (Barry, 1985; Barry and Duncan, 1984). It appears, therefore, that low concentration in the range of 5 to 40 g/kg DM would capture the nutritional benefits and avoid the negative effects of tannins.

Hydrolyzable tannins (HT) are easily degraded in the rumen and excreted in the urine (Hagerman et al., 1992). Nevertheless, HT when converted into absorbable low molecular weight metabolites, are toxic to ruminants or monogastrics (McSweeney et al., 1988; D'Mello, 1992; Zhi-Cheng, 1988). It can cause hemorrhagic gastroenteritis, necrosis of the liver, and kidney damage with proximal tubule necrosis. If liver or kidney damage is to occur from natural poisoning, it would be only when HT are released from plant material in the abomasum without being degraded in the rumen (Zhu and Filippich, 1995). Tannin toxicity from HT may occur in animals fed oak (*Quercus* spp.) and several tropical legumes (e.g. *Terminalia oblongata* and *Clideria hirta*). High mortality and morbidity was observed in sheep and cattle fed oaks and other tree species with more than 20% HT. Diets containing 29.9g/kg DM of HT did not compromise digestion in sheep but was able to cause signs of intoxication in sheep (McSweeney et al., 1988). Although phenolics are nominally toxic, in practice they rarely cause intoxication (Lowry et al., 1996). The most notable exception concerns the rapid breakdown of HT, when species containing up to 25% HT, are ingested in large amounts.

The presence of tannins is not the only way to prevent bloat. Some bloat-safe legumes such as sainfoin milkvetch do not contain tannins. However, mesophyll cells in these species are more resistant to mechanical rupture and do not allow a rapid release of soluble proteins. These legumes have strong cell walls, a high degree of tissue strength, or adaxial cuticle that prevents a rapid cell disruption.

Because cancer is such a devastating disease in humans and animals it is worthy of mention that tannins exhibit anticarcinogenic activity. Studies, such as the

one by Chen et al. (1996) or Bomser et al. (1996) both exemplify the potential long term benefit of having tannins in the animal diet.

Tannins in Plants and Pest Control

Tannins are among the most important secondary plant compounds involved in plant defense against insects and diseases. Tannins provide pest control primarily by preventing or inhibiting feeding on the plant by the pest or inhibiting growth of the pest on the plant, but can also be the direct cause of death for the pest. The deterrent action of tannins is facilitated by their astringent character and/or ability to inhibit enzymatic activity, thereby disrupting the digestive processes of the pest (Lege et al., 1993). With lepidoptera, it has been suggested that the effect of tannins may be to reduce concentration of surface-active phospholipids in the mid-gut and produced lipid or other dietary deficiencies (Ian de Veau and Schultz, 1992). Negative correlations were found between nymphal (-0.52) or egg (-0.75) populations of whitefly (*Bemisia tabaci* Genn.) and tannin content in cotton (Butter et al., 1992). Wang et al. (1995) by looking at poplar trees (*Populus* spp.) found a positive correlation between the resistance to *Batocera horsfieldi* and the content of tannins. In pine trees, tannins would be involved in the natural resistance of conifer seed cones to fungal degradation (Eberhardt and Young, 1994).

Condensed tannins, although useful in fighting various plant pests, cannot be responsible for all pest resistance. It is indeed logical to think that condensed tannins are not the only compounds related to an incredible array of resistance to pests found in plants. There are, therefore, many cases where tannins do not play any significant role in pest resistance. Examples include bruchids on *Vicia faba* seed coat (Desroches et al., 1995); willow beetle (*Phratora vulgatissima*) on *Salix* spp. (Kelly and Curry, 1991); artificial diets on *Trichoplusia ni* (Gonzalez-Coloma et al., 1994); psyllid (*Leucaena psyllid*) on *Leucaena* spp. (Wheeler et al., 1994).

Because tannins have a wide occurrence in nature, it is likely that various pests (and animals) have evolved to avoid the negative nutritional effects of tannins. It is interesting to note that some fungi have also evolved to detoxify tannins and that a symbiosis exists between various insects and these fungi that metabolize or detoxify tannins. (Dowd, 1992). Tannic acid has even been shown to be an attractant for nematodes at low levels and a nematacide at high levels (Hewlett and Hewlett., 1997).

In cotton, condensed tannins were thought to prevent feeding from cotton bollworm and tobacco budworm and may also convey host plant resistance to spider mite (Lege et al., 1993). However, the level or the type of CT needed to offer a good

control was not always conclusive (Smith et al., 1992). In a study on grass-feeding grasshoppers, tannins only exhibited a weak effect on these insects (Mole and Joern, 1994).

In light of the foregoing, it is apparent that there is a significant need for alfalfa plants having measurable endogenous tannin levels and a process for feeding of ruminants using alfalfa plants having measurable endogenous tannin levels. Such alfalfa plants and process would allow for alfalfa grazing without the possible significant negative economic impact caused by bloat, and would also provide other potential nutritional and health benefits to the grazing ruminants.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 illustrates a typical Radial Diffusion (RD) calibration set using tannic acid controls (Radial diffusion (cm^2) vs tannic acid (mg/well)). 20 µl/well of tannin standard (Tannic acid dissolved in 70% acetone + 0.1% ascorbic acid) was pipetted to wells in 0.1% BSA agar plates and allowed to incubate for 48 hours. RD in cm^2 was measured and compared to mg tannic acid.

Figure 2 illustrates the distribution of the ratings for Glandular Hair (GH) density and histochemical (DMACA-HCl assay) tannin ratings for 1274 selected Leafhopper (LH/GH) plants. The rating system is graduated on a descending numeric scale (9,7,5,3,1) from 9 = highest expression, 1 = lowest expression for both GH density and tannin rating. Within each GH subset (9,7,5,3,1) the leftmost tannin rating bar equals 9, following in descending numeric order to the rightmost tannin rating bar, which equals 1.

Figure 3 illustrates the effect of added tannic acid on foam volume of 4 alfalfa varieties.

Figure 4 illustrates the effect of polyvinylpyrrolidone (PVP) on foam volume of low tannin expressing alfalfa, a non-tannin alfalfa plant and birdsfoot trefoil. Greenhouse grown samples were tested for foam production using 3 grams fresh forage in 300 ml pH 5.6 phosphate-citrate buffer. Each plant was tested with and without the addition of 100 mg polyvinylpyrrolidone (PVP). Tannin estimate for plant 421 is 0.36% based on an RD assay. Plant 1054 tested very low for tannin. ND is a non-tannin expressing plant. Largest increase in foam production with the addition of PVP was seen on plant 421 and the trefoil (the 2 plants with highest tannin content).

SUMMARY

It is the object of the present invention to provide a method of identifying tannin-expressing alfalfa (*Medicago sativa* L.) plants. An additional object of the invention is the development of alfalfa plants having measurable endogenous tannin levels for bloat safety, protein protection, and improved ruminant health and nutrition and for increased pest resistance in such alfalfa plants, with the tannin levels measured by the use of a radial diffusion assay according to Hagerman (1987) and as modified and disclosed below. Another object of the invention is to provide a method for determining the optimal level of tannins in alfalfa plants for bloat safety in ruminants such as beef cattle, lactating dairy cattle and sheep.

The present invention provides methods of improving nutrition and health in ruminants by having ruminants graze stands comprising alfalfa plants having measurable endogenous tannin levels, or, alternately, feeding alfalfa silage or haylage of these alfalfa (*Medicago sativa* L.) plants to ruminants.

A further method, to reduce the protein degradation in alfalfa silage, or alternately, haylage, is provided in the present invention. Further, the present invention includes a method for controlling pests in alfalfa stands.

The discovery underlying the present invention makes it practical for alfalfa growers to grow a high yielding, high quality forage that will prevent bloat, improve the protein and amino acid availability to the ruminants and have health benefits for the animal.

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art.

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

Alfalfa Silage: chopped alfalfa that is then stored in upright or horizontal silos. Alfalfa silage can be separated into three groups on the basis of moisture level. High moisture silage or direct-cut silage has about 70% or more moisture. Wilted silage has about 60% to about 70% moisture. Low-moisture silage ranges between about 40% and about 60% moisture.

BSA	bovine serum albumin
CT	condensed tannin = proanthocyanidin
DM	dry matter
DMACA-HCl	4-dimethylaminocinnamaldehyde-hydrochloric acid
GH	glandular hair
Haylage	a type of alfalfa silage that is dried in the field to at least about 65%, and as low as about 40% moisture, before it is chopped and stored
HT	hydrolyzable tannin
LH	potato leafhopper
LIRD	low initial rate of digestion
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
RD	radial diffusion

Described herein are agronomic methodologies for identification, development and utilization of alfalfa plants having measurable endogenous tannin levels. The increased endogenous tannin levels of these alfalfa plants are levels measurable by the use of a radial diffusion assay according to Hagerman (1987), as modified and disclosed below. Alfalfa plants having measurable endogenous tannin levels include alfalfa plants having tannin levels of about 0.1% DM. The amount of tannin present in the alfalfa plants is the amount effective to reduce bloat. The amount of tannin can vary broadly. Generally the amount of tannin will be in the range of from about 0.1% DM basis to about 5% DM basis as measured by the radial diffusion assay. Other effective ranges are from about 0.2% DM basis to about 5% DM basis and from about 0.5% DM basis to about 5% DM basis as measured by the radial diffusion assay. Ranges are preferably from about 0.7% DM basis to about 5% DM basis as measured by the radial diffusion assay. More preferably, the range is from about 1% DM basis to about 5% DM basis as measured by the radial diffusion assay and most preferably, the range is expected to be from about 2% DM basis to about 4% DM basis as measured by the radial diffusion assay. Additional effective ranges are from about 0.1% DM basis to about 4% DM basis and from about 0.2% DM basis to about 4% DM basis as measured by the radial diffusion assay. One embodiment of the present invention is a method for identifying tannin-expressing alfalfa plants. Identification may be achieved by, but is not limited to, observing the alfalfa plant for GH pubescence and selecting those with high GH density. Determination of tannin expression is done by testing the selected plants using a DMACA-HCl histochemical assay, one of several methods known in the art for tannin determination, and further testing those plants expressing

tannins for tannin content using an RD assay. A related embodiment is the development of alfalfa cultivars having measurable endogenous tannin levels through plant breeding. This is accomplished by crossing tannin-expressing alfalfa plants with proprietary germplasm and intensively selecting for improved agronomics, improved GH expression and the related increased tannin levels. Development of alfalfa plants having measurable endogenous tannin levels is also accomplished by genetic engineering methods well known in the art, such as, but not limited to, interspecific protoplast fusion, gene insertion or induced mutations.

In one embodiment of the invention, alfalfa plants having measurable endogenous tannin levels are grown for grazing, hay, haylage or for silage to prevent bloat in ruminants such as beef cattle, dairy cattle, sheep or goats. In forage species, tannin content has been associated with bloat safety. It is presently believed, without wishing to be bound by any particular theory of mechanism of action, that tannins prevent stable foam formation, and therefore bloat, by precipitating soluble proteins and limiting gas production in the rumen. Because grazing of fresh alfalfa presents the highest risk of bloat in ruminants, this invention is especially advantageous for the grazing animal. The grower would not be restricted by the traditional bloat preventative management practices (mentioned above). This invention contemplates growing the new alfalfa plants in pure stands or as mixtures; using rotational grazing rather than continuous grazing; and grazing at optimal stage, that is pre-bud to first flower, instead of at full bloom stage. In order to ease the transition of the animal to the new diet, the invention contemplates the use of known and traditional accepted pasture management techniques; that is, to not turn hungry animals into the paddock and to limit access to the new pasture until the animals are fully adapted.

Another embodiment of the present invention comprises the use of alfalfa plants having measurable endogenous tannin levels to reduce protein degradation in the rumen and in alfalfa silage. Without using any additional processing steps, the grower using these alfalfa plants will see improvement of forage bypass protein from the rumen to the abomasum and the small intestine. Because the level of tannin will be in the range of about 0.2 to about 5% DM there will be no toxicity due to tannins. More preferred, the tannin levels will be in the range of about 2 to about 4% DM for an optimum protein protection. The tannin bound protein will also be useful to prevent protein breakdown in the alfalfa silage and reduce non-protein nitrogen fed to the ruminants.

An additional embodiment of the present invention includes the beneficial anticarcinogenic effects of tannins. Because tannins have been shown to prevent

cancer in animals, a diet richer in tannin level may help to prevent or control the development of this disease in ruminants.

A further embodiment of the present invention is the utilization of tannins as a plant defense mechanism. Because tannins are well known for their properties of discouraging pest feeding, the tannin-containing alfalfa should better control important alfalfa pests. Although not exclusive, the list of potential pests includes various species of mites, nematodes, aphids, whiteflies, and other insects in the orders of lepidoptera, coleoptera, hemiptera, homoptera and diptera.

An additional embodiment is a method for determining the bloat safety effect of these tannin-expressing plants. The bloat safety effect is measured by using an *in vitro* foam assay.

Yet another embodiment is the method for determining the optimal level of tannins in alfalfa plants for bloat safety in ruminants. The optimal level of tannins is determined by feeding ruminants alfalfa plants with increased endogenous tannin levels having known varying tannin levels, measuring the bloat safety effect and measuring the toxicity effect on the ruminants.

In all embodiments this invention relates to tannin produced in all the vegetative or reproductive tissues of the alfalfa plant including roots, crown, stems, petioles, leafs, flowers, pollen and seeds. The invention is described more fully below in the following examples. The materials, methods and examples are illustrative in nature and are not intended to limit the scope of the invention in any way.

EXAMPLE 1 Identification of Tannin-Expressing Alfalfa Plants

Using the DMACA-HCl histochemical assay tannins were detected in the glands of the glandular hairs of alfalfa variety derived from an experimental Pioneer Hi-Bred International, Inc. alfalfa variety, which expresses glandular hair resistance to the potato leafhopper (LH). Variability in the density of tannin containing hairs as well as variability in tannin concentration within hairs was noted.

Because of the discovery of tannins in hairs of the GH material, Step 1 in the variety development process was to select plants with high GH density. Greenhouse or field grown alfalfa plants were removed from the soil, and observed for pubescence. The ability to estimate GH density was greatly enhanced by holding the plants so that backlighting (sun or other light source) was available and a dark background was present. The use of a dissecting microscope was also effective.

GH selections were kept turgid by placing plants in water or a cooler (if plants are barerooted) or were transplanted to flats prior to further evaluation.

Step 2 in the process involved the identification of plants expressing tannins. Tannin expressing plants may be identified using a number of techniques as described earlier. A modification of the DMACA-HCl histochemical assay described by Li et al (1996) and a radial diffusion assay (Hagerman, 1987) were used because of their simplicity, allowing the rapid screening of large numbers of plants. Other tannin assays may be used in the program if they are reliable and efficient, or if they provide additional useful information.

DMACA-HCl Assay Protocol

1. A 0.5 cm stem section was collected from just below the node of the first fully expanded leaf, by cutting with a scalpel. Two disposable scalpels (Feather No. 11, Fisher) taped together worked well and provided uniform sample size.
2. Stem sections were placed, one per well, into 96 well plates (Dynatech Microtitre® Flat Bottom Plates).
3. 100 µl DMACA-HCl (3 g. DMACA in 500 ml cold methanol + 500 ml 6M HCl) was pipetted into each well.
4. Samples were allowed to stain for at least 4 hours at room temperature to ensure movement of stain throughout the sample.
5. Samples were rated for intensity of blue staining of tannin containing cells and given an overall rating for total tannin (intensity of staining plus frequency of tannin containing hairs) under a dissecting microscope.

The DMACA-HCl step may be omitted when screening germplasm resulting from previous tannin selection but was helpful as a quick and inexpensive screening especially in the early stages of selection.

Plants identified as expressing tannins in the DMACA-HCl assay were further tested for tannin content using a radial diffusion (RD) assay.

Radial Diffusion (RD) Assay Protocol

1. Agar plates were prepared basically as described by Hagerman (1987).
   A. 2 liters of Buffer A were brought to a boil, and 20 g 1% agarose (Agarose type 1:low EEO, Sigma) was added while stirring on a hot plate.
   B. The foil covered mixture was brought back to a boil and stirred until the agarose was completely dissolved.
   C. Agar was allowed to cool to 45°C in a water bath.
   D. 2 g. BSA (Sigma) was added [0.1% (w/v)], and mixture was gently stirred on stir plate until BSA was completely dissolved.

E. Agar was poured in 10.5 ml aliquots into standard 100 mm x 15 mm plates on a level surface and allowed to cool.

F. Wells were placed 1.5 cm apart using a No. 2 stopper punch which was connected to an aspirator by plastic tubing. Agar plugs were removed through aspiration, leaving wells with a capacity of approximately 20 µl.

G. Plates were stored at 4°C, until used, to prevent bacterial growth.

2. Plant Extraction and Testing.

A. Stem tips were clipped just below the first expanded leaf to ensure uniform sampling and provide approximately 100 mg fresh weight, and sample weight from each plant was recorded.

B. Samples were placed in tubes (Megatitre® 96 well plates, Sigma) and were immediately refrigerated.

C. 300 µl 70% acetone + 0.1% ascorbic acid was pipetted into each tube as an extraction solvent.

D. Two 5/32" ball bearings or two #4 copper-coated steel shots, were added to each tube to aid in tissue maceration.

E. Parafilm® was placed over tubes, tubes were capped, and caps were firmly pressed onto tubes using a manual or hydraulic press.

F. Samples were allowed to reach room temperature prior to grinding to prevent tube breakage.

G. Plates were placed on a shaker type grinder, (U.S. Patent Application Serial No. 08/713,507) set at 6 ½, and shaken at 1500 RPM for 30 seconds to macerate sample tissue.

H. Samples were allowed to extract for 1 hour at room temperature.

I. Four balanced plates per run were placed in a Jouan GR 4 22 Centrifuge, and were spun at 4000 RPM for 10 minutes.

J. Caps were removed from plates and 200 µl of supernatant from each tube was pipetted to a corresponding Microtitre® plate well for concentration of sample through freeze drying (at this point, samples could be directly pipetted to BSA plates if tannin content is high enough for adequate RD precipitation rings to develop, or samples could be dehydrated at room temperature or in a freezer or refrigerator). It is preferable to concentrate samples through freeze drying to minimize effects of heat and oxidation.

K. 20-25 µl of water or 70% acetone was pipetted to each well of freeze dried sample. The use of water to reconstitute the sample is preferred over 70% acetone because of the rapid evaporation of acetone.

L. Sample was stirred with the pipette tip to ensure that it was completely dissolved, and was pipetted to BSA agar plate wells. A second aliquot of each sample may be pipetted to wells after sample is absorbed by the agar if needed, to allow total sample to be used.

M. Plates were sealed with Parafilm® and stored at room temperature for 48 hours to ensure time for precipitation rings to reach equilibrium.

N. Diameter of precipitation rings (cm) was measured and recorded. For screening, samples may be rated on a 1-9 scale for ring diameter and intensity.

O. Plant sample results were compared to results obtained from controls to determine tannin estimates. Controls consisted of purified tannic acid in 70% acetone at 0.1% increments (20µl per well) from 0% - 1.0%.

RD precipitation rings were measured under a dissecting microscope. The area of the ring is linearly related to the amount of tannin placed in the well. To simplify calculations, the diameter squared was used instead of the area. The calibration lines had nonzero y intercepts because the wells into which the samples were dispensed have finite areas. The detection of the assay as reported by Hagerman is 0.025 mg tannin, with precision of ± 6% (relative standard deviation). Similar results were obtained by using tannic acid as the control. A regression was run on controls for each RD assay to determine the y intercept to allow for calculation of tannin estimates on plant samples. Regressions of ring diameter^2 x mg tannic acid consistently showed an RSQUARE of greater than 0.99. Figure 1 shows a typical RD calibration set using tannic acid controls.

Four thousand plants were screened for tannin expression using the DMACA-HCl technique. Tannins were not detected in the trichomes of a simple hair alfalfa experimental (58 plants), or in the hairs of a limited number of plants from commercially available alfalfa. Tannin staining trichomes were identified in all GH germplasm that were examined but the percentage of plants with tannin expressing trichomes, as well as hair density and intensity of staining, varied by source. Alfalfa populations previously selected for GH density as part of the LH resistance program exhibited high GH density. On average, between 20-40% of plants that rated high for GH density and LH resistance in the field in 1996, also rated high for tannin expression based on DMACA-HCl staining. The distribution of the ratings for GH density and histochemical tannin ratings for 1274 selected LH/GH plants is presented in Figure 2.

Tannin estimates on GH plants in our program have been as high as 0.73% DM (tannic acid equivalent). Tannin (tannic acid equivalent) estimates on a set of GH plants

assayed using RD are presented in Table 2. Since the presently described sample extraction procedure consists of only a single extraction, and only about 2/3 of the extraction solvent is recovered for use in the RD assay, and in light of reports that the use of tannic acid as a standard in RD assays may underestimate actual tannin levels in plants, it is believed that tannin estimates on our breeding material are very conservative.

Table 2. Radial diffusion (RD in cm^2) values and tannin (tannic acid equivalent) estimates on 27 greenhouse grown plants previously identified as being able to precipitate proteins (positive reaction) in an RD qualitative assay. RD assay was run using 0.1% BSA agar plates.

Plant #	Dry Matter (mg)	RD (cm2)	TN (mg)	TN %
614	20.0	1.000	0.114	0.57
618	22.5	0.903	0.099	0.44
621	25.0	1.000	0.114	0.46
625	27.5	1.000	0.114	0.42
636	27.5	1.103	0.130	0.47
648	25.0	0.490	0.035	0.14
657	20.0	0.490	0.035	0.17
695	22.5	0.563	0.046	0.21
697	25.0	1.440	0.183	0.73
703	27.5	0.810	0.085	0.31
709	22.5	0.903	0.099	0.44
726	27.5	0.856	0.092	0.33
736	25.0	1.440	0.183	0.73
741	22.5	0.903	0.099	0.44
742	25.0	0.640	0.058	0.23
743	25.0	0.423	0.024	0.10
754	20.0	0.810	0.085	0.42
759	22.5	1.210	0.147	0.65
767	22.5	0.423	0.024	0.11
775	20.0	0.856	0.092	0.46
789	22.5	1.000	0.114	0.51
802	20.0	0.423	0.024	0.12
814	22.5	1.210	0.147	0.65
824	25.0	0.810	0.085	0.34
833	22.5	1.000	0.114	0.51
835	25.0	0.723	0.071	0.28
850	25.0	1.000	0.114	0.46

EXAMPLE 2 Increasing the Endogenous Tannin Levels in Alfalfa Plants

Alfalfa germplasm expressing the glandular (GH) trait traces through public germplasm releases from Kansas State University (KS108GH5, KS94GH6)(Sorensen et al., 1986; Sorensen et al., 1985) and Purdue University (81IND-2) (Shade et al., 1986), to plant introductions from *Medicago glomerata*, *M. prostrata* and *M. glandulosa*. The germplasm releases are quite poor agronomically with very early fall dormancy, low yield potential, low disease and insect resistance and poor field appearance.

Plants identified as expressing desired tannin levels based on the RD assay were incorporated into the breeding program and used to transfer the GH trait to proprietary germplasm through several cycles of crossing, combined with intensive selection for improved agronomics and GH expression. Plants expressing desired tannin levels could also be developed through genetic engineering modifications well known in the art such as, but not limited to, interspecific protoplast fusion, gene insertion or induced mutations. This germplasm may be used in any crossing scheme normally used in alfalfa breeding in a recurrent selection program. A detailed review of alfalfa breeding methodologies can be found in "Alfalfa and Alfalfa Improvement", 1988, pp. 777-808. Typically, until the desired trait level is reached, selections within a population would be intercrossed, and progeny would be subjected to an additional cycle of selection for the trait, with the option of incorporating selections from other populations or for other traits. Evaluation for the selected trait is normally undertaken during the development process to monitor progress. When the desired trait level is reached, a thorough evaluation for the selected trait as well as other agronomically important traits is done. In the case of the selection for tannin in alfalfa, cycles of selection would be repeated until a level of about 0.2 to about 5% DM is reached, which is the range of tannin concentration shown to have beneficial effects in bloat prevention and protein degradation. Optimum levels to select for tannins in alfalfa plants would be about 2 to about 4%, with about 5% as the upper limit to incorporate into commercial alfalfa plants, to prevent toxic effects from heightened tannin levels.

To date, the most advanced material that has been quantitatively assayed using the RD method was the result of four cycles of RD tannin selection. All other material has been the result of only DMACA-HCL tannin selection. Each cycle of greenhouse crosses and cage seed increases has generally produced material with higher measurable endogenous tannin levels in progeny. Before beginning the RD screening, original populations were selected for high glandular hair expression and blue staining glands using the DMACA-HCL assay to identify those plants with highest tannin expression. The four experimental populations used are designated FD1, FD2, SD1,

SD2, with each population going through four cycles of RD screening. As noted above, the progeny produced has generally shown higher measurable endogenous tannin levels, including the progeny identified as SD2-4 in the following Table 3, which is alfalfa cultivar strain I98PS32, seeds of which have been deposited with the American Type Culture Collection (ATCC) under ATCC Accession No. 203052.

Radial Diffusion (RD) is thought to be a good indicator of tannin content since RD is used to measure protein precipitation, and tannins are very effective protein precipitants. Tannic acid and extracts from moderate-high tannin species such as sainfoin and big trefoil produce intense, solid disks in the RD assay. Diameter of disks are proportionate to tannin concentration in the sample. The scoring system used when rating RD assays is:

9 = very intense disk (protein precipitation similar to that seen with tannic acid and high tannin species)

8 = very intense open ring

7= intense disk

6= intense open ring

5 = moderately intense disk

4 = moderately intense open ring

3 = faint disk

2 = faint open ring

1 = no precipitation

Diameter of precipitation disk/ring is also measured for each sample. Plants producing the largest and most intense solid disk reaction are selected.

While there is a wide range of RD reactions seen when running the assay, the overall frequency of plants producing intense, solid disk reactions in RD has increased through cycles of selection (Table 3), with precipitation disks of nearly the intensity and diameter of sainfoin, especially in the cycle 3 and cycle 4 material, which would indicate tannin levels of 10% or more, which is higher than the levels of tannin expected in this material. In addition, staining of tannins from the same material using the DMACA-HCL assay has not reached the level of birdsfoot trefoil, which expresses from about 2% DM to about 5% DM tannin. Thus, while the RD test is believed to quantify tannins only, the test may be including tannins and an additional, unidentified precipitant. Those precipitants may also contribute to bloat safety.

Table 3. Radial Diffusion (RD) ratings from assays for each cycle of selection for the 4 rapid-cycle populations. The frequency of plants in each rating class, the total number of plants assayed per cycle and the average RD ratings are shown.

Population - Cycle	Frequency of Plants in Each Rating Class										
	9	8	7	6	5	4	3	2	1	Total	Avg. RD
FD1 – 1	41	22	95	86	74	0	139	0	518	975	3.11
FD1 – 2	5	5	87	31	287	29	183	11	368	1005	3.35
FD1 – 3	20	60	119	242	152	24	146	0	64	827	5.20
FD1 – 4	132	97	167	69	208	7	86	0	74	840	5.89
FD2 – 1	27	14	74	99	122	0	196	0	447	979	3.18
FD2 – 2	12	11	40	30	161	28	186	20	321	809	3.08
FD2 – 3	20	53	138	183	260	76	177	0	22	929	5.19
FD2 - 4	96	102	169	42	165	2	44	0	32	652	6.31
SD1 – 1	13	5	111	218	118	0	43	0	372	980	3.71
SD1 – 2	7	11	35	63	64	107	162	33	294	776	3.05
SD1 – 3	17	27	179	26	281	3	202	0	84	819	4.74
SD1 – 4	48	153	197	103	242	29	108	0	34	914	5.84
SD2 – 1	28	0	52	71	90	0	257	0	482	980	2.80
SD2 – 2	9	62	66	155	100	79	111	37	280	899	3.86
SD2 – 3	97	31	340	30	264	4	78	0	43	887	5.97
SD2 – 4	63	156	218	103	190	23	67	0	19	839	6.22

Selection for tannin content will continue in the greenhouse and in the field. Based on past experience in performing rapid cycles of recurrent selection, it is expected that 3-4 cycles of selection for tannin per year can be accomplished. Breeding work will include selection within tannin selected material as well as complementary strain crosses between tannin material and non-tannin material to incorporate the tannin trait into top-performing elite germplasm.

A spaced planted experiment containing tannin experimentals, along with GH varieties (not selected for tannin content) and non-GH varieties will be established in the field for continued evaluation for tannin content and agronomic characterization of the tannin experimentals. In addition, spaced planted nurseries containing tannin selected material will be established for additional tannin selection as well as selection for dormancy and field appearance.

Parent seed increases will be established with the goal of producing adequate seed for the establishment of sufficient acreage of the experimentals for conducting

several animal trials. Grazing trials will be established in several locations to determine optimum levels of tannins for ruminants and to verify the bloat safety characteristics of newly developed varieties in ruminants. Also, nutritional studies will include *in vitro* digestion (protein degradation) with rumen fluid and foaming properties of the forage. Finally, the tannin source found in alfalfa will be characterized to make sure that hydrolizable tannins cannot be detected or that their concentrations are at non-toxic levels.

EXAMPLE 3 Determining the Bloat Safety Effect of an Alfalfa Variety

A. Determining the Bloat Safety Effect In Vitro

In vitro Foam Protocol

1. The apical 10 cm of a sufficient number of stems with attached leaves to provide a 3 g sample were collected from each plant to be tested. For a replicated test on individual plants, enough stem material was collected so that the total sample will yield enough 3 g subsamples for the desired number of reps. For variety evaluation, 10 cm stem samples were collected from enough plants to represent the variety (a minimum of 25 plants per field plot) and were bulked for subsampling.
2. When bulk samples were used (and subsamples were needed), the sample was roughly chopped into approximately 2 cm sections using scissors. The rough sample was then finely chopped in a food processor so that uniform subsamples can be taken.
3. 3 g samples were placed in a blender with 300 ml of pH 5.6 Citrate-Phosphate buffer + 0.1% ascorbic acid, and blended on high speed for 1 ½ minutes. Blended samples can immediately be used for foaming, but we found that when comparing plants or varieties it was best to transfer the blended sample to resealable plastic bags (or other container) and to store samples overnight in a refrigerator.
4. Chilled samples were poured into a mixing bowl (1 quart) and whipped for 3 minutes at the highest setting with a mixer.
5. Contents were transferred to a 1000 ml graduated cylinder .
6. After 2 minutes the cylinder was shaken and tapped on the countertop to dislodge any large trapped air pockets.
7. Foam volume was recorded (total volume minus liquid volume). This value was considered the volume of stable foam.
8. Samples were run in duplicate with reference samples containing 50 mg polyvinylpyrrolidone (PVP) to estimate tannin content. PVP has been shown to

break the protein-tannin complex. A comparison of foam volumes with and without PVP showed tannin effects on foam production.

The in vitro foam assay presented above has been used as a possible method for estimating the bloat potential of forages (Kendall, 1966; Rumbaugh, 1969; Goplen et al., 1981; Kendall and Taylor, 1965), but based on literature review has been used little since the 1960's. The testing protocol is simple but because of the time required per sample, may not be as useful as other assays for screening large numbers of plants. The assay has been used for the evaluation of field and greenhouse plants and has shown good repeatability. Goplen et al (1981) reports that their experiments showed no correlation between foam volume and soluble protein or between foam volume and bloat incidence, but they were looking only at non-tannin expressing alfalfa.

In the present experiments, a clear relationship was established between tannin and foam volume when increasing amounts of tannic acid were included with fresh alfalfa. Foam volume decreased rapidly with each 1% increase in tannin, until approximately 5% tannin, when additional increases had little effect in reducing foam. Based on this data, it appears that the foam assay may be useful as a tool in the screening of tannin expressing plants to develop bloat safe varieties. Figure 3 shows the effect of added tannic acid on foam volume of 4 alfalfa varieties not previously selected for tannin expression. Figure 4 shows the effect of polyvinylpyrrolidone (PVP) on foam volume of low tannin expressing alfalfa, a non-tannin alfalfa plant and birdsfoot trefoil.

B. Determining the Bloat Safety Effect In Vivo

The bloat safety effect of alfalfa plants having measurable endogenous tannin levels is shown by grazing ruminants in stands comprising alfalfa plants having measurable endogenous tannin levels or feeding ruminants freshly cut alfalfa from alfalfa plants having measurable endogenous tannin levels and measuring the bloat safety effect on the ruminants (using, for example, the methodologies described by Hall et al., 1988; and Cheng et al., 1995, or other art-recognized methods).

EXAMPLE 4 Protein Degradation Reduction by Tannins

The effect of alfalfa plants having measurable endogenous tannin levels on protein degradation in the rumen is shown by grazing ruminants in pure stands of alfalfa plants having measurable endogenous tannin levels in animal trials and testing for reduced degradation of protein in the rumen and increased protein availability in the abomasum and the small intestine (using, for example, the methodologies described by

Broderick, 1995; Meissner et al., 1993; Poos-Floyd et al., 1985; Wood and Plumb, 1995; Tanner et al., 1994; and Waghorn et al., 1987, or other art-recognized methods).

The effect of alfalfa plants having measurable endogenous tannin levels on protein degradation in alfalfa silage or haylage and the resulting reduction of non-protein nitrogen fed to ruminants is shown by growing stands comprising alfalfa plants having measurable endogenous tannin levels, harvesting the stands as alfalfa silage or haylage and testing the alfalfa silage or haylage components grown from alfalfa plants having measurable endogenous tannin levels for reduced proteolysis with methods such as the ones used by Albrecht and Muck (1991), for example, or alternative methods available in the art.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, each of the disclosures of which is incorporated by reference in its entirety.

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DEPOSITS

It should be noted that the sample identified as SD2 – 4 in the preceding Table 3 is the alfalfa cultivar strain designated I98PS32, 2500 seeds of which have been deposited with the American Type Culture Collection (ATCC), Manassas, VA 20110 USA, under ATCC accession No. 203052, which were taken from the deposit maintained by Pioneer Hi-Bred International, Inc., 800 Capital Square, 400 Locust Street, Des Moines, Iowa 50309-2340 since prior to the filing date of this application. The subject alfalfa cultivar has been deposited under conditions that assure that access to the alfalfa cultivar will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto

under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

Further, the subject alfalfa cultivar deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of a deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the alfalfa cultivar. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject alfalfa cultivar deposits will be irrevocably removed upon the granting of a patent disclosing them.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 34, 35, line 27-36, 1-16

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

10801 University Blvd.
Manassas, Virginia 20110-2209
United States of America

Date of deposit	Accession Number
10 July, 1998 (10.07.98)	203052

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer PAUL F. URRUTIA — PFU INTERNATIONAL DIVISION 703 305 3681	Authorized officer

Form PCT/RO/134 (July 1992)

What is claimed is:

1. Alfalfa plants having measurable endogenous tannin levels.
2. Alfalfa seed produced by the plants of claim 1.
3. Alfalfa plants according to claim 1 wherein said tannin levels are measured by a radial diffusion assay.
4. Alfalfa plants according to claim 1 wherein said tannin levels are detected by a modified 4-dimethylaminocinnamaldehyde-hydrochloric acid assay.
5. Alfalfa plants according to claim 1 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.
6. Alfalfa seed produced by the plants of claim 5.
7. Alfalfa plants according to claim 1 wherein said tannin levels are in the range of from about 0.2% to about 5% on a dry matter basis, as measured by a radial diffusion assay.
8. Seed of an alfalfa cultivar having measurable endogenous tannin levels, designated strain I98PS32, representative samples having been deposited under ATCC Accession No. 203052.
9. A method of identifying tannin-expressing alfalfa plants, said method comprising identifying and selecting those alfalfa plants with high glandular hair density and subjecting said plants to an assay to determine tannin expression.
10. The method of claim 9, wherein the assay used is a modified 4-dimethylaminocinnamaldehyde-hydrochloric acid assay.
11. The method of claim 9, further comprising quantifying the tannin content of said plants by subjecting said plants to a radial diffusion assay.
12. A method of determining the presence of optimal levels of tannins in alfalfa plants for bloat safety in ruminants, said method comprising providing said alfalfa plants having known varying levels of tannins; feeding ruminants said alfalfa plants; and measuring the bloat safety effect on said ruminants.
13. The method of claim 12 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.
14. The method of claim 12 wherein said ruminants are beef cattle, lactating dairy cattle or sheep.
15. A method of breeding for alfalfa cultivars having measurable endogenous tannin levels, said method comprising crossing tannin-expressing alfalfa plants with other alfalfa plants through several crossing cycles; selecting for tannin expression; and selecting for improved agronomics.

16. Alfalfa seed produced by the method of claim 15.

17. The method of claim 15 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.

18. A method of providing improved nutrition and health to ruminants, said method comprising growing stands comprising alfalfa plants having measurable endogenous tannin levels, and having said ruminants graze said stands.

19. The method of claim 18 wherein said ruminants are beef cattle, lactating dairy cattle or sheep.

20. The method of claim 18 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.

21. A method of providing improved nutrition and health to ruminants, said method comprising growing stands comprising alfalfa plants having measurable endogenous tannin levels; harvesting said alfalfa plants to produce alfalfa silage; and feeding said alfalfa silage to ruminants.

22. The method of claim 21 wherein said alfalfa silage comprises haylage.

23. The method of claim 21 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.

24. The method of claim 21 wherein said ruminants are beef cattle, lactating dairy cattle or sheep.

25. A method of reducing protein degradation in alfalfa silage, said method comprising growing stands comprising alfalfa plants having measurable endogenous tannin levels and harvesting said stands of alfalfa silage.

26. The method of claim 25 wherein said measurable tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.

27. The method of claim 25 wherein said alfalfa silage comprises haylage.

28. A method of controlling pests in alfalfa stands, the method comprising planting, in fields susceptible to pests, seed of alfalfa plants having measurable endogenous tannin levels; and allowing stands comprising said alfalfa plants to grow.

29. The method of claim 28 wherein said tannin levels are in the range of from about 0.1% to about 5% on a dry matter basis, as measured by a radial diffusion assay.

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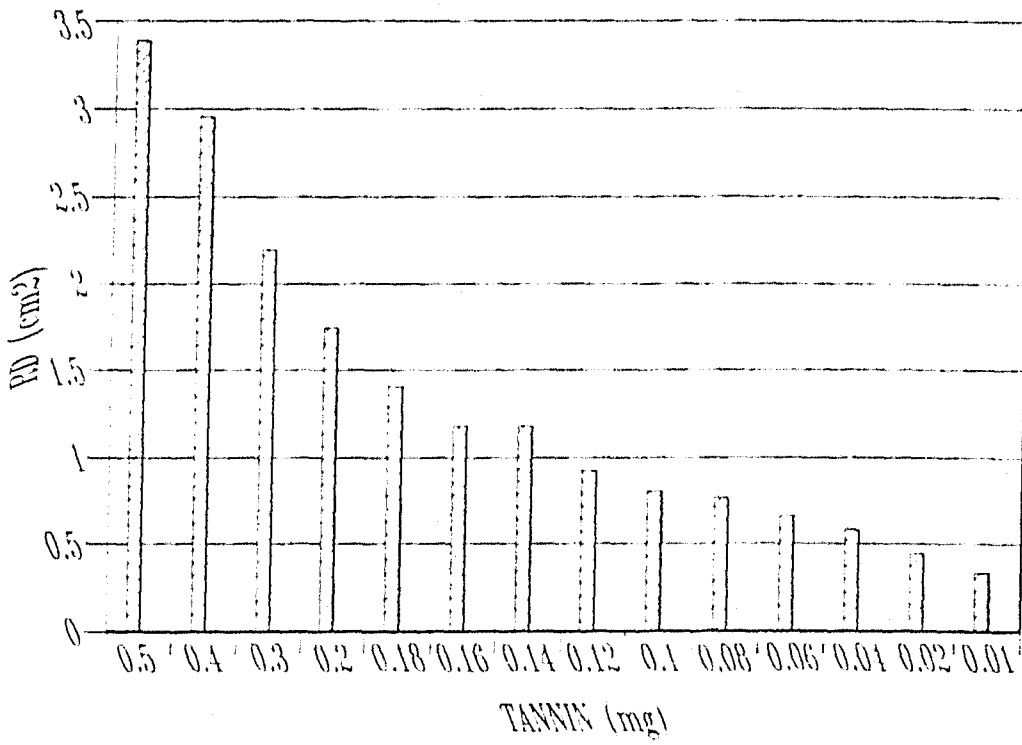
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2
1.5
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0
PD (cm2)
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TANNIN (mg)

FIG. 1

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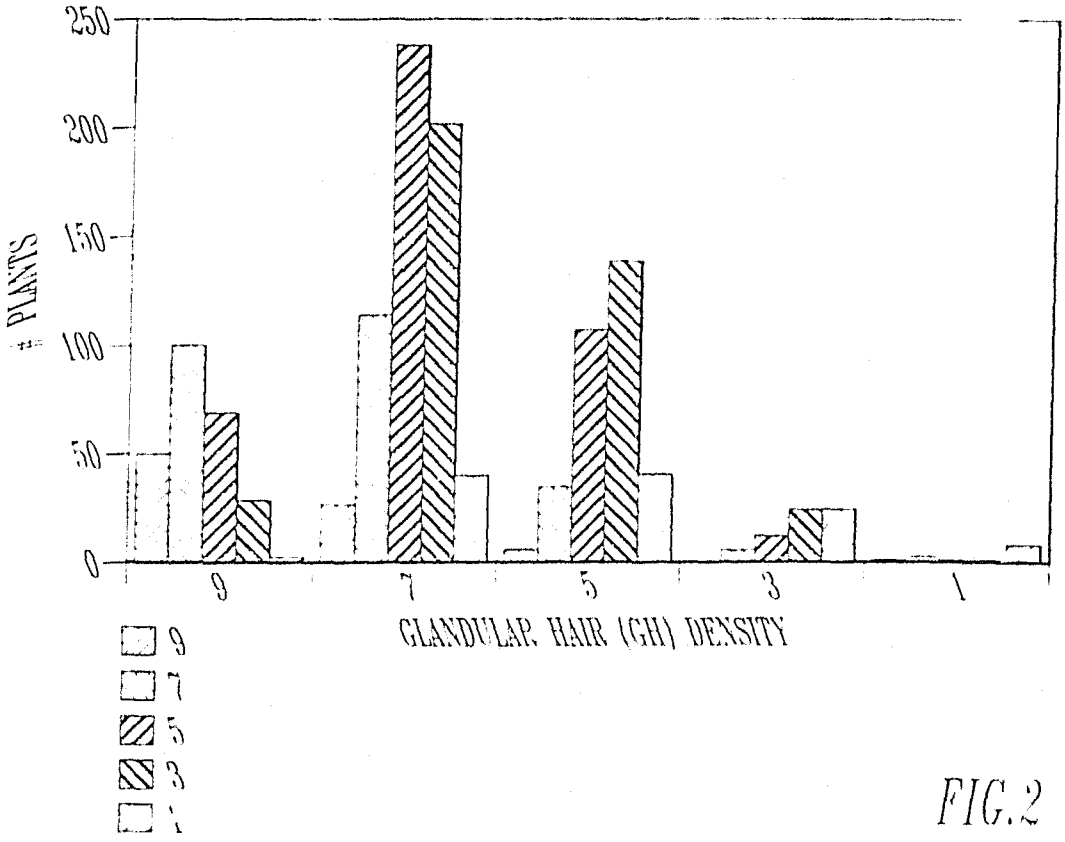
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200
150
100
50
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PLANTS
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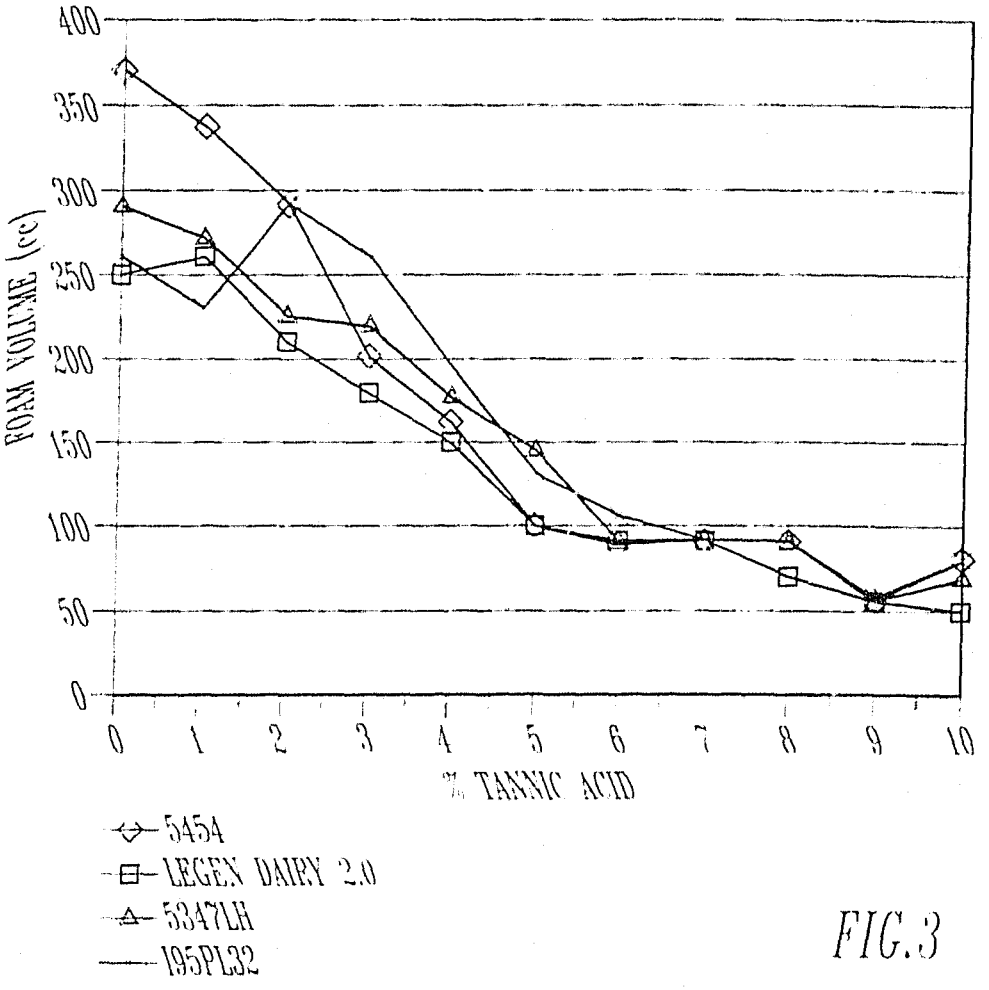
7
5
3
1
GLANDULAR HAIR (GH) DENSITY
9
7
5
3
1

FIG.2

3/4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350
300
250
200
150
100
50
0
FOAM VOLUME (cc)
0
1
2
3
4
5
6
7
8
9
10
% TANNIC ACID
5454
LEGEN DAIRY 2.0
5347LH
195PL32

FIG.3

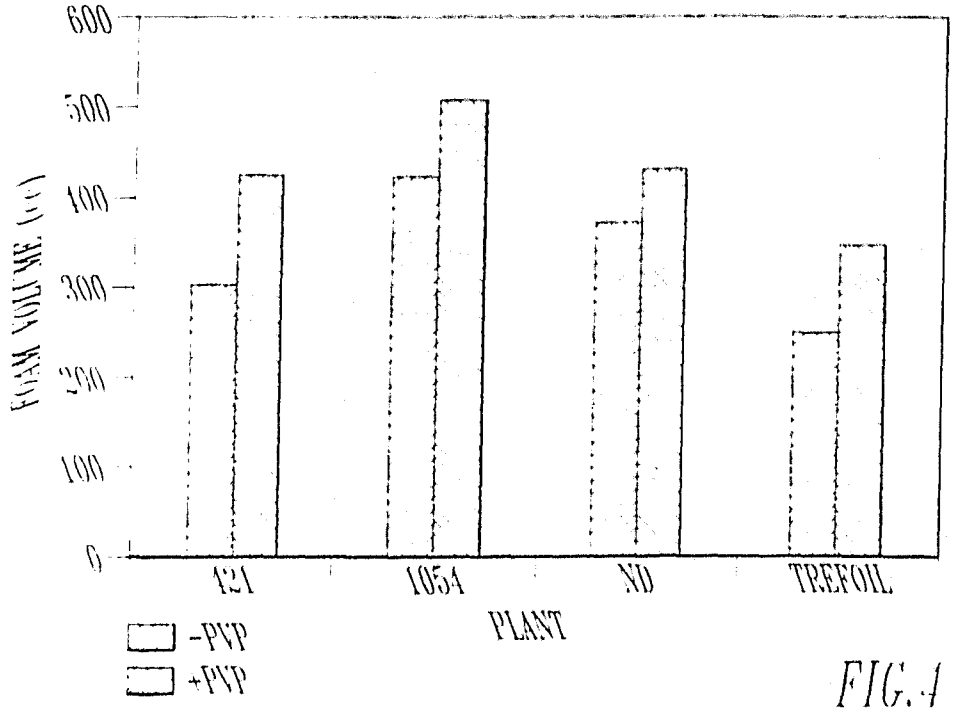

FIG. 1

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 98/17447

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SKINNER: "Protein degradability in a diverse array of alfalfa germplasm sources" CROP SCIENCE, vol. 35, no. 5, 1994, pages 1396-1399, XP002087855 see the whole document --- -/--	9

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 December 1998	04. 01. 1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fonts Cavestany, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ...nal Application No
PCT/US 98/17447

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CABA AN-97:59486 Journal of the Science of Food and Agriculture (1996) Vol. 72 num 4 pag. 483-492 "The condensed tannin content of a range of subtropical and temperate forages and the reactivity of condensed tannin with ribulose-1, 5-bis-phosphate carboxylase protein" JACKSON, F.S. et al. XP002087858 see abstract	15-17
X	DATABASE CABA AN-1998:106596 Seed production of lucerne. Proceedings of the 12th Eucarpia Meeting of the Group Medicago (Brno, Czech Rep.) 2-5 July 1996 pp. 185-188 "Breeding for a bloat-safe cultivar of lucerne" COULMAN, B. et al. XP002087859 see abstract	15,18,19
A	LENSSEN: "Forage quality of perennial glandular haired and eglandular medicago populations" CROP SCIENCE, vol. 28, no. 1, 1988, pages 168-171, XP002087856 see the whole document	9,12,15
A	DATABASE CABA AN-96:35807 Journal of the Science of Food and Agriculture (1996) Vol 70 num 1 pp. 89-101 "The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes" LI YUGUANG et al. XP002087860 see abstract	10
A	DATABASE CABA AN-95:478717 Journal of Animal Science (1995) Vol 73 num 9 pag. 2760-2773 "Desirable characteristics of forage legumes for improving protein utilization in rumiants" BRODERICK, G. A, XP002087861 cited in the application see abstract	18-27

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INTERNATIONAL SEARCH REPORT

Interr ...al Application No
PCT/US 98/17447

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

...ategory	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CABA AN-85:394204 Crop Science Vol 25 num 4 (1985) pag. 607-611 "Inheritance of density of erect glandular trichomes in the genus medicago" KITCH, L. W XP002087862 see abstract	28,29
A	DUKE, A.: "Medicago Sativa L." HANDBOOK OF ENERGY CROPS, - 1983 XP002087857 http://www.hort.purdue.edu/newcrop/duke_energy/Medicago_sativa.html Purdue University - Reported tannin contents of Alfalfa between 2.7 and 2.8 % see page 2, paragraph 2	13,17, 20,23, 26,29

1

...rm PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/17447

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-8
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(ii) PCT - Plant variety

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.