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in

Delgado I. (ed.), Lloveras J. (ed.).
Quality in lucerne and medics for animal production

Zaragoza : CIHEAM

Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 45

2001

pages 47-51

Article available on line / Article disponible en ligne à l'adresse :

<http://om.ciheam.org/article.php?IDPDF=1600055>

To cite this article / Pour citer cet article

Guines F., Julier B., Huyghe C., Poussot P. **Investigating variation for histological characters in alfalfa stems**. In : Delgado I. (ed.), Lloveras J. (ed.). *Quality in lucerne and medics for animal production*. Zaragoza : CIHEAM, 2001. p. 47-51 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 45)



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Investigating variation for histological characters in alfalfa stems

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SUMMARY – Alfalfa stem digestibility decreases as stems mature. Factors that limit digestibility are complex. Most research aimed at understanding differences in cell wall digestibility has focused on chemical limitations to enzymatic degradation of wall constituents. Anatomical factors may influence wall degradability. The aim of this experiment was to quantify histological characters in mature stems of 2 genotypes divergently selected for high and low digestibility. This quantification was used to localise the stem portion showing maximum variation. The aim was also to identify histological characters that could explain or give further information on the bases of differences for digestibility. Quantitative measurement of histological characters was carried out on stained cross sections using image analysis. Three stems per genotype were evaluated for histological characters and the rest of each genotype was used to determine NDF and Klason lignin content. Stems within each genotype did not show any significant differences for histological characters. The 2 genotypes were significantly different for cortex thickness, cortex area, xylem vessel area, xylem fibre area, xylem cell wall density, and xylem cell wall thickness. The maximum variation for histological characters was observed at the base of the stems making it possible to simplify the sampling to assess genetic variation. In future studies the available genetic variability for histological characters will be analysed within and among varieties in dense conditions.

Key words: Tissue, digestibility, quality, image analysis.

RESUME – "Recherche de variabilité pour les caractéristiques histologiques des tiges de luzerne". La digestibilité des tiges de luzerne diminue avec la croissance. Les facteurs qui limitent la digestibilité sont complexes. La majorité des travaux effectués sur la compréhension des différences de digestibilité sont basés sur la composition biochimique. Certains auteurs font l'hypothèse que la structure histologique des tiges pourrait jouer un rôle aussi important que la composition chimique dans la digestibilité. Le but de cette expérimentation était dans un premier temps de quantifier des facteurs histologiques dans des tiges matures de luzerne sur du matériel contrasté issu d'une sélection divergente pour la digestibilité. Cette quantification a été réalisée sur des coupes transversales de tiges colorées au Fasga, par analyse d'images via le logiciel Optimas. Un génotype ayant une bonne digestibilité et un autre de moins bonne digestibilité ont été choisis. Sur chaque génotype trois tiges matures ont été prélevées en début de floraison et ont été caractérisées par analyse d'images. Des mesures de teneur en NDF et en lignine Klason ont été réalisées sur le reste des tiges de chaque génotype par strate de 10 cm de hauteur. Au sein de chaque génotype les tiges n'ont pas montré de différences significatives entre elles pour les caractères histologiques mesurés. Des différences significatives ont été observées entre ces deux génotypes pour l'épaisseur et la surface du cortex, la surface moyenne des vaisseaux et des fibres du xylème, la densité surfacique des parois du xylème, et l'épaisseur des parois du xylème. Le maximum de variation a été observé en bas des tiges, ce qui permettra d'utiliser cette portion pour estimer la variabilité génétique disponible pour les caractères histologiques et pour la composition biochimique au sein de variétés et entre variétés en couverts denses.

Mots-clés : Tissus, digestibilité, qualité, analyse d'images.

Introduction

Alfalfa is a high yielding forage with a high protein content but a moderate digestibility. This is due to the low digestibility of the stem basis (Buxton *et al.*, 1987). Genetic differences for digestibility have been reported in alfalfa by several authors (Hill and Barnes, 1977; Buxton *et al.*, 1987; Julier *et al.*, 1996). Stems are the main component of an alfalfa canopy and contribute to a large part of forage yield. Stems also determine to a major extent of the whole genotype digestibility. Stem digestibility progressively decreases as stems grow (Lemaire and Allirand, 1993). Most research aiming at understanding differences in digestibility have focused on chemical limitations to enzymatic degradation of cell wall constituents. Wilson and Hatfield (1997) noticed that relationship of cell wall constituents to cell wall digestibility is not consistent across genotype parts, genotype among species, species, and particularly

across families. Alfalfa stem anatomy may be related to forage quality (Jewett and Barnes, 1994). Anatomical structure of cells and tissues significantly influences wall accessibility to rumen micro-organisms (Wilson and Mertens, 1995). Legume stems from a wall degradability point of view are composed of two populations of cells: The highly lignified xylem being 100% indigestible and the remainder that appears to be potentially 100% digestible (Wilson *et al.*, 1991). Jewett and Barnes (1992) noticed an important genetic variability for histological characters in alfalfa stems. The aim of this paper is to show that using image analysis to quantify histological characters makes possible to describe the evolution of histological characters along the mature stem, and to identify the stem portion expressing maximal differences between divergently selected genotypes.

Materials and methods

Two genotypes divergently selected for digestibility were chosen and evaluated in spaced plants for 2 cuts in 2000. Both genotypes showed 10 points of difference for NDF content with the same level of dry matter yield in the harvest 1999. Three mature stems per genotype were taken at the flowering stage and sections were cut each 5 cm from the apex to the basis of the stem to analyse histological characters along the stem.

In parallel chemical analyses were carried out each 10-cm long portion from the apex to the bottom of the remaining stems for each genotype. On the stem portions leaves were discarded. Stem samples were dried at 60°C and ground to pass a 1 mm sieve to determine the fibre (NDF) and Klason lignin (KL) contents. The position of each histological section allowed us to compare histological characters to chemical constituents for each 10-cm long portion and to compare stems from both genotypes.

For histological studies stem cross sections were fixed in a glacial acetic acid / 95° ethanol solution. 50 µm cross sections were stained with Fasca (Tolivia and Tolivia, 1987) giving a red staining for lignin and a blue one for cellulose. Semi automated image analysis was performed using the 6.1 version of Optimas™ (Media Cybernetics, 1996). Different histological characters were quantified: area of pith parenchyma, area of the whole cross section, area of the cortex, cortex thickness, distance between epiderma and pith parenchyma, lignified xylem proportion in the section, lignified cell wall thickness in the xylem and surface density of cell wall in the lignified xylem (defined as the surface of the lignified cell wall divided per the surface of the measurement area), area of vessel and fibre in the xylem.

Results and discussion

NDF and lignin content increased progressively from the apex to the basis of the stems from 30% and 4% at the top to 70% and 15% at the bottom of the stems respectively. The genotype selected for higher digestibility showed lower NDF and lignin content from the middle of the stem to the bottom. The top of the stem correspond to an elongation zone and the middle of the stem is characterised by a cambial activity resulting in secondary growth associated to an important lignin deposition in secondary wall of xylem tissues (Vallet, 1997). The analysis of variance did not show significant differences between the high and the low digestibility genotypes for the first cut but did for the second one (data not shown). Fig. 1 illustrates an example of the vertical evolution of a histological character: the surface density of the lignified cell wall in the xylem of both genotypes. This character ranged from 0.47 at the top to 0.65 at the bottom of the stem. The genotype with high digestibility showed a surface density of the lignified cell wall lower than the genotype with low digestibility all along the stem.

A principal component analysis was carried out on the 3 stems per genotype with 10-cm long portions. Figure 2 shows the representation of histological and chemical characters. Cortex thickness, whole section area, cortex area and distance between epiderma and pith parenchyma were well represented by the first axis, which expressed 46% of the variation. Those characters were closely linked to NDF and lignin content and are factors related to the secondary growth of the stems. Xylem cell wall thickness and xylem fibre area were well represented by the second axis, which explained 16% of the variation, and were not linked to NDF and Klason lignin contents.

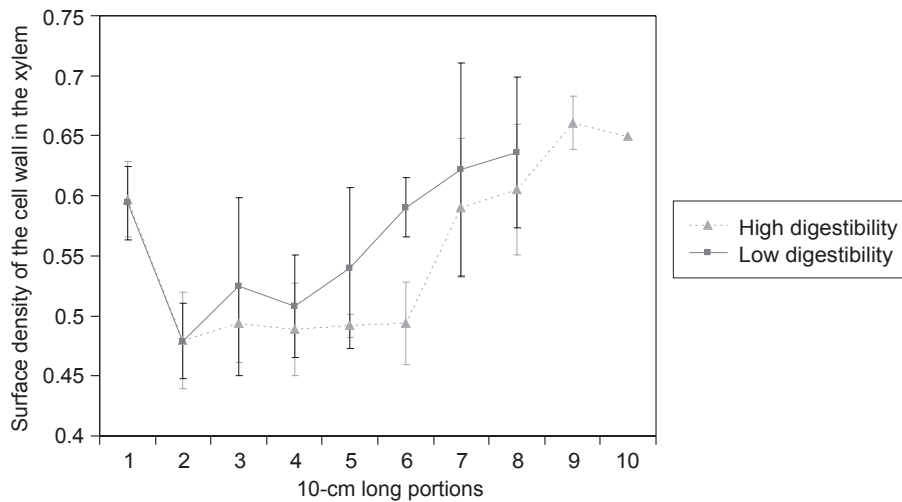


Fig. 1. Evolution of the surface density of the lignified cell wall in the xylem along the stem (portion 1 and 10 represent the top and the bottom of the stem respectively) for the genotypes with high and low digestibility.

Figure 3 illustrates the representation of stem portions of both genotypes. Portions belonging to the basis of the stems were well represented by the first axis explaining 46% of the variation. Stem bases of the genotype with high digestibility were well separated from stem bases of the genotype with low digestibility meaning that differences were maximised at the bottom of the stem.

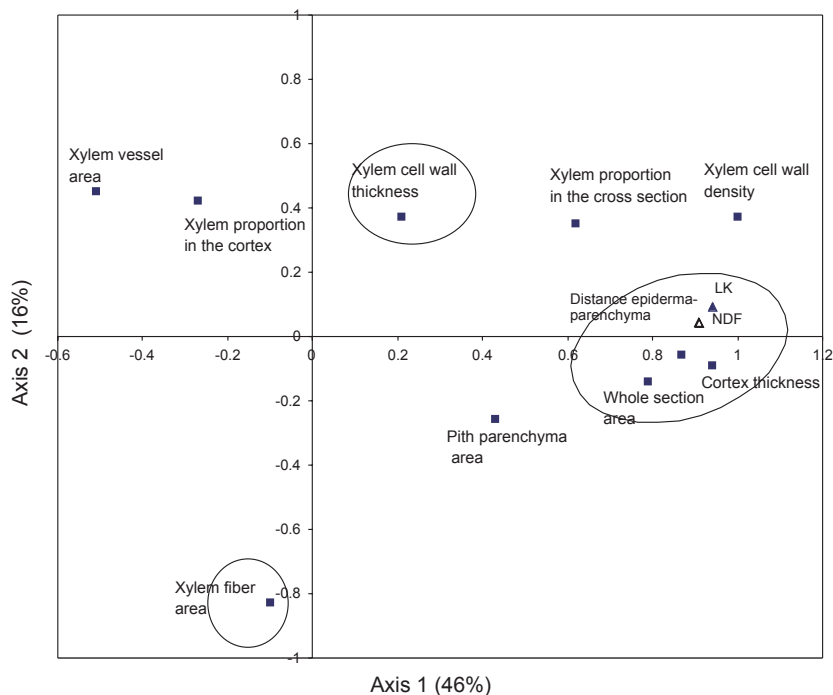


Fig. 2. Principal component analysis: representation of histological and chemical characters (on the 3 stems per genotype and with 10 cm long portions).

Caution have to be taken to distinguish differences resulting from genetics and those from differences in growth rate, taking into account that digestibility changes with plant height. An analysis of variance was carried out with genotype effect, portion effect, and interaction between genotype and portions. Stem effect was taken into account in the residual. The analysis showed significant differences

between genotype for cortex thickness, cortex area, surface density of the lignified cell wall in the xylem, xylem cell wall thickness (Table 1). Those characters were related to the secondary growth of the stem. The genotype effect was higher for all characters excepted for xylem proportion in the section, than the residual including stem effect. It showed that differences between genotypes was higher than differences among stems within genotypes. Portion effect was significant for all characters tested excepted for xylem fibre area.

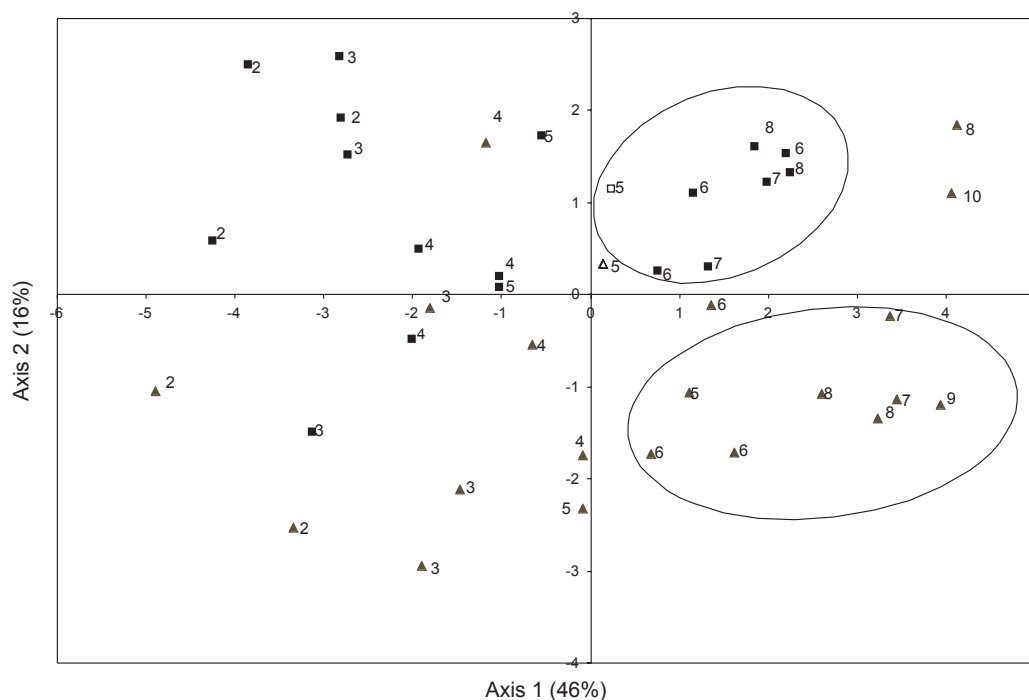


Fig. 3. Principal component analysis: representation of genotypes. Numbers represent 10-cm long stem portions numbered from 1 at the apex to 10 at the base of the stem (▲ corresponds to the genotype with high digestibility and ■ corresponds to the genotype with low digestibility).

Table 1. Analysis of variance (mean squares) for histological characters measured on two genotypes divergently selected for digestibility (with three stems per genotype and ten portions per stem)

Histological characters	Genotype	Portion	Genotype	Portion	Error
Characters related to the xylem (mean square $\cdot 10^{-4}$)					
Xylem proportion in the section	10	270***	190***		30
Xylem vessel area	6.9***	2.8***	0.3		0.7
Xylem fibre area	0.026**	0.002	0.0011		0.002
Surface density of cell wall in the xylem	93*	180***	15		16
Xylem cell wall thickness	0.43**	0.1*	0.1*		0.04
Characters related to stem morphology					
Whole section area	14.46	65.04***	12.27		5.74
Pith parenchyma area	5.07	22.43***	1.72		3.36
Cortex area	15.23***	25.09***	1.66		0.73
Cortex thickness	0.019***	0.0075**	0.001		0.001
Distance epiderma-pith parenchyma	0.09***	0.10***	0.001		0.005
d.f.	1	9	7		32

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conclusions

This was the first step of our study of genetic variation for histological characters. Results showed that it was possible to quantify histological characters in alfalfa stems with image analysis. Differences within genotypes (i.e. among stems) for all characters tested were lower than differences between genotypes divergently selected for digestibility. Significant differences were observed between genotypes for xylem vessel and fibre area, surface density of cell wall in the lignified xylem, xylem cell wall thickness, cortex area, cortex thickness and distance between epiderma and pith parenchyma. The maximum variation for histological characters was observed at the bottom of the stems. The top of the stem did not show variation for histological characters probably because the top of stem is characterised by an elongation zone with young tissues not lignified. The aim was also to identify histological characters, which could have explained genetic differences for digestibility. The effort has to be directed through characters showing no positive correlation with chemical constituents of digestibility, in order to find new elements, that could explain what NDF and lignin content could not. The available genetic variation for histological characters within and among varieties in dense canopies will be measured at the bottom of the stems.

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