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CHEMICAL VARIATIONS IN TENSION WOOD OF POPLAR TREE INDUCED BY INTERMITTENT BENDING, FERTILIZER AND HORMONE TREATMENTS

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ABSTRACT

Tree growth is influenced by various environmental factors that lead to anatomical, physical and chemical changes in their wood. Reaction wood is one of the tree's reactions that make many restrictions in wood usages. Reaction wood in broadleaf is called tension wood. This study was aimed to stimulate the formation of tension wood in two-year-old seedlings of *Populus alba* by using intermittent bending, nitrogen fertilization and gibberellin hormone. The application of different treatments increased the content of cellulose compared to the control sample. Meanwhile, the bent specimens had more increase while the straight specimens had no significant difference in the statistical grouping. The content of lignin decreased in all treatments compared to the control sample. The cellulose/lignin ratios obtained from ATR-FTIR (Attenuated Total Reflectance-Fourier Transform Infrared) analysis of wood sawdust and chemical composition measurements were almost close to each other and were higher in the treated samples than in the control. The degree of crystallinity obtained from XRD (X-ray Diffraction) measurements showed that all samples under intermittent bending had a significantly higher degree of crystallinity than the control sample, while this increase was not significant in all straight samples compared to the control sample. In general, it can be concluded that intermittent bending treatment had a

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greater effect on the stimulation and changes of chemical properties of tension wood in poplar and the application of nitrogen fertilization and gibberellin hormone increased this effect. The formation of gelatinous layer in the innermost part of the intermittent bent seedlings fiber cell wall was visible in light microscope images.

Keywords: Crystallinity, gibberellin, intermittent bending, nitrogen fertilization, poplar, tension wood.

INTRODUCTION

Nowadays, wood is considered as a vital material and one of the important raw materials of industries in countries where a small area is covered with forests (including Iran). Therefore, there is a need to better understand of the relationships between growth conditions, production physiology, wood production and biomass production, and wood characteristics to predict their effects on end-use properties and fiber production. Wood contains carbon-rich polymers such as cellulose, hemicellulose and lignin. Most of the carbon produced by photosynthesis in the tree is stored and stabilized in wood cells (Mellerowicz *et al.* 2001). Wood is used as a lignocellulosic raw material in the production of various products, but to compensate for the lack of this God-given gift, requires more planning and attention. Poplar plantation is one of the practical solutions to compensate for the lack of lignocellulosic resources. Poplar genus belongs to the willow family and has 40 species including *Populus alba*, *Populus nigra*, *Populus euphratica*, *Populus tremola*, *Populus euramericana*, *Populus deltoides* and other hybrid species. Poplar is one of the most widely used species in the papermaking industry and the need to produce its wood by plantation and, study of the factors that affect the of fibers biometrics, anatomical, physical and chemical properties of its wood is very important. The trees in natural forests and plantations are under different environmental conditions and many environmental factors stress the trees and affect their growth. Reaction wood is one of the responses of woody trees such as poplar to stresses and environmental factors. Reaction wood formed in broadleaves is called tension wood. Tension wood is formed on the upper side of the inclined trunk, leading to asymmetric growth characteristics and is used to direct the trunk in the correct position (Hellgren *et al.* 2004). In addition to a significant increase in cambium cell divisions, the tension wood has modified anatomy, and the fibers form an extra cellulose-rich inner gelatinous secondary cell wall layer (Andersson-Gunneras *et al.* 2006). Some researchers have considered the term of third cell wall for gelatinous layers formed in tension wood, arguing that 1) the gelatinous layer develops after the primary and secondary walls 2) and has a distinct composition, structure, and physical properties 3) and its formation is regulated by a series of transcription factors that are different from primary and secondary cell wall transcription factors (Clair *et al.* 2018, Gorshkova *et al.* 2012, Gorshkova *et al.* 2018). Although some studies have shown that in some species of tropical rainforest, the gelatinous layer is not formed in the tension wood (Clair *et al.* 2006), but further studies have shown that the gelatinous layer is formed in some of these species, but it is difficult to identify in mature wood where it is covered with final lignification (Roussel and Clair 2015, Ghislain and Clair 2017, Higaki *et al.* 2017). Tension wood is a rich source of cellulose that can be produced by planting trees with more tension wood, having more cellulose for many uses of it, including papermaking or biofuels. Due to the special applications of tension wood, which is due to its high content of cellulose, it can be used as a target product in forestry and increase its production during the formation of wood in the tree by stimulating the formation of tension wood. In poplar, tension wood is produced by asymmetric growth with accelerated cell division in the cambium region and a decrease in the number of vessel elements, and the production of special tension wood fibers containing the gelatinous cell wall layer (Jourez *et al.* 2001, Mellerowicz and Gorshakov 2012). The gelatinous layer in poplar mainly consists of crystalline cellulose with microfibrils oriented parallel to the fiber axis (Fujita *et al.* 1974, Clair *et al.* 2011). The cellulose crystallinity index measurement using XRD and its effect on cellulose performance showed that this method is easiest, which is also the most widely used method, gives us only the size of two heights in X-ray diffractogram and significantly higher crystallinity values than other methods. XRD and NMR methods have also been shown to provide more accurate measurements of cellulose crystallinity than other methods (Park *et al.* 2010).

The different environmental stresses caused tension wood in trees. In plants, stress equals stimuli that upset the biological balance. Stress occurs when an environmental factor affects the plant beyond normal. In general, more than one factor may be involved in the initial stimuli of reaction wood formation. Compression and tension stresses are the main stimuli. Most broadleaf trees respond to environmental forces, including bending due to gravity, wind, and snow, by producing special tension wood fibers to strengthen and change the direction of the lignified part of trunks and branches (Ruelle *et al.* 2009, Groover 2016). Also, nitrogen fertilizer has a significant effect on the quantity and quality of growth due to its participation in the construction of different

parts of plant cells. The quality of growth depends to some extent on the quality of structural changes, shape and physical and chemical properties of tissues

Therefore, it is necessary to study the effect of fertilizer on the growth changes of seedlings, especially changes in the chemical composition of tissues and cells, to use wood in the production of tension wood. Nitrogen fertilization has been shown to affect wood structure in poplar, for example, reducing wood density and cell wall thickness and, in some genotypes, increasing the proportion of tension wood (Luo *et al.* 2005). Plant hormones play an important role in controlling the activity of cambium and the derivation of cells from it in woody plants. Other internal factors influencing the growth of trees are hormones and enzymes. Hormones such as gibberellins are involved in the formation of tension wood and gravitational stress (Funada *et al.* 2008). This study was aimed to apply intermittent bending, NPK fertilizer (nitrogen, phosphate and potassium) and Gibberellin hormone to stimulate the formation of tension wood and, measuring the changes of crystallinity and chemical variations in two-year-old poplar. The hypothesis of this research is based that intermittent bending treatment can induce greater stress and cause more impact on the chemical properties of poplar wood.

MATERIALS AND METHODS

Plant materials and treatments

From a plant nursery, 24 healthy *Populus alba* seedlings have been selected and planted in research field of University of Tehran in Karaj. The seedlings were two-year-old. The height and diameter of the seedlings were measured. By applying bending treatment to some seedlings, they were divided into two groups of straight seedlings and bent seedlings and in each group, 4 treatments were applied: without chemicals, NPK fertilizer, Gibberellin GA3 and NPK+G. The direction of 45° bending treatment was changed alternately every month during the growing season. The NPK fertilizer (NPK: 30-5-5, Hortiland, s'Gravenzande, Netherland, 300 mL solution of NPK 40 g/L) treatment was applied twice at 1 May and 1 August 2017 and gibberellin hormone (GA3, Merck Chemicals industries, Darmstadt, Germany, 300 mL solution of gibberellin 500 mg/L) treatment was applied once 1 May 2017 during of the growing season to the soil around the seedlings. Straight seedlings without any treatments considered as control samples.

Sampling

Seedlings were cut at the end of growing season (30th of November, 2017) and then we measured their height and width. Wood cubes were cut from middle part of tree stem height and then these samples were debarked and used for measuring wood chemical composition and crystallinity. Because in the intermittent bending treatment, the seedlings were bent once in one direction and the next month in the opposite direction, the lower side and upper side were not fixed in each seedling. Therefore, sampling of tension wood in the whole growth ring was performed.

Wood chemical composition measurements

Measurement of cellulose and lignin content from tension wood regions of treated samples and the normal wood regions of control ones were prepared according to standard number TAPPI T264-cm-07 (2007) and TAPPI T222-om-15 (2015) regulation respectively. Also, chemical analysis of wood sawdust and wood cross sections samples with 30-micron thickness from tension wood regions of treated samples and the normal wood regions of control ones were performed by ALPHA Platinum ATR-FTIR made by Bruker company, Germany and the values of the obtained peaks were analyzed with OPUS software and finally the approximate ratio of cellulose to lignin was calculated (BRUKER, USA). The measurements were done from 3 position of each sample. After obtaining the spectra, the amounts of peaks related to the chemical composition of wood were measured using OPUS software, which is specific to the analysis of chemical spectra. ATR-FTIR peaks have been proposed for various chemical components of poplar wood (Chang *et al.* 2014). Peaks of 1505 cm⁻¹ and 1460 cm⁻¹ were considered for lignin and peaks of 900 cm⁻¹, 1160 cm⁻¹, 1315 cm⁻¹, 1370 cm⁻¹ and 1425 cm⁻¹ for cellulose.

Crystallinity measurements using X-ray diffraction (XRD)

The crystalline structure was investigated with the aid of the X-ray diffraction measurements (Bruker D8 advance Diffractometer using CuK α radiation with wavelength of 1,54016 Å). Data were collected over the range $2\theta = 10 - 800$ with a step interval of 0,01 °C at 25 °C temperature. Sawdust samples from tension wood regions of treated samples and the normal wood regions of control ones in 3 replicates were used to XRD measurements

Sections preparation and light microscopy

The stem cross-section specimens (30 microns in thickness) were cut using a rotary microtome (Histoline MRS 3500) from poplar stem and were double stained with 1:1 safranin /astrablue (Gartner and Schweingruber 2013). The stained micro-sections were studied using Olympus light microscope and taken images were used for measuring the anatomical properties using Image-J Software. Double staining with safranin /astrablue was used to demonstrate the presence of a G-layer in fibers. Safranin stains lignified tissues red, and astrablue stains unlignified cell walls blue. Lignified tissues were red mixed while unlignified cellulosic cell wall layers, and gelatinous layer, became blue in color.

Statistical analysis

The results obtained in this study were performed by SPSS Statistics Developer 21.0 (IBM, USA) software in a completely randomized design with 95 % confidence level for three biological replicates of each treatment.

RESULTS AND DISCUSSION

According to the results, significance of Levene's Statistics was 0,022 (sig<0,05) in C/L Ratio sawdust, so the test of the comparison of means with the assumption of homogeneity of variances was not performed whose results were shown in Table 2. The significance between the treatment groups was 0,681 (sig>0,05), so there existed no significant difference between groups. The significances of Levene's Statistic were higher than 0,05 (sig>0,05) in other tests (Table 1), thus the test of comparison of means with the assumption of homogeneity of variances was performed. The significances between groups on other tests were lower than 0,05 (sig<0,05), so there existed a significant difference between groups (Table 2); therefore, the hypothesis of homogeneity of means at 0,05 level was rejected, i.e., at least two groups out of the eight participating groups in the present study had different means.

Table 1: Test of homogeneity of variances.

	Levene Statistic	df1	df2	Sig.
Cellulose Content	1,694	7	16	0,181
Lignin Content	0,778	7	16	0,614
Hemicellulose Content	1,030	7	16	0,448
C/L Ratio (chemicals)	1,443	7	16	0,256
C/L Ratio sections	0,638	7	16	0,719
C/L Ratio sawdust	3,334	7	16	0,022
Crystallinity	0,967	7	16	0,487

Table 2: Results of ANOVA test.

		Sum of Squares	df	Mean Square	F	Sig.
Cellulose Content	Between Groups	58,366	7	8,338	35,544	0
	Within Groups	3,753	16	0,235		
	Total	62,120	23			
Lignin Content	Between Groups	38,530	7	5,504	11,680	0
	Within Groups	7,540	16	0,471		
	Total	46,070	23			
Hemicellulose Content	Between Groups	25,233	7	3,605	14,044	0
	Within Groups	4,107	16	0,257		
	Total	29,340	23			
C/L Ratio (chemicals)	Between Groups	0,837	7	0,120	25,117	0
	Within Groups	0,076	16	0,005		
	Total	0,913	23			
C/L Ratio (sections)	Between Groups	2,788	7	0,398	125,947	0
	Within Groups	00,051	16	0,003		
	Total	2,839	23			
C/L Ratio (sawdust)	Between Groups	0,131	7	0,019	0,688	0,681
	Within Groups	0,434	16	0,027		
	Total	0,564	23			
Crystallinity	Between Groups	48,076	7	6,868	24,899	0
	Within Groups	4,413	16	0,276		
	Total	52,490	23			

Chemicals measurements

The results of the chemical analysis of wood samples obtained from different treatments by the TAPPI standard method are presented in Figure 1.

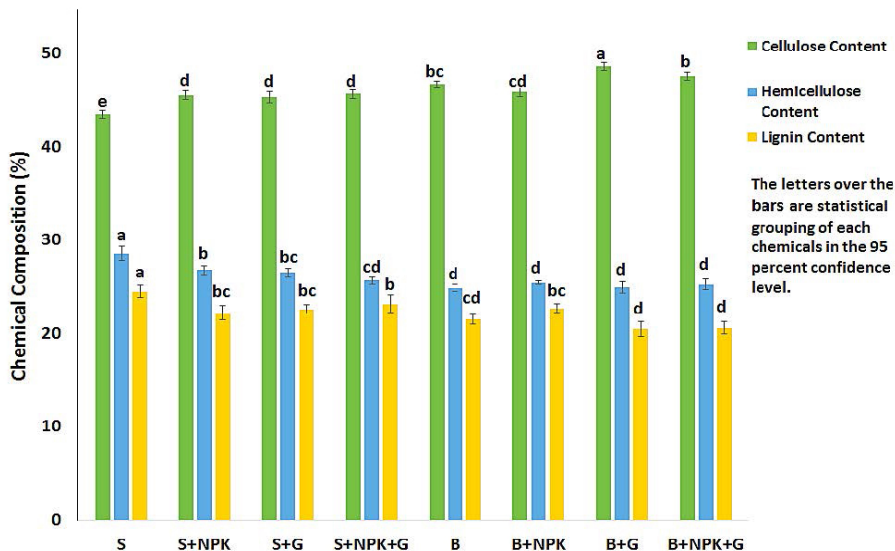


Figure 1: Wood chemical composition (green: Cellulose Content; blue: Hemicellulose Content; yellow: Lignin Content) of poplar (*Populus alba*) seedlings (*S*: straight seedlings; *B*: bent seedlings; *NPK*: N-Extra fertilization; *G*: Gibberellin hormone GA3).

The average content of cellulose in different treatments was higher than the control sample. In general,

different treatments of bent seedlings had a higher content of cellulose, lower content of hemicellulose and lower lignin than different straight treatments. The highest content of cellulose is related to intermittent bending + GA3 and intermittent bending + NPK + GA3 treatments with 48,6 % and 47,6 %, respectively. Application of GA3 in straight seedlings had almost the same effect as NPK fertilizer in increasing cellulose and decreasing lignin and hemicelluloses, but in bent seedlings, application of GA3 resulted in the highest content of cellulose and the lowest content of lignin and hemicellulose. Application of NPK fertilizer to straight seedlings increased the content of cellulose and decreased the content of hemicellulose and lignin compared to the control sample. Studies by Pitre *et al.* (2007a) on poplar seedlings showed that NPK fertilization increased the content of cellulose and decreased hemicelluloses and lignin. Novaes *et al.* (2009) reported an increase in cellulose and hemicelluloses and a decrease in lignin by applying NPK to poplar seedlings. Villegas *et al.* (2014) also observed an increase in cellulose and a decrease in lignin by applying NPK to poplar seedlings.

Figure 2 shows the ATR-FTIR spectra analyzed using OPUS software. Each peak in the spectrum represents a chemical composition present in the wood (Olsson *et al.* 2011, Chang *et al.* 2014). By measuring the area under each peak, the value of the associated chemical composition can be obtained.

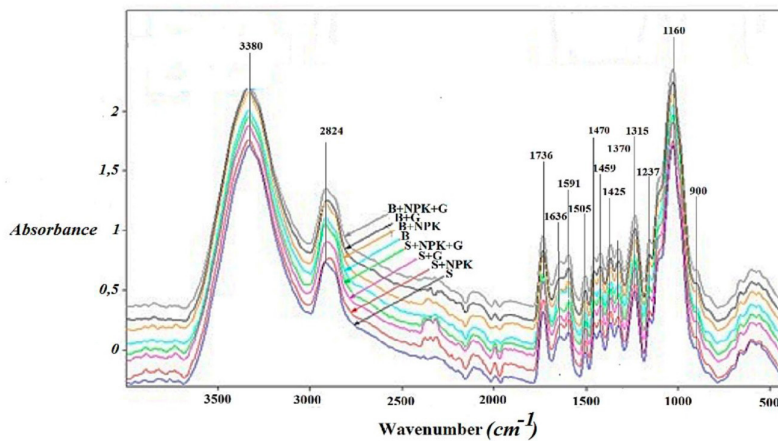


Figure 2: Spectra of different treatments of poplar wood (*Populus alba*) chemicals using ATR-FTIR.

The ratio of cellulose to lignin of different treatments was shown Figure 3. It can be seen that the ratio of cellulose to lignin obtained from chemical analysis data is higher in the treated samples than the control sample and almost all the intermittent bent samples showed a higher increase than the straight samples compare to control samples. The results showed that in both cross-sectional samples and wood sawdust samples, the ratio of cellulose to lignin in the treated samples was higher than the control samples but the differences in sawdust ratios were not significant. Straight treatment + NPK + GA3 in wood sawdust samples and intermittent bending treatment in cross-sectional samples with 2,15 and 3,69 showed the highest cellulose to lignin ratio, respectively. These results are slightly different from the ratios obtained from the measurement of wood compounds by chemical methods, but in both chemical analysis and ATR-FTIR method the treated samples showed higher cellulose to lignin ratio than the control sample. These observations show the effect of bending and NPK fertilizer treatment more strongly and also GA3 treatment on reducing lignin content and increasing cellulose and stimulating the formation of tension wood. Pitre *et al.* 2007b and Pitre *et al.* 2010 obtained similar results in relation to bending and NPK fertilizer treatment.

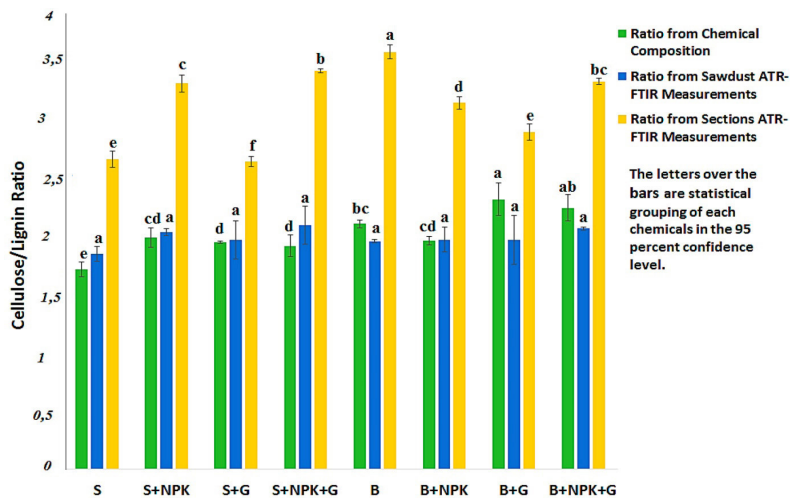


Figure 3: Cellulose/Lignin ratio of poplar wood (*Populus alba*) using chemical composition results and FT-IR measurements (*S*: straight seedlings; *B*: bent seedlings; *NPK*: N-Extra fertilization; *G*: Gibberellin hormone GA3).

Crystallinity measurements

Figure 4 shows the spectra obtained from the XRD device data for measuring the degree of crystallinity. As shown in Figure 5, the degree of crystallinity in the treated samples increased compared to the control sample, but this increase was not significant in different treatments of straight seedlings. Also, the application of NPK fertilizer to straight seedlings increased the degree of crystallinity. The degree of crystallinity in all treated samples increased compared to the control sample, but NPK fertilizer in seedlings under intermittent bending led to a decrease in the degree of crystallinity compared to the only bent seedlings. Bending resulted in a significant increase in the degree of crystallinity. GA3 increased the degree of crystallinity to a lesser extent in straight seedlings and to a greater extent in intermittent bending seedlings. The results did not correspond to the results of the study of *Eucalyptus* by Washusen *et al.* (2001). A recent study noted that the production of tension wood may be significantly reduced where fertilization is carried out. This may be because the increase in diagonal growth in response to fertilizer application leads to the stability of the tree trunk and the mechanism by which trees cope with the internal stresses generated by the wind.

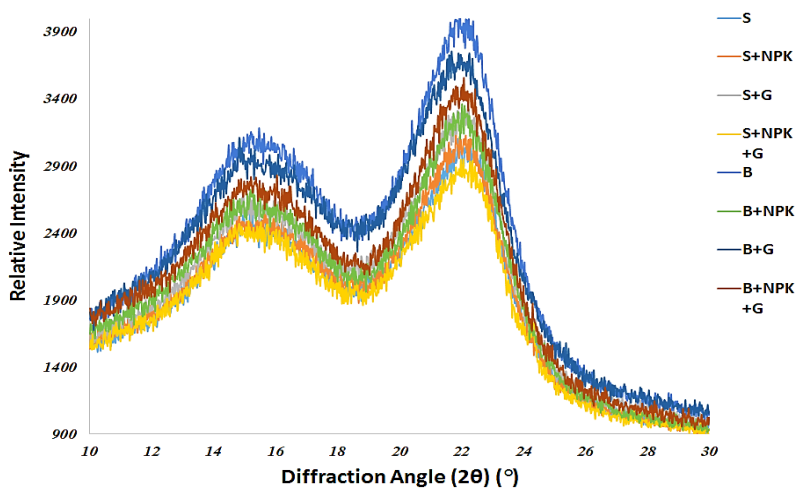


Figure 4: Crystallinity XRD diffraction patterns of poplar (*Populus alba*) wood samples (*S*: straight seedlings; *B*: bent seedlings; *NPK*: N-Extra fertilization; *G*: Gibberellin hormone GA3).

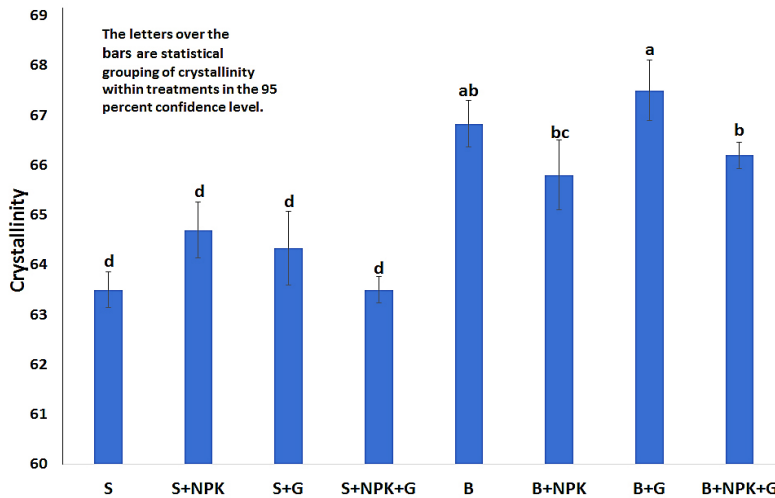


Figure 5: Crystallinity of poplar wood (*Populus alba*) using XRD measurements (S: straight seedlings; B: bent seedlings; NPK: N-Extra fertilization; G: Gibberellin hormone GA3).

In the present study, the application of NPK fertilizer led to increased crystallinity and formation of tension wood with gelatinous fibers, and due to environmental conditions and summer high temperature, the tree may have used all methods to cope with stresses. Bending led to a significant increase in the degree of crystallinity, which was consistent with the results of Foston *et al.* (2011) and Sawada *et al.* (2018). NPK fertilizer in intermittent bent seedlings reduced the degree of crystallinity compared to the just bent condition. The structural parameters of cellulose such as cellulose crystallinity, matrix-cellulose masses, microfibrils and cellulose crystallites show changes in tension wood. Although the effect of these changes on the properties of tension wood remains unclear, some researchers have been reported, the size of cellulose crystallite is in larger tension wood (Ruelle *et al.* 2009). The effect of GA3 on changes in crystallinity has been less studied, but in the present study, GA3 increased the degree of crystallinity to a lesser extent in straight seedlings and a greater extent in intermittent bending seedlings. This may also be due to the formation of tension wood with gelatinous fibers as well as an increase in the cellulose content of samples subjected to GA3.

Tension wood and gelatinous layer formation

Bending treatments in many cases lead to the eccentricity and asymmetry of a growth ring (Clair *et al.* 2006, Clair *et al.* 2011, Hellgren *et al.* 2004). However, in this study, the micro-cross-sections observation of the specimens showed that the alternate bending during the growth season prevented the eccentricity and asymmetry of the growth rings despite the formation of tension woods and gelatinous fibers (Figure 6e, Figure 6f, Figure 6g, Figure 6h).

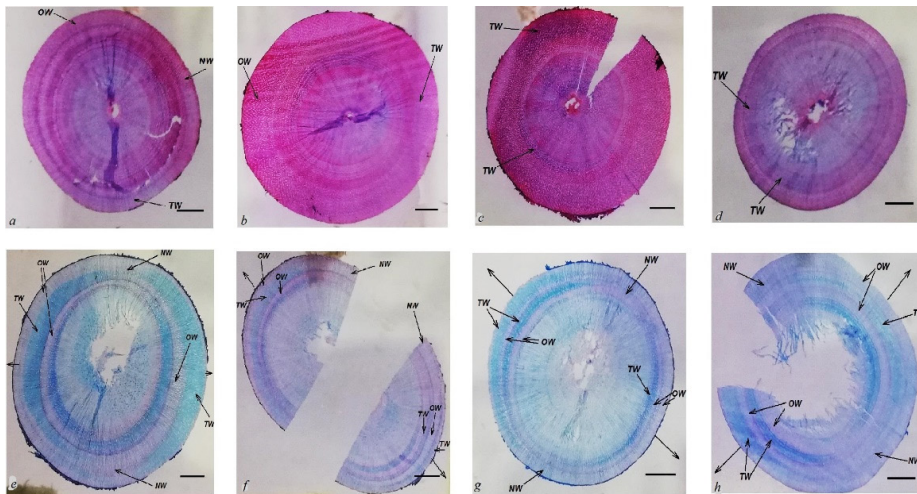


Figure 6: Cross-sections of specimens; Gorgij *et al.* 2022 (a: straight control seedling; b: S+NPK; c: S+G; d: S+NPK+G; e: bent control seedlings; f: B+NPK; g: B+G; h: B+NPK+G; TW: tension wood; NW: normal wood; OW: opposite wood).

The gelatinous layers are visible in the innermost part of the bent seedling fibers cell wall, while the control seedlings did not form the gelatinous layer. Further differences are observed showing a much thicker cell wall with G-layer formation in the TW sample. Based on these images, the gelatinous layer in the cell wall and cellulosic material content seems to be significantly increased in the tension wood. For tension wood, an increase in the availability of cellulose at the plant cell level has been proposed to be beneficial for enzymatic deconstruction (Foston *et al.* 2011).

CONCLUSIONS

These results indicated that this intermittent bending treatment, especially in the case with NPK fertilizer and GA3 application, had the greatest effect on the stimulation of tension wood and the intensity of tension wood formation was higher in these samples. It can be concluded that intermittent bending treatment, due to the possibility of high stress in the tree, has forced it to show the greatest response, especially in terms of changes in anatomical and chemical properties. NPK fertilizer and GA3 also had significant reactions in the mentioned cases, especially with intermittent bending treatment. In addition, NPK fertilization increased the diameter and height of the tree, which can be concluded that it is one of the biomechanical methods of the tree to cope with stress and increase its strength. According to the results, the hypotheses of this study are confirmed that the intermittent bending treatment could show the greatest effect on anatomical and chemical changes as well as the formation of tension wood. Both straight and artificially intermitted bent seedlings underwent changes in these characteristics.

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