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Evaluation by Video Microscope of Ruminal Digestion of Orchardgrass Tissues with Different Extents of Lignification

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Abstract

Video microscopy permits direct viewing of forage tissue degradation without any pretreatment. The present study was undertaken to investigate the relationship between the progress of lignification of orchardgrass tissues with maturity and *in vitro* degradation of them using a video microscope system. Orchardgrass was harvested weekly at a wide range of stage of maturity (Vegetative-Prebloom) and stored frozen until used. About 0.3 mm-thick cross sections from the 2nd internode from the soil were handcut and adhered to slides. After certain hours of incubation (a 6-hour interval), the sections were stained for lignin with acid phloroglucinol and evaluated for the relative degree of staining and degradation. For continuous observation, other slides in a vessel filled with rumen fluid and buffer were observed through wrapping film. Relative cross-sectional area of leaf sheath to stem decreased with maturity level. In the youngest grass, only vascular bundles of leaf sheath were lignified intensively. Then, the epidermis and vascular bundles of the stem were lignified. Parenchyma tissue was lignified from the outside inward with maturity. In grass younger than the heading stage, most tissues except vascular bundles of leaf sheath degraded within 12h of incubation. In mature stems, only unlignified inner parenchyma tissues degraded within 24h of incubation. By continuous obsevation, the following processes of degradation were recorded : cell walls of inner parenchyma turned vague and melted, and vascular bundles remained as peninsulas. Many protozoa crowded around the degrading parenchyma.

Key words : Orchardgrass, Digestibility, Lignin, Rumen, Video microscope

Introduction

The nutritive value of forage depends on its voluntary intake by animals and digestibility. These factors vary widely with the state of forage maturity. The nutritive value of forage has been studied mainly in relation to chemical composition of the forage.

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However, morphological information is limited on changes occurring with the progress of maturity of forage and ruminal digestion of forage tissues. The relationship between histochemical reactions and *in vitro* rumen degradation has been studied with light microscope[1,2,5,8], and scanning electron microscope (SEM) permitted further detailed observation of digestion by ruminal microbes[3,4,7]. Light microscopy is not appropriate for surface observation, and SEM requires pretreatment before observation. Recently, a video microscope system, a fiberscope with an objective lens, has been developed. The system permits direct viewing of forage tissue degradation without any pretreatment.

The present study was undertaken to investigate the relationship between the lignification of orchardgrass tissues during the first growth of the year and their *in vitro* degradation, using a video microscope system.

Materials and Methods

Orchardgrass was harvested weekly at a wide range of maturity stages (Vegetative -Prebloom) at a field on the University Farm near Sapporo, Hokkaido. Plant samples for chemical analysis were air-dried at 60 °C and samples for histochemical examination and *in vitro* rumen degradation were stored frozen until used.

For histochemical and degradative observation, about 0.3-0.5 mm-thick cross sections from the second internode from the soil were handcut and adhered to slides. Each slide had sections from 5 samples of different stages of maturity. Six slides with neighbor sections from the same internode were prepared. One slide was used for intact-tissue observation and the other was observed after incubation (6, 12, 18, 24 or 30h)[1]. During incubation, the slides were immersed in a coloration vessel filled with rumen fluid and McDougall's buffer. Histological reaction for lignin with acid phloroglucinol was examined with a video microscope system (Mitsubishi Kasei, Microwatcher VS-30H). Images from the video system were recorded, and micrographs were obtained by a video printer (Sony, CVP-M3).

For continuous observation, another slide with a section was immersed horizontally into a vessel with rumen fluid and buffer. The vessel was covered with wrapping film, and the section was able to be observed through the film. An image was recorded every 2 hours for 30 hours.

Air-dried samples were ground through a 1 mm screen and analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL)[6]. Hemicellulose and cellulose were determined by subtracting ADF from NDF and ADL from ADF[9]. Ash in the ADF was regarded as silica.

Results

Cutting date, grass length and NDF, ADF and ADL contents of the orchardgrass are shown in Table 1. These values increased with progress of maturity. The components of NDF are shown in Table 2. The proportion of hemicellulose in the cell wall constituents decreased, and the proportion of lignin increased with maturity, while the proportion of cellulose and silica did not change remarkably.

Figure 1 illustrates the progress of lignification during the first growth. Lignified

tissues were stained red by acid phloroglucinol. Until June 4th, no mesophyll or parenchyma tissue of leaf sheath or stem was lignified, while vascular bundles in the leaf sheath were lignified intensively and epidermis and vascular bundles of stem were lignified slightly. Then, parenchyma tissue of stem lignified from the outside inward with maturity. Clear reaction for lignin was not detected in mesophyll tissue of leaf sheath.

Table 1 Cutting date, grass length and fiber contents of the orchardgrass

Cutting date	Grass height (cm)	NDF	ADF	ADL
		(%)		
May, 28	55	54.1	31.3	2.3
June, 4	79	60.6	34.8	2.7
June, 11	101	65.4	38.8	3.5
June, 18	129	65.4	41.6	5.2
June, 25	135	67.7	42.3	4.9

Table 2 Composition of NDF (%)

Cutting date	hemicellulose	cellulose	lignin	SiO_2
May, 28	43.4	50.3	4.3	3.1
June, 4	43.6	50.3	4.4	2.5
June, 11	41.6	51.5	5.4	2.4
June, 18	38.2	52.3	7.9	3.4
June, 25	39.1	52.2	7.2	3.0

Proportions of cross sectional area for leaf sheath and stem, and percentages of cross sectional area of epidermis plus vascular bundles and parenchyma tissues are shown in Table 3. These tissues were scored by stain intensity with the acid phloroglucinol. The relative sectional area of leaf sheath to stem decreased with maturity. Unlignified mesophyll tissue and parenchyma tissue decreased remarkably, and the proportion of intensively lignified tissues increased with progress of maturity.

Table 3 Proportions (%) of cross sectional area of leaf sheath and stem, and the percentage of tissues with the histological reaction to acid phloroglucinol

	Leaf sheath			Stem					
		Ep+Vb	Pc(−)		Ep+Vb	Pc(−)	(+−)	(1+)	(2+)
May, 28	47.6	12.2	87.8	52.4	10.5	89.5	—	—	—
June, 4	34.2	8.8	91.2	65.8	15.5	84.5	—	—	—
June, 11	35.1	7.8	92.2	64.9	20.2	39.5	—	40.3	—
June, 18	24.0	28.0	72.0	76.0	26.5	18.5	—	27.5	54.0
June, 25	21.3	49.8	50.2	78.7	26.2	—	23.0	16.1	60.9

Ep+VB: Epidermis plus vascular bundle, sclerenchyma rings may be included. Histological reaction for the tissues were 1+ (younger grasses than June 4) or 2+.

Pc: Parenchyma, Histological reactions were shown in parentheses ranked from −to 2+.

Tissues after certain periods of *in vitro* degradation are illustrated in Figure 2. In grass younger than heading stage (June 4th), most tissues except vascular bundles of leaf sheath degraded within 12 hours of incubation. In the older grasses, epidermis, vascular bundles and outside lignified parenchyma tissue of stem remained, and unlignified tissue on the inward side degraded within 24 hours of incubation. Besides the vascular bundles and thin epidermal layer, most leaf sheath tissues degraded even in the most mature grass.

Percentages of degraded area of cross sections of stem are shown in Table 4. Data for grasses of May 28th and June 4th exceeding 12 or 18 hours of incubation are missing because undegraded tissues including vascular bundles effused into the rumen fluid-buffer solution. It appeared that degradation of the digestible tissues was completed in 24 hours. Maximum percentages of degraded area decreased remarkably with the progress of maturity.

Table 4 Percentage of cross sectional area of degraded tissues in the stem

	Hours of incubation				
	6	12	18	24	30
May, 28	88.5	—	—	—	—
June, 4	62.4	91.1	—	—	—
June, 11	25.3	30.3	34.5	42.0	42.0
June, 18	25.6	25.6	30.4	36.9	36.9
June, 25	12.5	18.7	18.7	18.7	18.7

Figure 3 shows typical examples of continuous observation (June 11th at 16 hours of incubation and June 25th at 10 hours of incubation). Bubble formation disturbed clear observation. However, the process of degradation of the same portion of sections could be observed. The typical progression was as follows: cell walls of inner parenchyma turned vague and the melted. Vascular bundle tissues remained as peninsulas. Many protozoa crowded around the degrading parenchyma tissues.

Discussion

Plant morphology in relation to ruminal degradation has been studied by light microscopy and SEM[1-5,7,8]. However, no study using video microscopy has been reported in this field. The video microscope system used in the present study was useful for observing and recording morphology of grass without any pretreatment. This system permits the observation of living objects such as protozoa which are impossible to observe by SEM.

Both ADL content and cross sectional lignified area of orchardgrass increased with progress of maturity. However, these values were not parallel, especially in late-cut grasses. This might be due to the difference of the analyzed part of the plant and of analytical technique.

A strong reaction occurred with acid phloroglucinol in the cell walls of vascular bundles of leaf sheath, and in the epidermis, sclerenchyma and vascular bundles of stem. Parenchyma tissues lignified from outside inward with progress of maturity. The results were similar to those of bermudagrass, sorghum and barley straw[2,3,5,7]. In the present

study, most stained tissues were indigestible. Tissues degrading within 24 hours of incubation showed no or faint reaction for lignin by acid phloroglucinol. Akin *et al.*[2,5] reported that acid phloroglucinol stained aromatic aldehydes deep red and demonstrated the complexes most recalcitrant within plant cell walls to microbial degradation. The values of unstained or slightly stained areas of cross sections of stems (Table 3) were close to the values of degraded area of corresponding samples over a wide range of maturity (Table 4). These results support the view that acid phloroglucinol is useful for detecting tissues indigestible in the rumen.

Continuous observation of the process of degradation confirmed the results of the intermittent observation of stained sections which were described above. This method is suitable for observing the process of degradation of the same part of a section, however, it is not suitable for comparing many samples at the same time. In the early trials, rumen fluid was centrifuged (1000 rpm, 5 min) so as to be able to view it clearly. A small part of the precipitate was introduced by injection syringe in the later trials. This was useful for observing the behavior of rumen protozoa. Many protozoa crowded around the degrading parenchyma tissues. Bubble formation often disturbed the observation.

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References

1) Akin, D. E. 1982. Section to slide technique for study of forage anatomy and digestion. Crop Sci., **22**: 444-446.
2) Akin, D. E., N. Ames-Gottfred, R. D. Hartley, R. G. Fulcher, and L. L. Rigsby, 1990. Microspectrophotometry of phenolic compounds in bermudagrass cell walls in relation to rumen microbial digestion. Crop Sci., **30**: 396-401.
3) Akin, D. E., W. W. Hanna, and L. L. Rigsby, 1986. Normal-12 and brown midrib-12 sorghum. I. Variations in tissue digestibility. Agron. J., **78**: 827-832.
4) Akin, D. E., W. W. Hanna, M. E. Snook, D. S. Himmelsbach, F. E. Barton, II, and W. R. Windham, 1986. Normal-12 and brown midrib-12 sorghum. II. Chemical variations and digestibility. Agron. J., **78**: 832-837.
5) Akin, D. E., M. T. M. Willemse, and F. E. Barton, II, 1985. Histochemical reactions, autofluorescence, and rumen microbial degradation of tissues in untreated and delignified bermudagrass stems. Crop Sci., **25**: 901-905.
6) Goering, H. K. and P. J. Van Soest, 1970. Forage fiber analysis (Apparatus, reagents, procedures, and some applications). USDA. Agric. Handbook **379**: 1-9.
7) Goto, M., O. Morita, and A. Chesson, 1991. Morphological and anatomical variations among barley cultivars influence straw degradability. Crop Sci., **31**: 1536-1541.
8) Hanna, W. W., W. G. Monson, and G. W. Burton, 1976. Histological and *in vitro* digestion study of 1- and 4-week stems and leaves from high and low quality bermudagrass genotypes. Agron. J., **68**: 219-222.

9) Okamoto, M. and Y. Hirose, 1972. Studies on the digestion of forage in the rumen using *in vitro* rumen fermentation technique. I. Changes of *in vitro* digestion with advancing maturity of forage. Jap. J. Zootech. Sci., **43**: 499-505.

要　　約

最近，光ファイバーの先端に高倍率のレンズを装着した「ビデオ顕微鏡」が開発された。これは手軽に拡大映像が得られ，焦点深度も深く，光学顕微鏡とSEMの利点を合わせ持つと考えられる。そこで，これを用いて牧草組織の人工ルーメン内消化の様相を観察した。出穂前から開花前までの5段階の生育期のオーチャードグラスを刈り取り凍結保存した。根元から第2節と第3節の節間をカミソリで0.3 mm程度の厚さに輪切りにし，以下の二つの方法で観察した。第一の方法は切片を両面テープでスライドグラスに張り付け，人工ルーメン中に浸漬し，6時間毎に取り出し，フロログルシン・塩酸法で木化組織を染色観察した。第二の方法は人工ルーメン内に切片を張り付けたスライドグラスを水平に固定し、全体をラップで覆い，その上から連続観察できるようにした。ただし，ビデオテープレコーダには2時間毎に記録した。

断面積に占める葉梢の割合は若いほど高く，茎の割合は生育の進行にともない増加した。若い草では維管束のみ木化していたが，生育につれて柔組織も表皮側から木化した。出穂前では12時間後には葉梢の維管束を除いて消化されたが，生育の進んだ茎では24時間後でも木化されないかその度合の低い柔組織のみ消化された。第二の方法では，まず内側の未木化柔組織の細胞壁が不明瞭になり，やがて溶解してヘドロ状になり、維管束が半島状に残される様相が観察される。時には中間の柔組織が軟化し，穴が開いてこれが拡大する様子が観察された。なお，プロトゾアを加えると盛んに分解されていると思われる部分に集まり活動する様子が観察される。

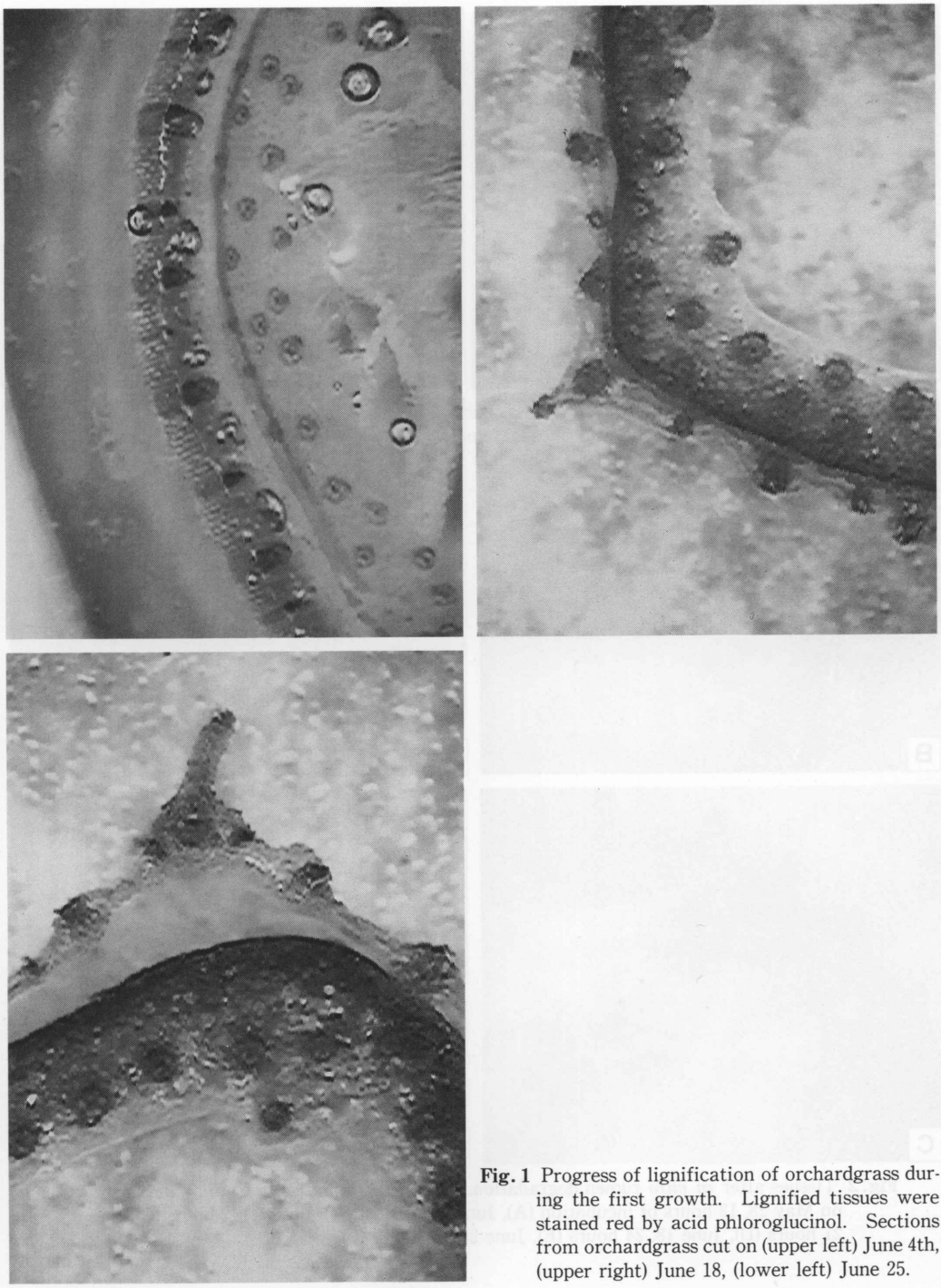

Fig. 1 Progress of lignification of orchardgrass during the first growth. Lignified tissues were stained red by acid phloroglucinol. Sections from orchardgrass cut on (upper left) June 4th, (upper right) June 18, (lower left) June 25.

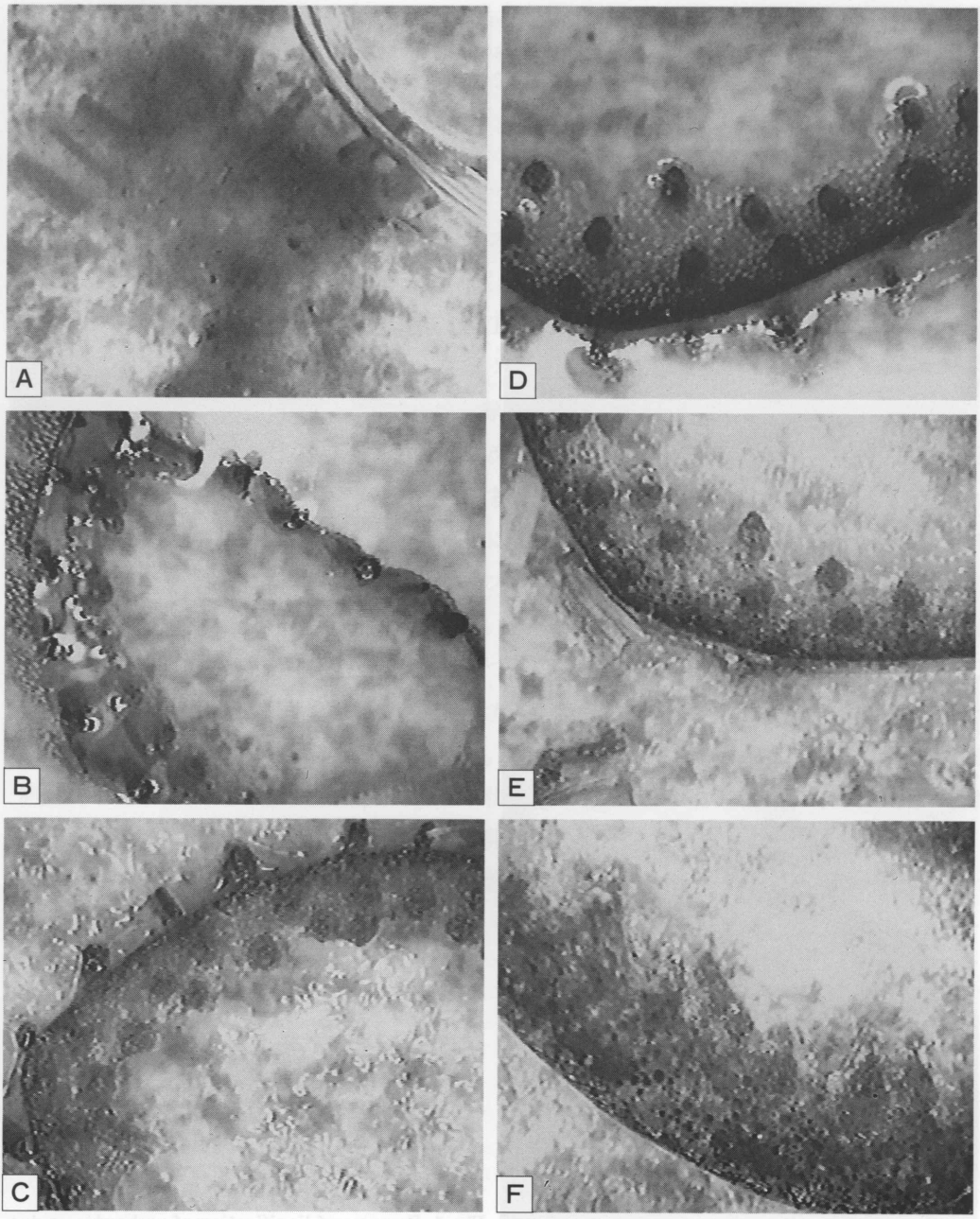

Fig. 2 Tissues after *in vitro* rumen degradation. Leaf sheath and stem from orchardgrass cut on May 28, 12 hours of incubation (A), June 4, 12 hours (B), June 11, 12 hours (C), June 18, 21 hours (D), June 18, 24 hours (E), June 25, 24 hours (F).

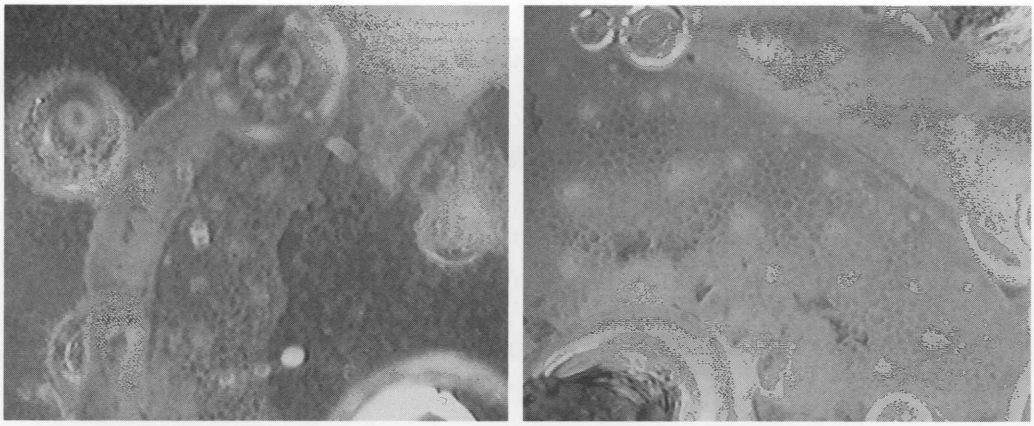

Fig. 3 Typical records from continuous observation. Left: Section of leaf sheath and stem from orchardgrass cut on June 11, 16 hours incubation. Some parenchyma tissues are melting. Large protozoa can be seen as white blips. Right: June 25, 10 hours. Inner parenchyma is turning to be vague.