Role of Water Activity in the Spoilage of Alfalfa Hay

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ABSTRACT

Moisture sorption isotherms of alfalfa stems and leaves obtained from the first and third cutting of the season were constructed. Important differences in the relationship between moisture content and water activity values for alfalfa stems and leaves were observed. The extent of those differences was influenced by the initial moisture content and maturity of the alfalfa plant. Water activity, not moisture content, determined the nature of fungal growth on alfalfa stems and leaves. At water activity of 1.0 (moisture content, 51.7 to 65.3%), fungal growth was characterized by extensive mycelial development. Reducing the water activity to .93 (moisture content, 28.1 to 41.7%) dramatically increased fungal fruiting body development and reduced mycelial development. Even though alfalfa stems are nutritionally inferior to alfalfa leaves, fungal fruiting bodies appeared sooner on the stems at water activity values less than .81. Addition of a rich nutrient source, yeast extract, to the stems and leaves did not change that relationship. Analysis of the experimental results reported showed that alfalfa stems have a greater spoilage potential than alfalfa leaves. Implications of these findings to the spoilage process and the nutritional quality of baled alfalfa hay are discussed.

INTRODUCTION

Alfalfa, *Medicago sativa* L., is the leading legume crop in the United States because it is a productive, nutritious, and palatable perennial. Typical hay-making practices involve drying alfalfa to a moisture content of less than 15%. Harvesting procedures at this low moisture content increase leaf loss, thereby reducing the nutritional quality of the feedstuff. In order to increase the nutritional value of alfalfa hay, it can be baled at a higher moisture content (9). However, this practice results in spoiled hay unless an effective preservative is used.

Although several studies have examined the microbial and biochemical changes in spoiled hay, no published reports have used the principle of water activity (a_w) or examined the role of alfalfa stems and leaves in the spoilage process of alfalfa hay (7, 8, 10). Food microbiologists and scientists now recognize that a_w is more closely related to the chemical, biological, and physical properties of foods and feedstuffs than is moisture content (16, 20). The realization that moisture content is an unreliable indicator of spoilage potential was well recognized by 1930. By that time it was recognized that foods such as egg solids and dry milk would readily spoil at 12% moisture content, whereas cereals would remain stable to 14% and dried fruits to 18% (14).

Water activity is a measure of the water that a microorganism can use for growth. All of the water found in a feedstuff is not available to the microorganism because of the way it is "bound", which makes the water unavailable.

Water activity is defined as the ratio of the vapor pressure of water over a substrate to that of pure water at the same temperature and pressure. Numerically, a_w is equal to the equilibrium relative humidity divided by 100 (17). This definition enables the study of the relationship between water activity and moisture content. When a range of a_w values and the corresponding moisture contents are depicted graphically, the resulting construct is called a moisture sorption isotherm (MSI).

Because of the increasing interest in the practice of baling alfalfa hay at higher than normal moisture contents, a clearer understanding of the spoilage process is necessary to

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develop ways to control it. The objectives of this study were 1) to examine the relationship between a_w and moisture content for alfalfa stems and leaves and 2) to study the effect of a_w on the growth of mold on alfalfa stems and leaves under conditions that reproduced as closely as possible actual hay making conditions.

MATERIALS AND METHODS

Alfalfa Stems and Leaves

First-cut and third-cut alfalfa (*Medicago sativa* L.) stems and leaves were air dried to 10 (low moisture) and 30% (high moisture) approximate moisture contents. Stems were cut to a length of 2.5 to 5.0 cm.

Preparation of Humidity Chambers

Humidity chambers were prepared using distilled water and saturated salt solutions in distilled water. Humidity chambers consisted of sealed 3.8-L glass jars. Saturated salt solutions were prepared by using the procedure of Magan and Lacey (13). All solutions were equilibrated at 25°C for at least 72 h before use. Conditions used to establish each equilibrium relative humidity (ERH), and the corresponding a_w values, were as follows: 1) distilled water, 100% ERH, a_w 1.00; 2) potassium sulfate, 97% ERH, a_w .97; 3) potassium nitrate, 93% ERH; a_w .93; 4) sodium benzoate, 88% ERH, a_w .88; 5) potassium chloride, 84.5% ERH, a_w .845; 6) ammonium sulfate, 80% ERH, a_w .80; 7) sodium chloride, 75.5% ERH, a_w .755; 8) potassium iodide, 69% ERH, a_w .69; 9) sodium bromide, 57.5% ERH, a_w .575; 10) magnesium chloride, and 33% ERH, a_w .33 (13). All salts used met American Chemical Society standards and were purchased from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Co. (Fair Lawn, NJ), or J. T. Baker Chemical Co. (Phillipsburg, NJ).

Equilibration of Alfalfa Stems and Leaves at Various Relative Humidities

Approximately .5 g of alfalfa stems or leaves were placed in an aluminum weighing dish, which was then placed into a humidity chamber. There were three samples per treatment in each humidity chamber. All dishes were weighed daily until equilibrium was established or until mold growth interfered with moisture adsorption. This problem occurred at an ERH of 100%. After equilibration was obtained, moisture content of the material was determined by drying for 2 h at 130°C (1).

Effect of Water Activity on the Nature of Fungal Growth

Alfalfa stems and leaves obtained from the third cutting of the season were prepared as described for the equilibration experiments. Each humidity chamber contained a sample representing stems at either 30 or 10% moisture and leaves at either 30 or 10% moisture. Equilibration was monitored by weighing. Fungal growth was monitored by unaided visual observation. The presence of fungal fruiting bodies was confirmed by microscopic examination. Pictures were taken using Kodachrome 64 (Eastman Kodak Co., Rochester, NY) and close-up filters (Rolev Corp., Port Washington, NY) on a 35-mm Yashica camera, model FD-X (Yashica Co., Ltd., Tokyo, Jpn).

Effect of Nutrient Content and Nutrient Availability on the Growth of Mold on Alfalfa Stems and Leaves

Crude protein content was determined by air drying the stems and leaves overnight at 60°C. The dried sample was ground to fine powder using a Wiley Mill (Arthur A. Thomas, Philadelphia, PA), equipped with a 20-mesh screen. The ground sample was digested by placing .25 g of sample along with 4 ml of concentrated sulfuric acid (Fisher Scientific Co., Fair Lawn, NJ) in a digestion flask. After boiling 4 min, 20 ml of 50% hydrogen peroxide (Hach Co., Loveland, CO) were added. Boiling was continued for 1 min. The digested sample was analyzed for CP by determining Kjeldahl nitrogen (1). Acid detergent fiber and ash content were determined by using the procedure recommended by the AOAC (1).

Nutrient availability was examined by mixing .1 ml of a .1% yeast extract (Difco Laboratories, Detroit, MI) solution with .25 g of third-cut alfalfa leaves or .50 g of third-cut alfalfa stems. As a control, .1 ml of sterile tap water was added to .25 g of third-cut alfalfa leaves or

TABLE 1. Relationship between moisture content and water activity values for alfalfa stems and leaves obtained from the first cutting of the season.

Water activity value	Low moisture[1]				High moisture[2]			
	Leaves[a,b]	SD	Stems[a,c]	SD	Leaves[c,d]	SD	Stems[b,d]	SD
	(% moisture)							
.330	8.3	.1	8.4	.3	7.8	.6	8.0	.5
.575	11.8	.4	11.9	.1	11.9	.4	11.9	.6
.690	15.1	.5	14.9	.6	14.8	1.7	14.4	.7
.755	18.7	.5	16.8	.7	18.1	.2	17.2	.3
.800	22.3	1.2	20.8	.6	21.2	.4	19.5	.4
.845	24.8	.3	21.8	1.3	26.2	.6	23.6	1.1
.880	30.1	.9	26.4	.5	29.3	1.0	28.6	.7
.930	35.6	.6	33.0	3.0	36.0	.5	33.7	1.3
.970	52.9	2.2	28.2	.2	51.4	2.2	45.0	1.9
1.000	62.6	2.1	51.7	2.3	61.8	.3	50.0	1.2

[a,b,c,d]Moisture sorption isotherms with the same letter are different ($P<.05$).

[1]Initial moisture content for leaves and stems was 11 and 13%, respectively.

[2]Initial moisture content for leaves and stems was 33 and 32%, respectively.

.50 g of third-cut alfalfa stems. Leaves have a larger surface area than stems and, therefore, required more solution to moisten the surface uniformly. Initial moisture content of the leaves was 27 and 10% and that for the stems was 30 and 9%. Stems and leaves were placed in aluminum weighing dishes, which were placed into humidity chambers of 80.0 and 75.5% ERH. Equilibration was monitored by daily weighing. Samples were examined daily for fungal fruiting bodies. The presence of fungal fruiting bodies was confirmed by microscopic examination.

Data Analyses

The experimental determination of the moisture sorption isotherms involved two replications with three samples at each water activity value per experiment. A dependent t test was used to determine the probability value at which the moisture sorption isotherms were significantly different ($P<.05$). The analysis was performed using the statistical computer program, Systat (Systat, Inc., Evanston, IL).

RESULTS

Moisture Sorption Isotherms for First-Cut Alfalfa Stems and Leaves

Statistical analysis (Table 1) revealed 1) there was no difference ($P>.05$) in the low and high moisture isotherms for alfalfa leaves, 2) the MSI for alfalfa stems and leaves at the same initial moisture content were different ($P<.05$), and 3) initial moisture content did not affect ($P>.05$) the moisture isotherm of alfalfa stems.

Moisture Sorption Isotherms for Third-Cut Alfalfa Stems and Leaves

Statistical analysis (Table 2) revealed 1) initial moisture content did not affect ($P>.05$) the moisture isotherm of alfalfa leaves, 2) the low moisture but not the high moisture isotherm of alfalfa stems was different ($P<.05$) from that of alfalfa leaves, and 3) initial moisture content did affect ($P<.05$) the moisture isotherm of alfalfa stems.

Comparison of the Moisture Sorption Isotherms of First-Cut and Third-Cut Alfalfa Stems and Leaves

Statistical analysis revealed differences in the moisture sorption isotherms of first-cut and third-cut alfalfa stems and leaves, including 1) there was no difference ($P>.05$) in the moisture isotherms for first-cut and third-cut alfalfa leaves, regardless of the initial moisture content, 2) the low moisture isotherm for third-cut alfalfa stems was different ($P<.05$) from that of

TABLE 2. Relationship between moisture content and water activity values for alfalfa stems and leaves obtained from the third cutting of the season.

Water activity value	Low moisture[1]				High moisture[2]			
	Leaves[a]	SD	Stems[a,b,c]	SD	Leaves[c]	SD	Stems[b]	SD
	(% moisture)							
.330	8.3	.4	8.2	.4	8.3	.5	9.0	.2
.575	11.9	.3	11.7	.6	11.2	.3	13.0	.2
.690	15.4	.4	13.6	.3	14.2	.2	15.6	.4
.755	18.0	.2	15.4	1.1	17.0	.1	18.0	.2
.800	20.6	.3	17.5	.6	19.2	.2	21.6	.1
.845	24.6	.6	20.9	.6	22.1	1.1	24.9	1.2
.880	31.6	.4	23.9	1.0	29.1	1.0	28.8	1.2
.930	41.7	.5	28.1	.6	40.8	1.1	35.4	.6
.970	53.3	.6	34.7	1.0	52.9	.3	42.4	.4
1.000	63.3	2.0	51.7	2.7	65.3	1.5	53.3	2.5

[a,b,c]Moisture sorption isotherms with the same letter are different ($P<.05$).

[1]Initial moisture content for leaves and stems was 10 and 9%, respectively.

[2]Initial moisture content for leaves and stems was 27 and 30%, respectively.

first-cut alfalfa stems, and 3) the high moisture isotherm for third-cut alfalfa stems was not different ($P>.05$) from that of first-cut alfalfa stems.

Effect of Water Activity on the Time Required for the Appearance of Fungal Mycelia and Spores

Decreasing the a_w value increases the time required for the visually unaided appearance of fungal spores on third-cut alfalfa (Table 3). At a_w value of .755, the average time required for the visual appearance of fungal fruiting bodies on alfalfa stems and leaves was 62.5 and 105.5 d, respectively (Table 3). Microscopic examination of the fungal fruiting bodies indicated that they were the asexual stage of the genus *Aspergillus*.

Effect of Nutrient Content and Nutrient Availability on the Development of Fungal Fruiting Bodies on Alfalfa Stems and Leaves

The CP, ADF, and ash contents for alfalfa stems were 14.9, 53.0, and 7.2% (Table 4), respectively. For alfalfa leaves the CP, ADF, and ash contents were 34.1, 21.5, and 9.5% (Table 4), respectively.

Yeast extract, when added to alfalfa stems and leaves, did not affect the time required for the visual appearance of fungal fruiting bodies (Table 5). The average time required for the visual appearance of fungal fruiting bodies at a a_w of .80 on alfalfa leaves was 28.8 d, whereas that for alfalfa stems was 23 d. At a_w of .755 the average time required for the visual appearance of fungal fruiting bodies for alfalfa leaves and stems was 56.3 and 46.8 d, respectively. The difference between the values reported in Tables 3 and 5 is probably due to the temporary increase in the a_w of the alfalfa during experimental preparation. Brief exposure to high a_w conditions can have a dramatic effect on fungal spore germination and subsequent mold development (2, 3).

Effect of Water Activity on the Nature of Fungal Growth on Alfalfa Stems and Leaves

Water activity had a dramatic influence on the nature of fungal growth on alfalfa stems and leaves (Figure 1). At a a_w of 1.00 (Figure 1), mycelial growth was much greater than spore formation. As the a_w is reduced, there is a shift from mycelial growth to the synthesis of fungal fruiting bodies (compare parts of Figure 1).

DISCUSSION

Water activity is used to describe the amount of water available to a microorganism growing on an organic substrate, such as alfalfa

hay. Water activity has been established as a more reliable indicator of spoilage potential than moisture content (16). Understanding the relationship between moisture content and a_w (the moisture sorption isotherm) is essential to understanding the spoilage process in alfalfa hay.

The experimental results reported suggest there are differences in the MSI for alfalfa stems and leaves. The extent and nature of those differences is influenced by plant maturity and initial moisture content.

The initial moisture content (low vs. high) did not appear to affect the relationship between moisture content and a_w (Table 1) for alfalfa stems and leaves obtained from the first cutting of the season. A similar response was seen with third-cut alfalfa leaves (Table 2). However, initial moisture content affected the MSI of third-cut alfalfa stems (Table 2). The low MSI was below the high MSI. This difference is called hysteresis, and is often seen in food and feedstuffs (16). The difference in the response between first-cut and third-cut alfalfa stems to initial moisture content may be due to the difference in stem structure. First-cut stems are hollow but third-cut are "solid" (11).

There were significant differences in the MSI of alfalfa stems and leaves (Tables 1 and 2). Alfalfa stems and leaves are chemically different (Table 4). Snow et al. (21) showed that differences in the MSI various feedstuffs were primarily due to differences in their proximate composition. More specifically, it was the relative ratio of soluble carbohydrates, fiber, and protein that affect the shape and level of the MSI. The results of this study generally support their conclusions. However, even though first-cut and third-cut alfalfa stems are chemically similar (Table 4), their respective MSI can be significantly different. Thus, it appears that both structure and initial moisture content can affect the MSI of alfalfa stems.

Moisture content at a particular a_w value reported in this paper is in general agreement with those reported for other types of hay (5, 6, 12, 24, 25). Differences in temperature and technique make definitive comparisons difficult. However, at a_w greater than .90, the corresponding moisture content in this study was significantly greater for alfalfa stems and leaves than previously reported (5, 6). Wright (24) showed that if equilibrium is not established,

TABLE 3. Number of days required for the unaided visual appearance of fungal mycelium and fruiting bodies on third-cut alfalfa stems and leaves at various water activity values.[1,2]

Moisture	Water activity value	Fungal mycelium	SD	Fungal fruiting bodies	SD
High					
Stems	1.000	4	0	6	0
Leaves		4	0	5	0
Low					
Stems		3	0	10	1.4
Leaves		3	0	N[4]	. . .
High					
Stems	.970	5	0	5	0
Leaves		5	0	5	0
Low					
Stems		5	0	5	0
Leaves		4	0	5	0
High					
Stems	.930	ND[3]	. . .	6	0
Leaves		ND	. . .	7	0
Low					
Stems		ND	. . .	7	0
Leaves		ND	. . .	7	0
High					
Stems	.880	ND	. . .	7	0
Leaves		ND	. . .	7	0
Low					
Stems		ND	. . .	7	0
Leaves		ND	. . .	8	0
High					
Stems	.845	ND	. . .	11	0
Leaves		ND	. . .	13	.7
Low					
Stems		ND	. . .	11	0
Leaves		ND	. . .	17	0
High					
Stems	.800	ND	. . .	21	.7
Leaves		ND	. . .	24	.7
Low					
Stems		ND	. . .	24	.7
Leaves		ND	. . .	23	0
High					
Stems	.755	ND	. . .	60	1.4
Leaves		ND	. . .	97	2.8
Low					
Stems		ND	. . .	65	1.4
Leaves		ND	. . .	114	5.6

[1]Average of two replications.

[2]Initial moisture contents for leaves and stems was 10 and 9%, respectively, for low moisture and 27 and 30%, respectively, for high moisture alfalfa.

[3]ND = Not determined.

[4]N = None detected.

TABLE 4. Nutrient analysis of first-cut and third-cut alfalfa stems and leaves.[1]

Alfalfa	CP[2]	SD	ADF[2]	SD	Ash[3]	SD
First-cut						
Low moisture						
Stems	11.4	.1	48.9	.7	7.8	.1
Leaves	30.9	.2	19.1	.5	11.3	.1
High moisture						
Stems	11.0	.4	47.2	.5	8.6	.2
Leaves	31.0	.1	18.9	.5	12.2	.1
Third-cut						
Low moisture						
Stems	12.7	.3	52.4	5.9	6.3	.1
Leaves	31.4	.6	19.9	.1	9.9	.4
High moisture						
Stems	17.1	.4	53.5	0	8.1	0
Leaves	36.7	3.6	23.1	.5	9.0	.2

[1]Percentage dry matter basis.

[2]Average of three replications.

[3]Average of two replications.

[4]Initial moisture content for leaves and stems was 11 and 13%, respectively, for low moisture and 33 and 32%, respectively, for high moisture alfalfa.

[5]Initial moisture content for leaves and stems was 10 and 9%, respectively, for low moisture and 27 and 30%, respectively, for high moisture alfalfa.

the moisture content at high a_w values will be significantly underestimated.

Water activity and not moisture content had a dramatic influence on the time required for the appearance of fungal fruiting bodies on both alfalfa stems and leaves. For example, alfalfa stems at a moisture content of 19.4%, which corresponds to an a_w of .80, 22.5 d were required for fruiting bodies to appear. Decreasing the moisture content by 2.7% changes the a_w value to .755. This increased the time for the appearance of fungal fruiting bodies by 40 d. Thus, minor, apparently insignificant changes in the moisture content of alfalfa hay can dramatically affect the spoilage of that hay. This observation becomes more critical when one considers the immense variability in the moisture content of alfalfa at baling time.

Water activity also affected the nature of fungal growth on both alfalfa stems and leaves.

TABLE 5. Number of days required for the unaided visual appearance of fungal fruiting bodies on alfalfa stems and leaves treated with water or yeast extract at reduced water activity values.[1]

	.800 Water activity				.755 Water activity			
	Water	SD	Yeast extract	SD	Water	SD	Yeast extract	SD
High moisture[2]								
Leaves	27	.7	29	.7	57	0	59	0
Stems	24	.7	22	0	50	0	49	.7
Low moisture[3]								
Leaves	30	0	29	.7	56	0	56	0
Stems	22	0	24	0	43	.7	45	0

[1]Average of two replications.

[2]Initial moisture content for leaves and stems was 27 and 30%, respectively.

[3]Initial moisture content for leaves and stems was 10 and 9%, respectively.

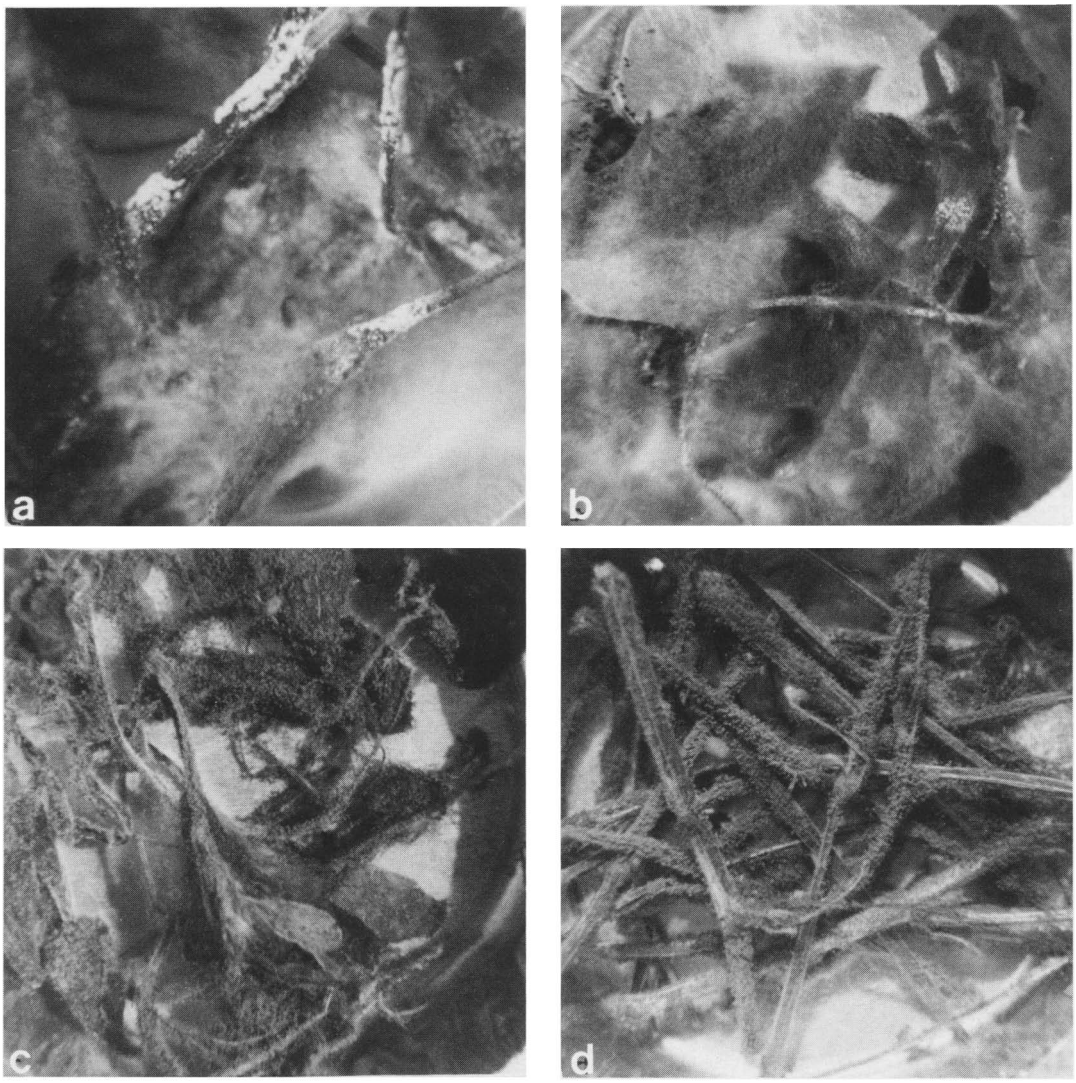

Figure 1. Extent of fungal growth after 23 d of storage on: a) alfalfa stems at a water activity value of 1.00, 19 d of growth; b) alfalfa leaves at a water activity value of .97, 18 d of growth; c) alfalfa leaves at a water activity value of .93, 17 d of growth; and d) alfalfa stems at a water activity value of .88; 16 d of growth.

At a a_w of 1.00, which corresponds to a moisture content of 51.7 to 63.3% (Table 2), fungal growth was characterized by extensive mycelial development (Figure 1a). Reduction of a_w to .97, corresponding to a moisture content range of 53.3 to 34.7%, (Table 2), increased fruiting body production by the growing molds (Figure 1b). At a_w of .88, which corresponds 31.6 to 23.9% moisture content (Table 2), fungal mycelia were no longer visible to the unaided eye (Figure 1d). It is well-known that any environmental condition that inhibits mycelial growth enhances spore formation (4). The obvious effect of a_w on the nature of fungal growth correlates well with the observed fungal growth patterns seen in spoiled alfalfa hay. At times, mold growth in hay is characterized by extensive mycelial growth. Other times only "dust", which is fungal spores, is seen. The former condition occurs at a very high a_w and minor

decreases from that condition result in the latter. The nature of fungal growth would determine the nutritional equality of the feedstuff. Rapid vegetative growth, which would result in more cell mass, would generate more heat than reproductive growth. As the amount of heat produced increases, loss in nutrient content becomes greater.

In addition to differences in the MSI for alfalfa stems and leaves, there was a difference in the time required for the visually unaided appearance of fungal fruiting bodies. Even though the nutritional quality, as measured by protein, fiber, and ash content of third-cut alfalfa stems, is less than that of alfalfa leaves (Table 4), fungal fruiting bodies appeared sooner on the stems than on the leaves at reduced a_w values (Tables 3 and 5). This differs from observations reported by Snow (19), who reported that added nutrients accelerated the formation of fruiting bodies by an *Aspergillus* sp. Differences in experimental systems make definitive comparisons difficult. Snow (19) used plain and nutrient gelatin as the growth substrate, but the present study used a natural product.

In a natural product like alfalfa hay, it is entirely possible that nutrient content is not the determining factor affecting fungal growth and development. Differences in chemical composition as measured by nutritional analysis may result in subtle alterations in the physical and chemical environment in which the mold is growing (22). Indeed, growth and sporulation of fungi on standing crops and stored grain are largely dependent on environmental factors (18).

The role of nutrient availability was examined by mixing a .1% yeast extract solution with alfalfa stems and leaves. Thus, the fungal spores had for their growth and development a rich supply of readily available nutrients. The presence or absence of yeast extract did not appear to affect the appearance time of fungal fruiting bodies on alfalfa stems and leaves. Fungal fruiting bodies were observed sooner on alfalfa stems than on leaves (Table 5).

The results of this study do not mean that nutrient availability is not important. Although yeast extract is often used to supplement media used to culture fungi (15), it is very possible that yeast extract does not supply the factor(s) necessary to enhance fungal growth and development on alfalfa stems and leaves. Indeed, the importance of nutrient availability was demonstrated by the observation that fungal growth on alfalfa stems usually begins at very specific sites. It was repeatedly observed that fungal growth on alfalfa stems usually started at the internodes or at breaks in the stem. Presumably, these are sites where the moisture and soluble nutrients necessary to initiate and maintain growth are readily available. Provided the a_w value is high enough, the mold spreads from these sites along the stem (unpublished observations). Waite (23) reported that mold growth on grass hay usually started at breaks or internodes.

The results of this study indicate that alfalfa stems and leaves should be thought of as different entities when considering the spoilage process in alfalfa hay. Even though alfalfa stems do not appear to be as high in nutritional quality as alfalfa leaves, the former appear to have a greater spoilage potential. This difference was displayed in two ways 1) at any moisture content, alfalfa leaves will usually have a lower corresponding a_w value and 2) at reduced a_w, fungal fruiting bodies will appear sooner on alfalfa stems than alfalfa leaves. In addition, if alfalfa stems do contain a factor(s) that enhance fungal development, then the "sweating" process, which is the release of moisture from the stem, not only increases the a_w value in the bale but also permeates the bale with a factor or factors that stimulate fungal development.

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