Gibberellin mediates the development of gelatinous fibres in the tension wood of inclined Acacia mangium seedlings

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INTRODUCTION

In response to environmental forces and to a gravitational stimulus, angiosperm trees form a special type of secondary xylem, known as tension wood, on the upper side of leaning stems. Tension wood generates a strong tensile force, which can pull a large inclined stem into the vertical or near vertical position, along the grain of the upper side of the living stem (Okuyama et al., 1994; Yoshida et al., 1999, 2000; Yamamoto et al., 2002; Clair et al., 2006, 2011; Ruell et al., 2006; Coutand et al., 2007).

A gravitational stimulus induces modifications in the anatomical structure of the wood of angiosperm trees, and tension wood is generally characterized by the formation of gelatinous fibres with a thick inner gelatinous layer with high cellulose and low lignin content (Wardrop, 1964; Timell, 1969; Barnett and Jeronimidis, 2003; Déjardin et al., 2010). The cellulose microfibrils in the gelatinous layer are oriented parallel or nearly parallel to the longitudinal axis of the fibres (Prodhan et al., 1995a, b; Funada et al., 2008). Not only does a gravitational stimulus generally induce the formation of gelatinous fibres but it also induces modifications in the anatomical characteristics of other elements of wood, such as modifications of the number and size of rays, vessels and fibres in Populus deltoides (Kaeiser and Boyce, 1965). In Populus euramericana ‘Ghoy’, a gravitational stimulus induces modification of the anatomical structures of all woody elements, namely wood fibres, vessel elements and ray parenchyma cells (Jourez et al., 2001). Eucalyptus gunii responds to a gravitational stimulus by the formation of gelatinous fibres and a reduction in the number of vessel elements in the region of the tension wood (Toghraie et al., 2006). The tension wood region had greater fibre wall thickness and wider vessel diameter compared with opposite wood in Eucalyptus globulus (Aguayo et al., 2010). In Hevea brasiliensis, the tension wood region had gelatinous fibres, narrower vessels, a lower vessel area percentage and a higher percentage of fibres (Mokugawa et al., 2008).

Plant hormones have been shown to be involved in the formation of tension wood and gravitropism (Little and Pharis, 1995; Mellerowicz et al., 2001; Barnett and Jeronimidis, 2003; Pilate et al., 2004; Kwon, 2008) and there is evidence that gibberellin plays an important role in the formation of tension wood (Nakamura et al., 1994; Baba et al., 1995; Yoshida et al., 1999; Du et al., 2004; Funada et al., 2008). In a previous study, we showed that the application of gibberellin stimulated the formation of tension wood and the negative gravitropism of stems of
tilted seedlings of *Acacia mangium* (Nugroho et al., 2012b). By contrast, the application of inhibitors of the biosynthesis of gibberellin inhibited the return to vertical growth and suppressed the formation of tension wood. Thus, it was apparent that gibberellin was required for induction of the formation of tension wood and, as a consequence, for the gravitropism of angiosperm trees.

The purpose of the present study was to clarify the role of gibberellin in the development of gelatinous fibres and the changes in the anatomical characteristics of woody elements that occur in response to a gravitational stimulus, since gibberellin is known to play an important role in differentiation of xylem (Aloni, 1992, 2007; Aloni et al., 2000). We applied gibberellin and two inhibitors of the biosynthesis of gibberellin, namely, paclobutrazol and uniconazole-P, to the soil in which *A. mangium* seedlings were growing. We then tilted the pots of seedlings at an angle of 45° to the vertical. Two months later, we examined the anatomical characteristics of woody elements (wood fibres, vessel elements and ray parenchyma cells) in the tension wood and opposite wood of tilted stems. We found clear evidence of a role for gibberellin in the formation of gelatinous fibres in tension wood.

**MATERIALS AND METHODS**

**Plant material**

Twenty approximately 1-year-old healthy seedlings of *Acacia mangium* that had been grown from seeds and had uniform features were used for experiments. The seedlings were approximately 70 cm tall and were planted in pots (diameter 20 cm) filled with regosol soil in a greenhouse at the nursery of the Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

**Treatment of seedlings**

We applied gibberellic acid (GA3; Wako Pure Chemical Industries, Osaka, Japan) and two inhibitors of gibberellin biosynthesis – paclobutrazol (Bountym®; Flowable; Syngenta Japan, Tokyo) and uniconazole-P (Sumiseven® P; Sumitomo Chemical Corporation, Tokyo) – directly to the soil, as follows. Fifty millilitres of a solution in water of GA3 (0.01 %, w/w), paclobutrazol (1 % w/w) or uniconazole-P (0.01 % w/w) were applied to the soil around each seedling. For controls, 50 mL of distilled water was applied instead of the plant hormone or inhibitor. We butrazol (1 % w/w) or uniconazole-P (0.01 % w/w) were applied to the soil in which seedlings were moistened daily with approximately 200 mL of water. Seedlings were harvested 2 months later for analysis.

**Preparation and observation of transverse sections**

Ten-millimetre segments of stems, 10 cm above soil level, were removed from each seedling for analysis. Segments were fixed in 4 % glutaraldehyde in 0-1 M phosphate buffer (pH 7.3). Transverse sections 15 μm thick were cut from the segments on a sliding microtome (Yamatokohki, Saitama, Japan). Sections were stained with 0.1 % solution of safranin (Wako Pure Chemical Industries) and then with 1 % solution of Astra blue (Sigma-Aldrich, Steinheim, Germany). They were dehydrated in a graded ethanol series, mounted on glass slides, fixed in resin (Entellan New; Merck, Darmstadt, Germany) and covered with coverslips (Nugroho et al., 2012b). Images were recorded under a light microscope (Axiostar; Carl Zeiss, Oberkochen, Germany) with a digital camera (Nikon Digital Sight DS-5M-L1; Nikon Corporation, Japan) as described by Begum et al. (2012). Digital images of transverse-sectional areas of 35.3 × 10^3 μm^2 were recorded from regions of tension wood and opposite wood of inclined stems for measurement of apparent thickness of the gelatinous layers, the thickness of fibre walls and the radial diameters of wood fibres. Fifty wood fibres were measured in each sample. For measurements of the apparent thickness of the gelatinous layers, we defined the inner layers of cell walls that were stained blue by the combination of safranin and Astra blue as gelatinous layers (Nugroho et al., 2012b). Three digital images of transverse-sectional areas of 8.1 × 10^6 μm^2 from each sample were used for measurements of the diameters and frequencies (numbers of vessels mm^−2) of vessels in the tension wood and opposite wood. The apparent thickness of the gelatinous layers, the radial diameter of wood fibres, the thickness of wood fibre walls and the diameters and frequencies (numbers of vessels mm^−2) of vessels were determined with image-analysis software (ImageJ; National Institutes of Health, MD, USA). In the present study, the thickness of wood fibre walls was defined as the entire thickness of cell wall layers that included gelatinous layers.

In addition, we observed the *in situ* thickness of the gelatinous layers with resin-embedded wood blocks. One fixed sample per each group was chosen for resin-embedded sections. Fixed samples were washed in 0.1 M phosphate buffer, trimmed to 3 mm in length and then dehydrated in a graded ethanol series and embedded in epoxy resin (Begum et al., 2012). Transverse thin sections 1 μm in thickness were cut at distance greater than 30 μm below the primary surface of samples with glass knives on an ultramicrotome (Ultracut N; Reichert, Vienna, Austria) as described by Clair et al. (2005a). Sections were stained with Toulidine blue, mounted on glass slides, fixed in resin and covered with coverslips, and images were recorded under a light microscope with a digital camera.

**Preparation and observation of tangential sections**

Tangential sections 15 μm thick were cut from the outermost part of the tension wood and opposite wood of inclined stems on a sliding microtome. Sections were double-stained with a solution of safranin and Astra blue, dehydrated in a graded ethanol series, mounted on glass slides, fixed in resin and covered with coverslips. Three digital images of tangential-sectional areas of 558 × 10^3 μm^2 from each section were recorded under a light microscope with a digital camera for measurement of the height, width and frequency of rays. For calculations of the mean height and width of rays, 50 rays were measured in each section.

**Preparation and observation of macerated materials**

Small slices of wood were cut from the tension and opposite wood of stem segments of inclined seedlings on a sliding microtome. The slices of wood were macerated in Franklin solution, which is a mixture of equal volumes of glacial acetic acid and hydrogen peroxide, and heated at 60°C for 1 or 2 days (Kitin et al., 1999; Nugroho et al., 2012a). Images of wood fibres and vessel elements were recorded under a light microscope with a
digital camera. Lengths of wood fibres and vessel elements were measured with ImageJ. One hundred wood fibres and 30 vessel elements in each sample were measured at random.

Statistical analysis

Data were analysed with the statistical software Prism5 for Mac OS (GraphPad Software, USA). Effects of treatments on the anatomical characteristics of woody elements in tension wood and opposite wood were analysed by one-way analysis of variance followed by Tukey’s post hoc test. The significance of differences among treatments was accepted at $P < 0.05$.

RESULTS

Negative gravitropism of tilted seedlings and formation of tension wood

Figure 1 shows typical responses of the stems of control, $\text{GA}_3$-treated, paclobutrazol-treated and uniconazole-P-treated seedlings of $A. \text{mangium}$ to tilting of pots. Stems of control and $\text{GA}_3$-treated seedlings returned to the vertical position after 2 months of inclination. By contrast, the orientation of stems of paclobutrazol-treated and uniconazole-P-treated seedlings remained basically unchanged.

Tension wood in $A. \text{mangium}$ is characterized by the presence of gelatinous fibres. In this study, we defined tension wood as wood that consists of gelatinous fibres with inner gelatinous...
layers that were stained exclusively blue by Astra blue and safranin. As shown in Figs 2 and 3, all seedlings examined had formed gelatinous fibres on the upper side of inclined stems within 2 months after the pots were tilted. Paclobutrazole-treated and uniconazole-P-treated seedlings had narrower regions of tension wood on the upper side of inclined stems than compared with control and GA₃-treated seedlings (Fig. 2).

**Thickness of gelatinous layers**

Light microscopy of transverse sections 15 μm thick revealed that the apparent thickness of the gelatinous layers differed significantly among treatments \( (P < 0.0001) \). Paclobutrazole-treated and uniconazole-P-treated seedlings formed thinner gelatinous layers (Figs 3 and 4). The mean (± s.e.) apparent thicknesses of the gelatinous layers were approximately 2.3 ± 0.1 μm in control seedlings, 2.5 ± 0.3 μm in GA₃-treated seedlings, 1.3 ± 0.2 μm in paclobutrazol-treated seedlings and 1.5 ± 0.3 μm in uniconazole-P-treated seedlings.

A similar trend was found in transverse sections 1 mm thick from embedded samples of control, GA₃-treated, paclobutrazol-treated and uniconazole-P-treated seedlings (Fig. 5). In situ, the gelatinous layers in the tension wood of were thinner in paclobutrazol-treated and uniconazole-P-treated seedlings than in control and GA₃-treated seedlings.

**Morphology of wood fibres**

Two months after tilting the pots, the lengths of wood fibres in the tension wood of inclined stems differed significantly among treatments \( (P < 0.0001; \text{Fig. 6A}) \). The mean (± s.e.) lengths of wood fibres in the tension wood of inclined stems were 541 ± 7 μm in control seedlings, 553 ± 7 μm in GA₃-treated seedlings, 401 ± 9 μm in paclobutrazol-treated seedlings and 398 ± 6 μm in uniconazole-P-treated seedlings. The GA₃-treated seedlings had significantly longer wood fibres than control seedlings. By contrast, paclobutrazol- and uniconazole-P-treated seedlings had significantly shorter wood fibres than control seedlings. In the opposite wood of all seedlings examined, lengths of wood fibres ranged from 417 to 425 μm and no significant differences were found among treatments \( (P = 0.924; \text{Fig. 6A}) \).

The radial diameter of wood fibres of inclined stems did not differ significantly among treatments, either in tension wood or in opposite wood \( (P = 0.419; \text{P} = 0.615; \text{Fig. 6B}) \). The mean (± s.e.) radial diameters of wood fibres in tension wood and opposite wood of inclined stems were 10.8 ± 0.2 and 11.9 ± 0.2 μm in control seedlings, 10.2 ± 0.3 and 11.6 ± 0.2 μm in GA₃-treated seedlings, 10.7 ± 0.2 and 11.3 ± 0.5 μm in paclobutrazol-treated seedlings and 10.7 ± 0.2 and 11.5 ± 0.3 μm in uniconazole-P-treated seedlings.

The thickness of cell walls of wood fibres differed significantly among treatments in the tension wood \( (P = 0.005) \), while no significant differences were found in the opposite wood.
There were no significant differences among treatments in ray height and width in either tension wood or opposite wood (Fig. 9A, B). The mean (± s.e.) height and width of rays in tension wood were 86.8 ± 2.8 and 11.9 ± 0.4 μm, respectively, in control seedlings, 85.0 ± 4.9 and 11.7 ± 0.6 μm in GA3-treated seedlings, 80.2 ± 1.4 and 11.6 ± 0.6 in paclobutrazol-treated seedlings and 78.8 ± 1.9 and 11.6 ± 0.5 μm in uniconazole-P-treated seedlings. In the opposite wood, ray height ranged from 73.8 to 83.4 μm and ray width from 10.8 to 11.8 μm.

Ray frequency did not differ significantly among treatments in either tension wood or opposite wood (P = 0.054 and P = 0.083, respectively; Fig. 9C). Mean (± s.e.) ray frequency in tension and opposite wood of inclined stems was 60.8 ± 3.1 and 57.8 ± 3.3 mm⁻², respectively, in control seedlings, 63.4 ± 2.5 and 60.0 ± 2.6 mm⁻² in GA3-treated seedlings, 52.8 ± 3.3 and 51.4 ± 2.3 mm⁻² in paclobutrazol-treated seedlings and 53.6 ± 1.6 and 51.2 ± 2.7 mm⁻² in uniconazole-P-treated seedlings.

**Morphology of vessel elements**

Diameters of vessel elements were smaller in the tension wood of all inclined stems. In both tension wood and opposite wood, there were no significant differences among treatments in the mean length of vessel elements (P = 0.934 and P = 0.924, respectively), mean vessel diameter (P = 0.057 and P = 0.560, respectively) and mean vessel frequency (P = 0.105 and P = 0.929, respectively) (Fig. 7). The mean lengths of vessel elements in the tension wood and opposite wood of stems ranged from 208 to 226 μm. The mean (± s.e.) vessel element diameter and frequency in the tension wood of inclined stems were 35.8 ± 0.8 μm and 33.4 ± 1.5 mm⁻² in control seedlings, 33.0 ± 0.4 μm and 31.8 ± 2.2 mm⁻² in GA3-treated seedlings, 34.6 ± 1.0 μm and 38.0 ± 2.1 mm⁻² in paclobutrazol-treated seedlings and 36.4 ± 1.1 μm and 40.8 ± 4.1 mm⁻² in uniconazole-P-treated seedlings. In the opposite wood, the mean (± s.e.) diameter and frequency of vessel elements were 44.7 ± 1.0 μm and 51.8 ± 2.6 mm⁻² in control seedlings, 42.8 ± 0.4 μm and 51.0 ± 1.0 mm⁻² in GA3-treated seedlings, 44.3 ± 2.1 μm and 50.2 ± 1.2 mm⁻² in paclobutrazol-treated seedlings and 41.0 ± 3.6 μm and 50.8 ± 1.0 mm⁻² in uniconazole-P-treated seedlings.

**Ray morphology**

All rays were uniseriate in all samples examined (Fig. 8). In addition, we found that the axial parenchyma was predominantly paratracheal, scanty, vasicentric and aliform. There were no significant differences among treatments in ray height and width in either tension wood or opposite wood (Fig. 6C). The mean (± s.e.) cell wall thicknesses of wood fibres in tension wood and opposite wood of inclined stems were 3.2 ± 0.2 and 1.5 ± 0.0 μm in control seedlings, 3.2 ± 0.2 and 1.6 ± 0.2 μm in GA3-treated seedlings, 2.7 ± 0.2 and 1.4 ± 0.3 μm in paclobutrazol-treated seedlings and 2.7 ± 0.1 and 1.5 ± 0.1 μm in uniconazole-P-treated seedlings. Paclobutrazole-treated and uniconazole-P-treated seedlings formed thinner gelatinous layers. Scale bar = 25 μm.

**DISCUSSION**

The results described above show clearly that gibberellin plays an important role in the gravitropism of *Acacia mangium*, a typical woody plant. Two months after the pots had been tilted, control seedlings and GA3-treated seedlings returned to the upright position but stems of paclobutrazol-treated and uniconazole-P-treated seedlings did not do so. In a previous study, we found that a single application of GA3 via the soil to seedlings of *A. mangium* significantly stimulated the return to the vertical of stems in tilted pots (Nugroho et al., 2012b). Similarly, it has been reported that negative gravitropism of *Fraxinus*...
mandshurica var. japonica was enhanced after the application of gibberellin, while the application of uniconazole-P inhibited the upward bending of horizontal seedlings (Jiang et al., 1998a, b, 2006, 2008). Furthermore, treatment with gibberellin prevented the downward bending of branches of the weeping type of Prunus spachiana (Baba et al., 1995; Nakamura et al., 1994; Yoshida et al., 1999).

Negative gravitropism of inclined woody stems is closely related to the formation of tension wood. Tension wood generates a strong tensile force and thus contributes to the negative gravitropic movement of inclined woody stems (Okuyama et al., 1994; Yoshida et al., 1999, 2000; Yamamoto et al., 2002; Clair et al., 2006; Ruelle et al., 2006; Fang et al., 2008). In the poplar, larger numbers of gelatinous fibres per unit area of tissue were associated with higher longitudinal growth stress (Fang et al., 2008). Moreover, in our previous study we showed that the width of the region of gelatinous fibres was strongly correlated with the negative gravitropism of inclined A. mangium seedlings (Nugroho et al., 2012b). In the present study, we found that the apparent thickness of the gelatinous layers of non-embedded tension wood sections of paclobutrazol-treated and uniconazole-P-treated seedlings did not return to the vertical orientation.

In our previous study, we demonstrated that GA3 stimulated the formation of tension wood of tilted stems of A. mangium seedlings, while the application of either of two inhibitors of gibberellin biosynthesis, paclobutrazol and uniconazole-P, suppressed increases in the width of tension wood (Nugroho et al., 2012b). In the present study we found that the apparent thickness of the gelatinous layers of non-embedded tension wood sections of paclobutrazol-treated and uniconazole-P-treated seedlings
were thinner than those in control and GA3-treated seedlings (Figs 3 and 4). However, Clair et al. (2005a, b) reported that sectioning of non-embedded tension wood samples resulted in uncontrolled swelling of the gelatinous layers and in detachment of gelatinous layers due to tensile stress. The apparent thickness of the gelatinous layers from the non-embedded tension wood samples may not express the in situ thickness. We therefore made sections of embedded samples as described by Clair et al. (2005a) to obtain unswollen gelatinous layers. Microscopic observation on thin sections of embedded samples revealed that the in situ thickness of the gelatinous layers in the tension wood of paclobutrazol-treated and uniconazole-P-treated seedlings was less than in control and GA3-treated seedlings (Fig. 5). We concluded that in situ thickness of the gelatinous layers showed a similar trend to the apparent thickness of the gelatinous layers in 15-μm-thick transverse sections of non-embedded tension wood samples.

Our results suggest that application of inhibitors of gibberellin biosynthesis to the soil not only suppressed increases in the amount of gelatinous fibres but also negatively influenced the quality of the gelatinous fibres that developed. The thicker gelatinous layers in the tension wood of the inclined stems of control and GA3-treated seedlings might generate stronger tensile forces that bend the leaning stems upward. Fang et al. (2008) reported that in the poplar thicker gelatinous layers accompanied greater longitudinal growth stress, and concluded that the gelatinous layer plays a critical role in the generation of strong growth stress.

We found that application of GA3 stimulated the elongation of fibres in the tension wood of inclined stems. The extent of elongation of fibres was approximately 148% in control seedlings, 162% in GA3-treated seedlings, 86% in paclobutrazol-treated seedlings and 88% in uniconazole-P-treated seedlings, as estimated from the lengths of the vessel elements, which are generally similar to those of fusiform cambial cells (Romberger et al., 1993; Kitin et al., 1999). Our results support the hypothesis proposed by earlier investigators that gibberellin plays an important role in the formation and function of tension wood.
role in the cell elongation of wood fibres (Aloni, 1992, 2007; Aloni et al., 2000). Moreover, Ridoutt et al. (1996) reported that the elongation of wood fibres of *Eucalyptus globulus* was positively correlated with higher levels of endogenous gibberellins. Longer wood fibres were produced in the stems of transgenic hybrid aspen that harboured genes for the enhanced biosynthesis of gibberellins (Eriksson et al., 2000). Moreover, Israelsson et al. (2005) reported that, in *Populus tremula*, bioactive gibberellins and the associated biosynthetic genes were co-localized in regions of differentiating xylem cells; for example, regions of fibre elongation. A recent study by Dayan et al. (2012) revealed that gibberellin provides the specific signal for induction of the secondary differentiation of xylem fibres. By contrast, application of inhibitors of gibberellin biosynthesis, such as paclobutrazol and uniconazole-P, might reduce endogenous levels of gibberellins and thus suppress the elongation of wood fibres in the tension wood of inclined stems. There were no significant differences among treatments in the radial diameters of fibres in the tension wood of inclined stems. Similarly, none of the treatments induced any significant changes in the properties of fibres in the opposite wood. However, although there were no differences in terms of fibre diameter, the thickness of gelatinous layers and the thickness of fibre walls in the tension wood differed significantly among treatments. Paclobutrazol and uniconazole-P each suppressed increases in the thickness of gelatinous layers, and the cell walls of wood fibres in the tension wood of paclobutrazol-treated and uniconazole-P-treated seedlings were therefore significantly thinner than those of control and GA₃-treated seedlings.

Application of gibberellin and of inhibitors of gibberellin biosynthesis did not induce any changes in the lengths of vessel elements in the tension and opposite wood of the inclined stems. The mean lengths of vessel elements in the tension wood and opposite wood of stems ranged from 208 to 226 µm. Honjo et al. (2005) reported similarly that the lengths of vessel elements were relatively constant (approximately 200 µm) across the stems of *A. mangium*. The lengths of vessel elements are very similar to those of fusiform cambial cells (Romberger et al., 1993; Kitin et al., 1999) and our results thus indicate that a gravitational stimulus, the application of gibberellin and the application of inhibitors of gibberellin biosynthesis had no effect on the development of fusiform cambial cells. Furthermore, we found that the diameters of vessels and the frequencies of vessels in the tension wood and opposite wood did not differ significantly among treatments. Gibberellin provides the specific signal that induces the differentiation of xylem fibres, whereas auxin provides the specific signal that regulates the formation of vessels (Aloni and Zimmermann, 1984; Aloni, 1992, 2007; Aloni et al., 2000; Dayan et al., 2012). Our results therefore suggest that gibberellin might not be a specific regulator of vessel differentiation.

We found that gibberellin did not cause any changes in the differentiation of rays. The application of gibberellin and inhibitors of gibberellin biosynthesis did not affect the height, width or frequency of rays in the inclined stems of *A. mangium* seedlings. Lev-Yadun (2000) reported that the signal flow of auxin and that of ethylene influence ray differentiation and that auxin and ethylene appear to provide the main hormonal signals that control the initiation of ray formation and the regulation of the size and spacing of rays in the cambium (Lev-Yadun and Aloni, 1991, 1995; Aloni et al., 2000; Aloni, 2007). It therefore seems likely that gibberellin might play only a minor role in ray differentiation.

In summary, our results show clearly that the application of either of two inhibitors of the biosynthesis of gibberellin, namely paclobutrazol and uniconazole-P, suppresses increases in the thickness of gelatinous layers and the elongation of gelatinous fibres, while GA₃ stimulates the elongation of gelatinous fibres. In addition, neither gibberellin nor the gibberellin biosynthesis inhibitors had a significant effect on the anatomical characteristics of vessel and ray parenchyma cells in inclined seedlings of *A. mangium*. Thus, we can conclude that gibberellin is essential for the development of gelatinous fibres in the tension wood of inclined *A. mangium* seedlings and therefore in gravitropism in this typical woody plant.

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**LITERATURE CITED**


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