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EFFECT OF 2,6-DICHLOROBENZONITRILE (DCB) ON SECONDARY WALL DEPOSITION AND LIGNIFICATION IN THE STEM OF *HIBISCUS CANNABINUS* L.

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Abstract: Light and electron microscopic studies were carried out on the secondary xylem of actively growing shoots of *Hibiscus cannabinus* treated with cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB). Treatment with 20μM DCB induced differentiation of xylem fibres with thin secondary walls and parenchyma cells with abnormal wall thickening and lignification. At concentration above 50 μM resulted in the disappearance of cambial zone, inhibition of secondary wall deposition, lignification of primary walls, deformed vessel walls and dispersed lignin distribution in secondary walls. Transmission electron microscopic study revealed the initiation and formation of large intercellular spaces between the walls of differentiating xylem elements. Abnormal pattern of wall deposition and inhomogeneous lignin distribution was evident in fibres and vessel. The length and width of both fibres and vessel elements were reduced significantly even with lower concentrations of DCB.

Keywords: 2,6-dichlorobenzonitrile, secondary wall deposition, lignification, *Hibiscus cannabinus.*

Introduction

Dichlobenil is an herbicide commonly used to control weeds in gardens, lawns, near ornamental trees etc. It is a well-known inhibitor of seed germination and growth in plant roots and shoots. The growth inhibition in actively growing plants has been demonstrated to be mainly through hindering cellulose biosynthesis which affects the cell wall formation. In most of the studies, cell culture method has been adopted to elucidate the DCB effect on plant growth. However, the effect of DCB on cell wall formation in mature intact plants is not yet studied in detail. Since wood formation represent one of most complex system of cell wall formation which has directly related to cellulose biosynthesis, present study aimed to study the effect of DCB on secondary wall deposition during wood formation in *Hibiscus cannabinus*.

The presence of a rigid cell wall is a characteristic feature of plant cells which determines the size and shape of the cell and its structure directly related to cell function [EVERT, 2006]. The wall is secreted and assembled as a complex structure in a rhythmic manner. Therefore, the secondary growth of woody stem is considered as a dynamic process that integrates multiple developmental mechanisms [GROOVER & ROBISCHON, 2006]. Structural models have been proposed for the cell wall that emphasizes a defined organization of cellulose fibrils, hemicellulose, pectins and random intervening of cell wall polymers such as lignin [ALBETSHEIM & al. 1973]. Cellulose microfibrils are synthesized by cellulose synthesizing complexes embedded in the plasma membrane [DELMOR & ARMOR, 1995], whereas other polysaccharides of cell wall matrix are assembled in the Golgi apparatus and transported in secretary vesicles to the cell surface [DRIOUICH & al.

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1993; ISABELLAE & al. 2001]. From the developmental point of view, cellulose is considered as the principal component of plant cell wall and cellulose microfibril deposition has been controlled by cortical microtubules [BASKIN, 2005]. It is widely believed that the extent of primary wall radial expansion in cambial derivatives controls how much secondary wall deposition and lignification occurs subsequently [SAVIDGE, 2000]. Many studies have been carried out to understand the role of cellulose in development of cell wall through the inhibition of cellulose biosynthesis using 2, 6-Dichlorobenzonitrile (DCB). DCB is demonstrated as inhibitor of many processes involved in cell wall development such as formation of cellulose microfibrils [MIZUTA & BROWN, 1992], cell plate formation [BURON & GARCIA-HERDUGO, 1983; VOUGHN & al. 1996] and regeneration of cell walls of protoplast [ARAD & al. 1994] and cell cycle progression [ALVIN & JOSEPH, 2003]. The first indication for the existence of feedback mechanism regulating the polysaccharide composition of cell walls came from the study of tomato and tobacco cells adapted to DCB. There was a drastic reduction of cellulose and significant enrichment in pectin [SHEDLETSKY & al. 1992]. In contrast, cell walls from DCB adapted monocot cells did not show increased pectin contents, but normal to elevated amount of other non-cellulosic materials [SHEDLETSKY & al. 1992] showing fundamental difference between dicot and monocot cell walls, including the way they compensate for the reduction of their cellulose content. Though much information is available on the role of DCB on development and biochemistry of cell wall formation particularly from cell culture experiments, it is not know how the alternations in cellulose biosynthesis affect secondary wall structure and histochemistry in woody stems. Therefore, in the present study, we examine the effect of cellulose biosynthesis inhibitor DCB on secondary growth and cell wall structure of *Hibiscus cannabinus* (Kenaf), an important fibre yielding plant belongs to Malvaceae.

Materials and methods

Plant Materials. Kenaf, a fibrous plant native to east-central Africa, belongs to family Malvaceae. It is a common wild plant of tropical and subtropical Africa and Asia. It has been a source of textile fibre for products such as rope, twins, bagging etc. As a promising source of raw material for pulp, paper and other fibre products, Kenaf has been introduced and cultivated in several countries like China, Russia, Thailand, South Africa, Egypt, Mexico and Cuba. It is woody to herbaceous annual plant, mostly unbranched, fast growing with prickly stem. It was selected for the present study as it is grows rapidly with a straight stem having distinct and sufficient amount of secondary vascular tissue. Four month old plants having 4-5 feet height growing in the Botanical Garden of Sardar Patel University were used for the experiments.

Preparation of DCB solution. A stock solution of DCB (Fluka, Gemany) was prepared in DMSO. A final experimental concentration of 20, 50, 80 and 100 µM DCB prepared by dissolving stock solution in DMSO.

Treatments. DCB treatment was carried out at the 10^{th} to 11^{th} internodes of the main stem where secondary growth was prominent. A set of 6 plants were used for each treatment. To the cut end of stem, microtip was fixed by using stripe of parafilm. Then it was filled with DCB solution through the pointed tip using a syringe. For controls, microtip was filled with DMSO for one set and distilled water for another set of plants. At an interval of every 4 days, microtip was replaced after removing 2-3 mm tissue from the cut surface and the DCB solution was applied. Segments of stems measuring 3 cm below the

site of application were collected and fixed in 3% glutaraldehyde in phosphate buffer (*p*H 7.4) after 15 days of treatment.

Sectioning and staining. Samples were subjected to hand sectioning using double edge razor blades. The sections were stained with 0.05% Toluidine blue 'O' (Sigma, Germany) for general histology [BERLYN & MIKSCHE, 1976], Phloroglucinol (Sigma, Germany)/HCl method for lignin localization [GAHAN, 1984], 0.02% aqueous Ruthenium red (Sigma, Germany) for pectic polysaccharides [JOHANSEN, 1940] and 1% Congo red for cellulose [PRAT, 1993].

Measurements. The length and width of vessel elements and fibres were measured with an ocular micrometer scale mounted in a research microscope. Ray dimension and density were measured from tangential longitudinal sections using ocular micrometer scales, 1 mm and 1 mm^2 respectively. The fibre wall thickness and vessel density were also recorded from transverse sections using ocular micrometer scales. For each parameter 100 reading were taken from randomly selected elements from six replicants. Student t-test was carried out to determine statistically significant differences of anatomical parameters at a 0.05 confidence level using Sigmastat software (Version 3.5, San Jose, CA, USA).

Electron microscopy. For ultrastructural studies the samples were immediately fixed in 2.5% glutaraldehyde in phosphate buffer (*p*H 7.2) followed by 2% osmium tetroxide. After the routine dehydration and infiltration the samples were embedded in Spurr's resin [SPURR, 1969]. Semithin sections on glass slides were stained in 1% toluidin blue and photographed using a Zeiss microscope with Carl Zeiss (KS 300) Image Analyzer. For transmission electron microscopy (TEM), ultrathin sections on nickel and gold grids were subjected to potassium permanganate and periodic acid- thiocarbohydrazide-silver proteinate (PATAg) [THIERY, 1967] staining respectively and observed under TEM (Philips, Tecnai). Micrographs were taken using Keenview soft Imaging system.

Results

Control plants treated with either distilled water or DMSO showed as active cambium with 4-6 layers of differentiating xylem elements (Fig. 1A). Towards phloem, along with sieve elements, differentiation of parenchyma and phloem fibres were noticed. Phloem fibre bundles showed 2-3 groups of cells undergoing secondary wall deposition and were rich in crystalline polysaccharides (Fig. 1C). Centripetally cambial zone was followed by 3-4 layers of cells showing secondary wall deposition (Fig. 1C). Cell walls of majority of vessels completed secondary wall deposition and underwent lignification. The vessels were round to oval in shape in control plants (Fig. 1D). Acidic polysaccharides were observed in the walls of cambial cells, dividing and differentiating elements and its distribution was more prominent towards the cell corners. DCB treatment, even in low concentration resulted in changes in the structure of cells in the cambial zone and its derivatives towards xylem and phloem. At a concentration of 20 µM, cambial cell layers reduced to 1-2 and the cells close to cambial zone showed elongation of radial walls. Cambial derivatives towards phloem were characterized by the absence of phloem fibres and abnormal wall thickening and lignification of parenchyma cells (Fig. 1E). The phloroglucinol-HCl reaction of xylem derivatives showed fibres with thin lignified secondary walls underwent lignification. Vessel walls became deformed and wavy (Fig. 1E). Treatment with 50, 80 and 100 µM DCB resulted in disappearance of cambial initials by elongation of their radial walls followed by differentiation into parenchyma cells (Fig. 1F). Parenchyma cells with primary wall showed lignification. In those cells which showed a thin secondary wall, inhomogeneous lignin distribution was noticed (Fig. 1G).

Lignin distribution was more in the cell corners and primary wall whereas secondary wall showed dispersed pattern of lignin distribution. Thin walled vessels underwent lignification without much expansion (Fig. 1G). In deformed vessels, the walls bulged into the lumen (Fig. 1H). At a concentration of 80 and 100 µm thin walled xylem fibres showed large intercellular spaces (Fig. 1I).

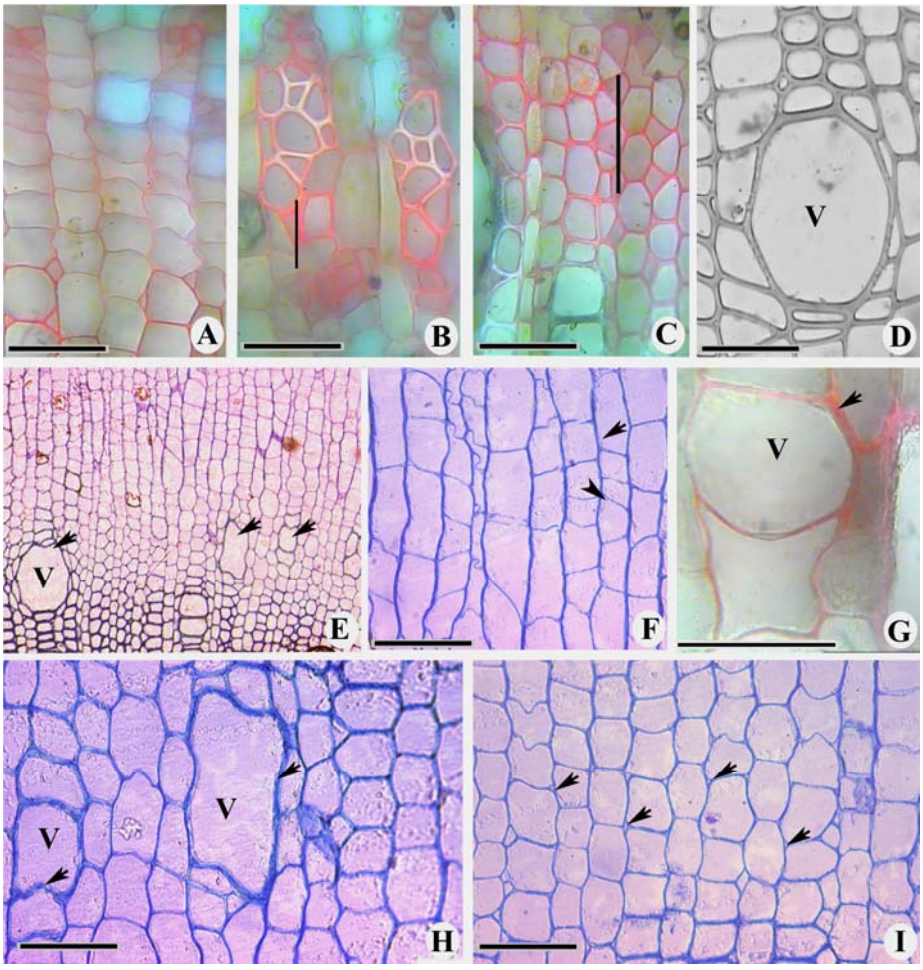

Fig. 1. (A-J) Transverse sections of stems of Kenaf treated with distilled water and DMSO (A-D) and with DCB (E-I): **A.** Cambial zone showing initial cells and differentiating elements. **B.** Phloem fibre bundles phloem fibres undergoing secondary wall deposition (vertical line). **C.** Xylem derivatives showing secondary wall deposition (vertical line) in the fibres. **D.** Stem showing thick walled vessel with a large lumen (V). **E.** cambial zone and differentiating xylem showing elongation of radial walls and deformed vessels (arrows) in the 50µm DCB treated stem. **F.** Cambial zone of 80 µm DCB treated stem showing oblique divisions (arrow head) and expansion of radial walls (arrow). **G.** Stem treated with 50 µm DCB showing partially lignified, thin secondary walls of vessels. **H.** Deformed vessel (V) in the 50 µm DCB treated stems showing poorly lignified wavy wall bulged into the lumen (arrow). **I.** Xylem derivatives of 80 µm DCB treated stem showing cells with intercellular spaces (arrows). Scale Bar = 50 µm

Transmission electron microscopic study revealed intact cell wall with relatively more lignin distribution in middle lamellae and S3 wall layers of fibres in control plants (Fig. 2A). Fibres in the DCB treated plants showed cell wall separation between adjacent cells and formation of large inter cellular spaces among differentiating xylem derivatives. Cell wall separation initiates at the cell corner middle lamellae and extends along the radial walls (Fig. 2B*).* Inhomogeneous distribution of lignin was observed in the middle lamellae region of fibres during early stages of lignification (Fig. 2C). The deformed vessel walls showed abnormal pattern of secondary wall deposition and lignification (Fig. 2D). The S1 layer became thicker and became more or less similar thickness of S2 layer while S3 layer remain thinner among three layers. The S2 layer showed more lignin distribution than S1 and S3 layer. This shows that the rhythm of secondary wall deposition and lignification were altered following DCB treatment.

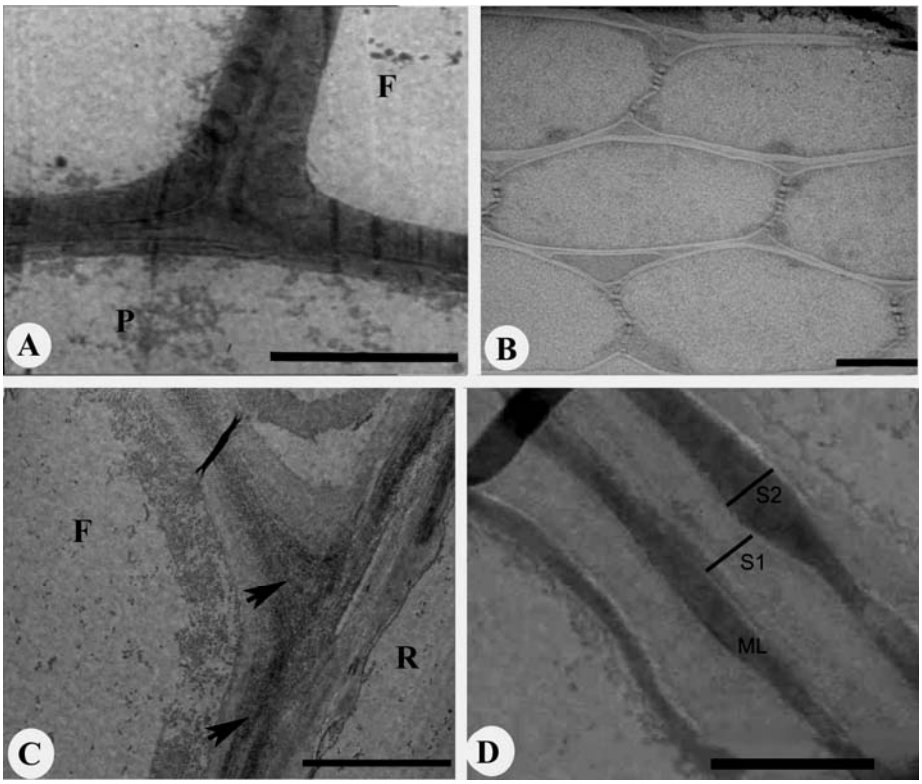

Fig. 2. (A-D) Transmission electron microscopic images from the secondary xylem of 50µm DCB treated stems of Kenaf showing intercellular space formation (A, B) and lignin distribution in xylem fibre wall (C) and deformed vessel wall (D).
A. The fibre and axial parenchyma of control stem showing the lignification pattern in cell wall ($KMnO_4$ staining) Bar = 5 µm. **B.** Radially elongated xylem derivatives showing separation of walls at cell corners (PATAg Staining) Bar = 2 µm. **C.** Fibre wall showing inhomogeneous lignin distribution during its early lignification stage ($KMnO_4$ staining) Bar = 2 µm. **D.** A magnified view of deformed vessel wall showing secondary wall layers and pattern of lignin distribution ($KMnO_4$ staining) Bar = 4 µm

Dimensions of fibre and vessel elements

There was no significant difference observed in fibre and vessel elements dimensions between plants treated with distilled water and DMSO (Tab. 1). DCB treated plants showed significant reduction in fibre length and width. Fibres became shorter and narrow with increasing concentration of DCB (Tab. 1).

DCB treatment also resulted in the reduction in length and width of vessel element. Vessel elements exhibited considerable variations in their dimensions from 20 µM concentration of DCB treatment and they became shorter and narrow with higher concentrations of DCB (Tab. 1).

Tab. 1. Dimensional characteristics of vessel element and fibres in control and DCB treated plants of Kenaf

Characte-ristics	Control				DCB Treatment							
	DW		DMSO		20µm		50µm		80µm		100µm	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fibre dimensions												
Length	1170	140	1192	205	1014	121	1008	114	1003	153	971	219
Width	26	4	26	4	25	3	24.5	3.4	24.2	3.2	21	3.6
Vessel element dimensions												
Length	617	105	612	117	499	129	469	128	462	95	402	150
Width	93.5	16	92	12	77	13	74	14	70.6	13.7	68	10.7

Discussion

Biosynthesis and deposition of cell wall polysaccharides are believed to be the fundamental building blocks for development of plant cells. The present study reveals that the inhibition of cellulose biosynthesis by DCB has significant impact on secondary growth by altering the activity and cell wall structure of cambial cells. The division and differentiation of vascular cambial cells are the key factors determining secondary growth in higher plants [EVERT, 2006]. The expansion of radial walls and abnormally oriented tangential walls in the cambial zone cells indicates that the deposition of cellulose microfibrils has been altered after DCB treatment. Primary cell walls of plants are known to be composed of α-cellulose with high degree of crystallinity and microfibrils that are parallel to the elongation axis which determines the characteristic shape of the cell [KATAOKA & KONDO, 1999]. The orientation and crystallinity of cellulose microfibrils may have been changed by the action of DCB. There are various reports on the effect of DCB on microtubules and orientation of cellulose microfibrils. The microtubules are proposed to be guide the deposition of the cellulose synthase complex in the plasma membrane during secondary wall synthesis [GARDINER & al. 2003; WIGHTMAN & TURNOR, 2008; RAJANGAN & al. 2008]. On the other hand, HIMELSPACH (2003) reported that, DCB can deviate and disrupts microtubule orientation in root tips; however, the cellulose synthesizing machinery can re-establish well-ordered microfibril deposition indicating a self-assembly mechanism that have little reliance on cortical microtubule orientation. The altered cellulose biosynthesis by DCB or by genetic modification [PAGANT & al. 2002; SUGIMOTO & al. 2003] also shows that microfibril lose its parallel orientation indicates the importance of the certain rate of cellulose production possibly related to synthase complex density. In the presence of DCB, the primary wall cellulose synthase complexes (CSCs) appeared aggregates at sites within the plasma membrane and

sites may represent insertion site of CSC from intercellular compartment [DEBOLT & al. 2007]. The expansion and abnormal wall thickening of radial walls sites of phloem parenchyma can be such aggregation sites of CSCs.

The structural changes in the secondary wall of xylem elements are a further evidence for the inhibition of cellulose biosynthesis by DCB. Xylem fibre with thin secondary walls shows that cellulose deposition has been greatly reduced following DCB treatment. RAYMOND & al. (2009) reported that DCB has a significant impact on secondary wall deposition by slowing down the movement of CSCs beneath the regions of secondary wall formation. Reduction of secondary wall formation has been followed by the early lignification of cell walls. Though there is a significant reduction in wall thickness, the fibre walls remain rigid after lignification. However, the vessel walls became wavy and deformed. The proportion of cell wall layers altered and S2 layer showed relatively more lignin deposition than S1 and S3 layers. Earlier reports have also shown that the cell walls from dichlobenil habituated cells possess a multi-lamellate structure lacking a proper middle lamellae and were thicker and more irregular [ENCINA & al. 2001, 2002; GARCIA-ANGYULO & al. 2009]. SABBA & al. (1999) reported that long term DCB habituation results in the replacement of normal cellulosic wall with one enriched in both unesterified and highly esterified pectins. The pectins in the habituated cells appear to be arranged in prominent strands or lamellae and occur across the entire expense of the wall giving multi-lamellate structure to the cell wall and are mainly composed of polygalactouronic acid [SABBA & al. 1999; ENCINA & al. 2001]. Therefore, the abnormal wall thickening and lignification pattern might be associated with the altered chemical composition induced by DCB. Compared to fibres, the process of secondary wall deposition and lignification is faster in vessel elements. Therefore, DCB induced inhibition of secondary wall deposition followed by early lignification of thin walled vessels which already underwent expansion results in destabilized walls with poor mechanical strength. Cellulose is considered as a principal component of plant cell walls as it plays dynamic role in maintaining cell size and shape through controlling cell expansion and elongation [BASKIN, 2005]. The present study also reveals a significant reduction in length and width of fibres and vessel elements treated with DCB. This confirms that there is a positive correlation exists between cellulose microfibrils and elongation and expansion of cells.

The present study demonstrates that cellulose inhibition may induce changes in composition of other cell wall polysaccharides and polyphenols. Earlier studies reported that there is a considerable increase in pectic polysaccharides in tobacco cells adapted to DCB [SHEDLEZSK & al. 1990]. On the other hand, there was no change in pectic polysaccharides in DCB adapted monocots [SHEDLEZSK & al. 1992]. Therefore, ISABELLA & al. (2001) suggested that there is fundamental difference that occurs between dicots and monocot cell walls in feedback mechanism following inhibition of cellulose biosynthesis. Our results also indicate altered chemical composition particularly in pectic polysaccharides resulting in the formation of wide intercellular spaces in differentiating xylem elements. This demonstrates a further complex relationship between biosynthesis of crystalline and acidic polysaccharides. In addition, the high lignification even in primary walls of cells in cambial zone and phloem shows that there may be a close association that exists between flux of carbohydrates for biosynthesis of cellulose and polyphenols. The increased carbohydrate reserves following inhibition of cellulose biosynthesis may have diverted to the phenolic pathway which leads to the lignification of cell walls. However, the inhomogeneous distribution of lignin in the cell walls indicates that the changes in the

orientation of cellulose microfibrils may affect the linkage of cellulose-lignin monomers within cell walls.

Conclusions

The present study suggests that inhibition of cellulose biosynthesis has a significant effect on secondary growth through changes in the cell wall structure, histochemistry, shape and dimensions of cells in the stem of DCB treated *Hibiscus cannabinus*. The major changes induced by DCB are decrease in secondary wall thickness, abnormal distribution of lignin in cell wall layers, formation of intercellular spaces among differentiating xylem and reduction in dimensions of vessel elements and fibres. The study demonstrates that DCB influences the structure of secondary xylem through its impact on distribution pattern of major cell wall polymers cellulose and lignin.

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