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Lignin associated anatomical changes at different growth stages of tall fescue (*Festuca arundinacea* Schreb.)

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Abstract

Lignin is a phenolic heteropolymer that limits the nutrient availability in ruminants from forages by acting as physical barriers to microbial enzymes and interfering with the cell wall polysaccharides digestion. Maule staining of Tall fescue internode sections was done at four different growth stages. The staining of internodal sections revealed progressive increase in lignification from first node palpable stage to spikelet emergence stage. Maximum lignin deposition was observed at spikelet emergence stage and minimum at first node palpable stage. A shift in colour from yellow to red has been observed from first node palpable stage to spikelet emergence stage suggesting an increase in syringyl (S) lignin deposition.

Key words: Lignin, *Festuca arundinacea*, Maule staining, anatomical change.

Forages are the major supporting feature of sustainable agriculture and constitute one of the major resources for the successful animal production system. Due to shortage of feed and fodder resources in the country, animal production has become an uneconomical and unattractive venture for the farming community. The forage grasslands have been the major source of livestock feeding and represent about 26% of the land area and 70% of the agricultural area at global scenarios (Capstaff and Miller 2018). Forage crops generally belong to the grass family or are the herbaceous legumes, however tree legumes such as *Acacia* and *Leucaena* species also fulfills the demand of forage for animals (Muir *et al*. 2011; Capstaff and Miller 2018). The digestibility of forages exhibit extreme variation and as the materials of cell wall is the largest component, cell wall digestibility becomes the primary determinant in efficacy and productivity of animals. During the process of maturation, cell walls of plant tissues endure changes in chemical composition and structural organization. The aerial parts such as leaves and stems exhibit variation in types, proportions and composition of chemical constituents that affects the cell wall digestibility of crops, lignin being one of the major factor (Jung 2012). Lignin is an imperative component impregnated in the cell walls of forages that has been recognized as the key factor restraining the digestibility of forage crops (Vogel and Jung 2001). Lignin being essentially undigestible inhibits the fermentation of forage cell wall polysaccharides in the rumen of cattle (Kondo *et al.* 1998) resulting in quality reduction of the forage crop.

Tall fescue (*Festuca arundinacea* Schreb.) is a perennial, cool-season bunchgrass grown for pasture, hay, silage and turf (Mian *et al.* 2005). It belongs to the genus '*Festuca*,' which is one of the largest genera under the Poaceae family, containing over 500 grass species (Hand *et al.* 2012). It is a good source of nutritional components such as protein (9.33-12.54%), NDF (63.10-71.40%), ADF (46.06-53.06%), hemicellulose (11.23-19.46%) with higher dry matter digestibility (47-55.80%) (Katoch *et al.* 2013). However, digestibility of tall fescue has been criticized due to deposition of certain limiting factors which limit the intake potential and energy availability from this crop. The most important constraint on the digestion of plant cell wall is lignin (Vogel and Jung 2001). Lignification of forage tissues limits the amount of digestible energy available to livestock, resulting in an incomplete utilization of celluloses and hemicelluloses by ruminant animals (Casler *et al.* 2002).

Lignins are complex phenolic heteropolymers associated with the polysaccharidic components of the plant cell wall. In forage grasses, the major constituents

of lignin are guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol. Lignification in plants is an important developmental process that provides rigidity to the cell wall and helps in plant support (Jones *et al.* 2001; Boerjan *et al.* 2003). The lignin deposition mostly occurs in the sclereids, fibers and tracheary elements (Jones *et al.* 2001; Ferreira *et al.* 2017). In spite of its important role in the plant survival, lignin is a major constrain in the digestibility of animal feeds. The lignin content as well as composition affects the cell wall degradability of forages (Vogel and Jung 2001). The anatomical structure of lignified cell and tissues in grasses are important in determining the extent as well as rate of fiber digestion by affecting the accessibility of rumen microorganisms (Wilson and Mertens 1995; Wilson and Hatfield 1997).

Maule staining is usually used to distinguish the gymnosperms and angiosperms wood due to difference in the chemical structure of lignin. Maule reagent uses potassium permanganate, a strong oxidizing agent capable of cleaving -C-C – linkages between adjacent –CHOH groups in polysaccharides to –CHO groups which is further oxidized to –COOH group. Potassium permanganate can oxidize lignin and gets reduced to manganese oxide (Crocker 1921). The manganese oxide formed interacts to form complex with lignin (Hepler *et al.* 1970). In order to determine the extent, timing and composition of lignin Maule staining of Tall fescue internodes was done to analyze the anatomical changes associated with lignin deposition at four different growth stages.

Materials and Methods

Plant material

Tall Fescue (*Festuca arundinacea* Schreb.) plants were raised and maintained in pots under controlled conditions at the research farm, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur. The fresh tissues at first node palpable (S1), second node palpable (S2), third node palpable (S3) and spikelet emergence (S4) stage were collected from pot grown plants for histochemical analysis of lignin deposition.

Histochemical analysis

Tall fescue internodes were selected for dissection of sections. The histochemical analysis was carried out using Maule staining technique (Nakano *et al*. 1992). The sections were hand cut and engrossed in neutral potassium permanganate (1%) solution for 5 minutes followed by rinsing with double distilled water. Decoulorization of sections were carried out by placing them in 3% HCl solution for 3 minutes followed by washing with double distilled water and neutralization with 14.8 M ammonium hydroxide solution. The photographs of sections were observed using Olympus inverted Microscope attached with colour camera.

Results and Discussion

Histochemical staining of Tall fescue internodes was carried out at four different growth stages. The staining results revealed a gradual increase in deposition of lignin with the progression of Tall fescue growth from first node palpable stage (S1) to spikelet emergence stage (S4). The staining of the Tall fescue internodal sections at first node palpable stage (S1) exhibited minor yellow coloured staining around the vascular bundles and in the outer epidermis and faint yellow colour in the cortical region as visualized under microscope (Figure 1).

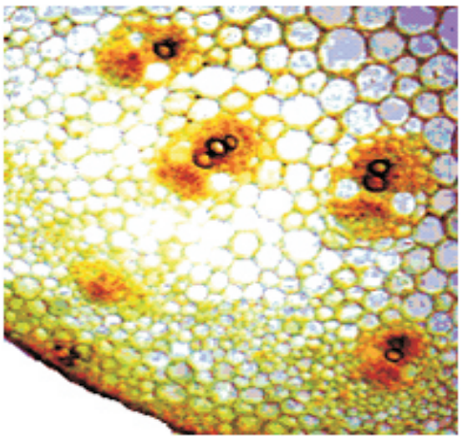


Figure 1: Maule Staining of Tall fescue internodes at first node palpable stage (S1). The yellow colour around the vascular tissues suggests deposition of G and H lignin.

The section of Tall fescue internodes at second node palpable stage (S2) exhibited a shift of colour to yellow-brown was observed (Figure 2). The sclerenchymatous region was found to exhibit lignification in addition to the vascular bundle regions.

The increase in colour intensity both around the vascular bundles and in sclerenchymatous region was observed in the sections of third node palpable stage (S3) (Figure 3).

The internodal sections at the spikelet emergence stage (S4) exhibited a deep red colour stain throughout the vascular bundle and sclerenchymatous cell regions (Figure 4).

Figure 2: Maule Staining of Tall fescue internodes at second node palpable stage (S2). The yellow and brown colour stain suggests lignin deposition around the vascular tissues as well as in the sclerenchymatous region.

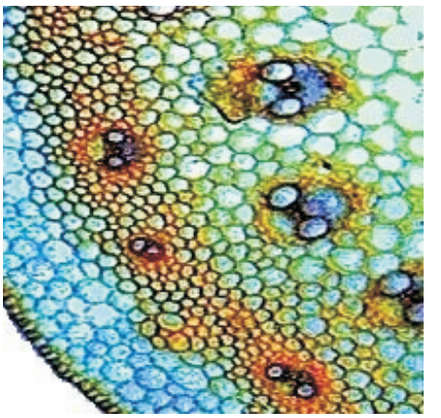


Figure 3: Maule Staining of Tall fescue internodes at third node palpable stage (S3). The brown colour stain suggests lignin deposition around the vascular tissues as well as in the sclerenchymatous region. An increase in colour intensity exhibit enhanced lignin deposition.

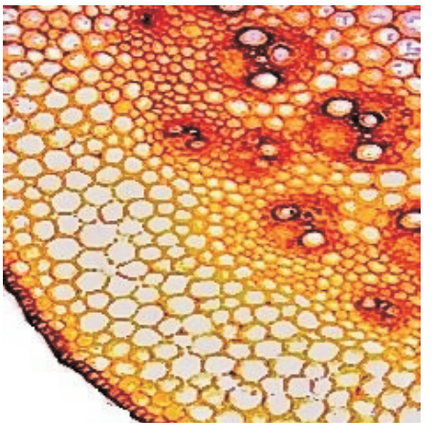
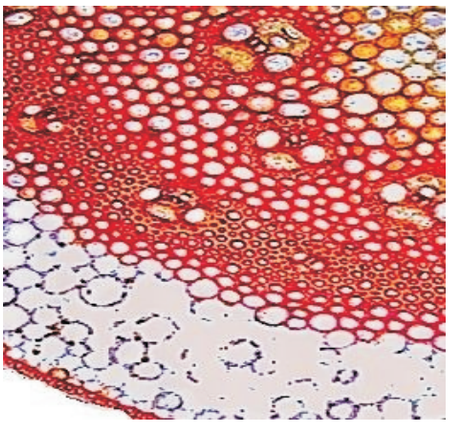


Figure 4: Maule Staining of Tall fescue internodes at spikelet emergence stage (S4). The red colour stain represents deposition of syringyl (S) lignin.



The lignins are detected by Maule's test in either red or brown depending on the type of lignin (Patten *et al.* 2007). The lignin polymer rich in syringyl (S) unit stain red, whereas, guaiacyl (G) and hydroxyphenyl (H) unit stains brown (Ferreira *et al.* 2017). Maule staining has been specifically used for detection of syringyl (S) lignin and also for distinguishing guaiacyl lignin from syringyl lignin (Donaldson 2009). The staining procedure has also been used in determination of cellular specificity, distinct subcellular localization of lignin deposition as well as composition of lignin (Ferreira *et al.* 2017).

In our study, a gradual increase in stain colour was observed in the nodal sections of Tall fescue with progression of growth stages. The increase in colour intensity of Maule stain with the progression of growth stages from first node palpable stage to spikelet emergence stage suggests that the deposition of lignin starts at the first node palpable stage, however, the prominent lignin deposition was observed at second and third node palpable stage with maximum deposition at spikelet emergence stage (Figure 1-4). The increase in lignin deposition may be due to increased activities of lignin synthetic enzymes with progression of growth (Buxton and Fales 1994), however, the increase may also be due to enhanced partitioning of plant dry matter to more lignified tissues (Cone and Engels 1990). Most of the lignification was observed in the region of epidermis, vascular tissues and sclerenchyma. Grasses generally exhibit higher concentrations of lignin in epidermis, xylem and sclerenchyma (Buxton and Redfearn 1997; Moore and Jung 2001). In our study, the internodal sections of Tall fescue were stained in yellow, brown and red with progression of growth stages which may be due to reduction of potassium permanganate by lignin resulting in formation of manganese oxides (Kutscha and Gray 1972) which forms complexes with lignin (Hepler *et al.*1970). The yellow colour stain at first node palpable stage (S1), yellow brown at second node palpable stage (S2) and brown colour at third node palpable stage (S3) may be due to deposition of guaiacyl (G) and *p*-hydroxyphenyl (H) lignin at initial vegetative phases of growth and development in Tall fescue (Figure 1-3). The shift in colour from yellow at first node palpable stage (S1) to red at spikelet emergence stage (S4) suggests an increase in syringyl (S) lignin deposition and decrease in guaiacyl (G) lignin deposition at reproductive stage of Tall fescue (Figure 1-4). The results are in support with the fact that monocots lignin is mostly composed of G and S units of comparable levels (Baucher *et al.* 1998). Similar findings on increase in lignin content and shift of lignin types has also been reported in case of transgenic tobacco (Sewalt *et al.* 1997), Norway spruce (Soukupova *et al.* 2000), *Medicago sativa* (Patten *et al.* 2007), *Arabidopsis thaliana* (Mitra and Loque 2014) and *Neolamarckia cadamba* (Li *et al.* 2019). Lignin deposition in the cell wall structure of grasses is an important constrains in its digestibility and anatomical limitations in the digestibility have been considered as a vital issue in matured grass stems (Wilson and Hatfield 1997). In Tall fescue, anatomical changes are closely associated with lignin deposition which increases with progression of growth stages with maximum deposition of syringyl (S) lignin at reproductive stage. Understanding the process and timing of lignification as well as composition of lignin will provide an insight for improvement and establishment of Tall fescue as forage crop.

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