

ROLES OF AUXIN AND GIBBERELLIN IN GRAVITY-INDUCED TENSION WOOD FORMATION IN *AESCULUS TURBINATA* SEEDLINGS

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SUMMARY

The lowest nodes of 6-week-old *Aesculus turbinata* seedlings were treated with uniconazole-P, an inhibitor of gibberellin (GA) biosynthesis, or a mixture of uniconazole-P and GA₃ in acetone solution. To the seedling stems, an inhibitor of auxin transport (NPA) or inhibitors of auxin action (raphanusanin or MBOA) were applied in lanolin paste. The seedlings were tilted at a 45° angle and kept for 10 weeks before histological analysis. Decreases in both normal and tension wood formation followed the application of uniconazole-P. The application of GA₃ together with uniconazole-P partially negated the effect of uniconazole-P alone. The application of NPA inhibited tension wood formation at, above, and below the lanolin-treated portions. The treatment of raphanusanin or MBOA also resulted in decreases in tension wood formation at the treated portions. The inhibitory effects of these chemicals applied on the upper side of tilted stems or around the entire stem were greater than on the lower side. The application of uniconazole-P in combination with raphanusanin, MBOA or NPA showed synergistic effects on the inhibition of tension wood formation. The results suggest that both auxin and GA regulate the quantitative production of tension wood fibers and are essential to tension wood formation.

Key words: *Aesculus turbinata*, auxin, gibberellin, MBOA (6-methoxy-2-benzoxazolinone), NPA (*N*-1-naphthylphthalamic acid), raphanusanin, tension wood, uniconazole-P.

INTRODUCTION

Plant hormones, auxins in particular, have been intensively studied to reveal the mechanisms related to gravitropism and reaction wood formation in woody plants. It is postulated that whereas compression wood formation, which usually happens on the lower side of an inclined stem of gymnosperms, is associated with a supra-optimal level of auxin, tension wood formation is induced by a deficiency of auxin, usually on the upper side of an inclined stem of woody angiosperms (Timell 1986). A higher concentration of auxin on the lower than on the upper side of inclined hardwood shoots has been detected by several investigators, e.g. in *Populus alba* (Nečesany 1958),

P. robusta (Leach & Wareing 1967) and *Salix viminalis* (Lepp & Peel 1971). A number of investigations on the effect of endogenous auxins and auxin antagonists on tension wood formation support the above postulation. These include applications to hardwood stems of indoleacetic acid (IAA), naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D) that have inhibited tension wood formation if treated on the upper side, of IAA and 2,4-D that have resulted in tension wood formation on the untreated side, and of auxin antagonists including triiodobenzoic acid (TIBA) and 2,4-dinitrophenol that have induced tension wood formation on the treated point (Timell 1986). However, several investigators have reported different or contrary results. Wareing et al. (1964) were unable to confirm an endogenous auxin difference between upper and lower sides of *Populus* and *Salix* branches. Moreover, the application of morphactin IT3456 (2-chloro-9-hydroxyfluorene-9-carboxylic acid) to the upper side of inclined *Aesculus hippocastanum* shoots was followed by a suppression of tension wood formation on this side (Smoliński et al. 1974; Phelps et al. 1975).

Besides auxins, gibberellins (GAs) are another group of plant hormones that are essential to cambial growth in both woody angiosperms and gymnosperms (Aloni 1979; Little & Pharis 1995). The possible role of GAs in tension wood formation has been investigated for years. Denhard and Feucht (1971) observed that the formation of tension-wood-like fibers in vertical *Prunus avium* shoots was stimulated by GA application. The application of GA₃ to growing shoots of drooping mutants of *P. spachiana* and *P. persica* caused resumption of vertical shoot growth and promoted the formation of tension wood on the upper side of shoots (Nakamura et al. 1994, 1995; Baba et al. 1995). However, it was also reported that application of GA₃ to the upper side of horizontal-positioned *Acer rubrum* seedlings had no effect on either gravitropic bending or tension wood formation (Cronshaw & Morey 1968). In horizontal shoots of *Populus alba*, GA₃ application in combination with IAA did not affect wood formation (Blum 1971). Jiang et al. (1998a, b) found that the application of uniconazole-P, (E,S)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazolyl)-pentenol, a GA biosynthesis blocker, inhibited both wood formation and negative gravitropic bending of horizontally positioned *Fraxinus mandshurica* var. *japonica* stems, but applying GAs with it negated the inhibitory effects, whereas tension wood formation was not significantly affected in either of the cases. Therefore, the roles of auxin and GAs in regulating the differentiation of tension wood fibers and negative gravitropic bending of hardwood tree stems are still obscure.

To further understanding of the roles of auxins and GAs in gravitropism and reaction wood formation in woody angiosperms, we applied several kinds of plant growth retardants that can either inhibit the basipetal transport of IAA, e.g. NPA (*N*-1-naphthylphthalamic acid), or inhibit the action of auxins, e.g. raphanusanin (3-(methylthio)methylene-2-pyrrolidinethione) and MBOA (6-methoxy-2-benzoxazolinone) (Hasegawa et al. 1987, 2000; Hoshi-Sakoda et al. 1994; Hashimoto & Shudo 1996), or inhibit GA biosynthesis (Izumi et al. 1988; Oshio et al. 1990; Izumi & Oshio 1991) so that more information about the interactions of auxins and GAs would be obtained.

MATERIALS AND METHODS

Aesculus turbinata Blume (Japanese horse-chestnut) seedlings were grown from seeds sown on May 13, 2000, in $7 \times 7 \times 19.5$ cm paper pots containing sand and peat (2/1, v/v) in a greenhouse in the nursery of Tottori University. On June 29, a total of 135 seedlings, of which the average stem heights and diameters at 2.5 cm above the ground level were 20.5 cm and 7.5 mm, respectively, were selected for their uniformity of size and development. Two different experiments were performed simultaneously: 30 seedlings for Experiment 1 to determine the effects of NPA, 105 seedlings for Experiment 2 to determine the effects of raphanusanin and MBOA. Both experiments were also subjected to a series of GA-related treatments to determine the effects of applied GA₃ and uniconazole-P.

In Experiment 1, the 30 seedlings were divided into three groups: 1) 10 seedlings treated with 5 μ l of 60% acetone (denoted plain acetone) as control, 2) 10 seedlings treated with 40 μ g of uniconazole-P in 5 μ l of 60% acetone to lower the levels of endogenous GAs, and 3) 10 seedlings treated with a mixture of 40 μ g of uniconazole-P and 40 μ g of GA₃ in 5 μ l of 60% acetone to determine the effect of exogenous GA₃. Those solutions were dripped onto the lowest node of the seedlings with a micropipette on June 29 and repeated once a week until the end of the experiment.

On July 6 at seven days after the initiation of GA-related treatments, the seedlings of each group were tilted at an angle of 45° and divided into two subgroups for NPA treatments. A 2-cm-long band of epidermis was removed from the stem circumference in each seedling using a scalpel at 3 to 5 cm (denoted 4 cm) below the lowest node (Fig. 1A). Five seedlings of one subgroup were treated with plain lanolin and the remaining five seedlings were treated with a 10 mg g⁻¹ NPA lanolin paste. The paste-applied portions were wrapped with a piece of aluminum foil to prevent them from environmental influences.

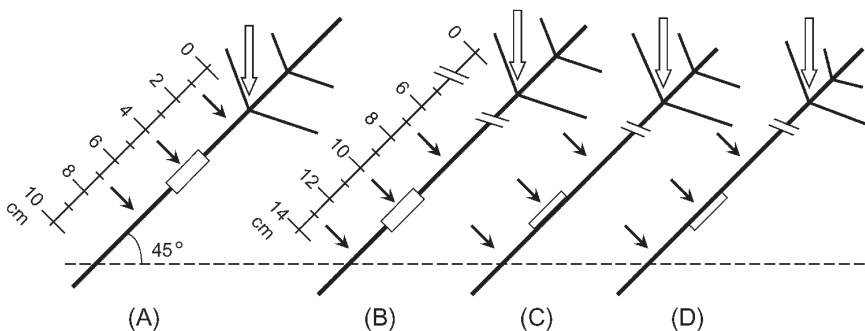


Fig. 1. Diagrams showing the locations of lanolin paste applications (rectangles) and the transverse sections taken for histological determination (solid arrows). Broad arrows indicate where the acetone solution was applied. Drawings: (A) illustrates circumferential applications of NPA and plain lanolin in Experiment 1; (B) through (D) illustrate applications of raphanusanin and MBOA to the circumference, upper side and lower side, respectively, in Experiment 2. All seedlings were tilted at 45° to induce tension wood formation.

In Experiment 2, the 105 seedlings were divided into three groups: 1) 35 seedlings for control, 2) 35 seedlings for uniconazole-P application, and 3) 35 seedlings for the application of uniconazole-P together with GA₃. Those solutions were applied on June 29 as described in Experiment 1.

On July 6, the seedlings of each group were tilted at 45° and divided into seven sub-groups for treatments of raphanusanin or MBOA as: 1) 5 seedlings, stems treated with plain lanolin around the entire stem, 2) 5 seedlings, stems treated with 0.16 mg g⁻¹ raphanusanin lanolin paste (c. 10⁻³ M) around the entire stem, 3) 5 seedlings, stems treated with the raphanusanin paste on the upper side of the stem, 4) 5 seedlings, stems treated with the raphanusanin paste on the lower side of the stem, 5) 5 seedlings, stems treated with 0.165 mg g⁻¹ MBOA lanolin paste (c. 10⁻³ M) around the entire stem, 6) 5 seedlings, stems treated with the MBOA paste on the upper side of the stem, and 7) 5 seedlings, stems treated with the MBOA paste on the lower side of the stem. The treatments were applied at 9 to 11 cm (denoted 10 cm) below the lowest node (Fig. 1B, C, D). The paste-applied portions were wrapped as described above.

All seedlings in both experiments were kept at a 45° angle for the next 10-week treatment period, during which they were watered everyday and fertilized once a week in a greenhouse.

On September 14, the seedlings were harvested and their stem segments were fixed in 70% ethanol and sectioned transversely at 10–15 µm thick through each paste-applied zone (at 4 or 10 cm below the lowest node), 3 cm above it and 3 cm below it with a sliding microtome (Fig. 1). The sections were stained with safranin and fast green solutions, and mounted in Eukitt. For each section, xylem tissues on the upper side were investigated microscopically. Tension wood fibers were recognized on the basis of having thickened, blue-stained and unligified gelatinous layers (Zimmermann & Brown 1971). The area of tension wood fibers and the total area of newly formed xylem tissues on the upper half were measured with a light microscope (Olympus BHS 323) in combination with a video micrometer (Olympus Flovel VM-31, Flovel Co. Ltd. Japan).

The statistical analysis of variance was applied to each two-way data set, and the multiple comparisons between treatments were performed by the method of Scheffé's test for two-way classification (Scheffé 1959; ESUMI 2000).

RESULTS

In both experiments, the application of uniconazole-P through an acetone solution significantly reduced the quantity of total wood formation. This inhibitory effect was not observed when GA₃ was added to the uniconazole-P solution in Experiment 1, in which the samples were sectioned at 1, 4, and 7 cm below the lowest node (Fig. 1A; Fig. 2, upper). In Experiment 2, however, the similar effects of GA₃ were not statistically significant in most observed samples, which were sectioned at 7, 10, and 13 cm below the lowest node (Fig. 1B, C, D; Fig. 3 & 4, upper). Application of NPA through lanolin paste enhanced total wood formation at and above the application site, even though the enhancement at 3 cm above was not statistically significant. Applications of raphanusanin or MBOA in the present concentrations, however, had not resulted in

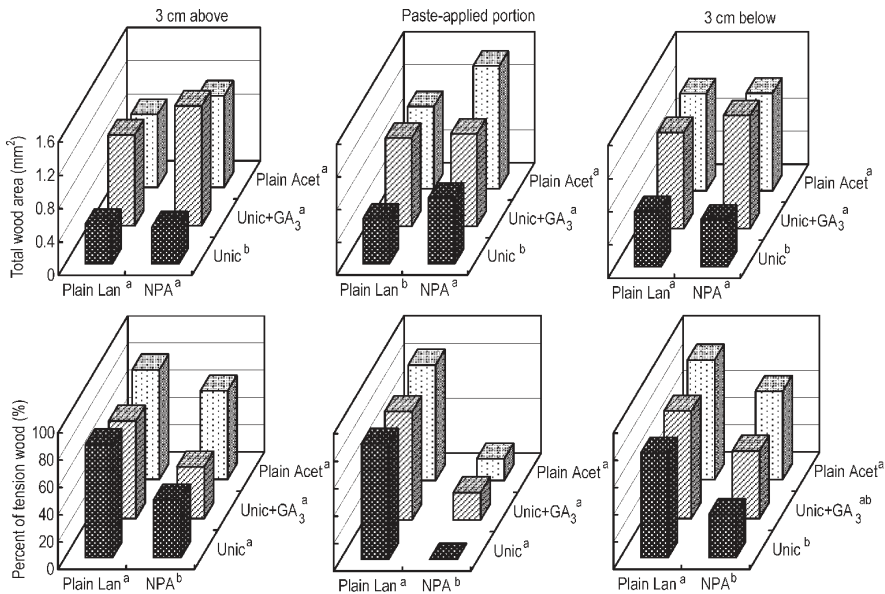


Fig. 2. The effects of NPA, uniconazole-P and GA₃ through combined treatments on the total area of newly formed xylem and the percentage of tension wood in it on the upper half of transverse sections of the tilted stems. The seedlings were ringed either with NPA lanolin paste or with lanolin only as control (Plain Lan in figures), in combination with the treatments of acetone solutions containing uniconazole-P (Unic in figures), uniconazole-P + GA₃, or neither of them (Plain Acet in figures), separately. The treatments without common superscript letters in these and subsequent figures are significantly different at P < 0.05 using Scheffé's test for two-way classifications.

any significant differences in the amount of newly formed wood, regardless of the side to which the paste was applied.

In the seedlings treated only with plain lanolin and acetone solution, typical tension wood fibers formed on the upper side along the tilted stems (Fig. 5A). Applications of NPA, raphanusanin, MBOA and uniconazole-P inhibited the formation of tension wood tissues in both quantity and intensity.

In Experiment 1, the application of 10 mg g⁻¹ NPA lanolin paste around the entire stem greatly inhibited the formation of tension wood fibers in the application zone (Fig. 2 & 5C). This inhibitory effect was relatively lower in zones above and below the applied portions. The percentage of tension wood area in the upper half of newly formed xylem tissues in transverse sections showed that tension wood formation was significantly decreased in all investigated zones of the NPA-treated seedlings. In the seedlings treated with uniconazole-P solution, tension wood formation was completely inhibited at the NPA-treated zone (Fig. 5D). Even at 3 cm above and below the treated portion, very few tension wood fibers developed in these seedlings. These results showed that there was a synergistic inhibitory effect of NPA and uniconazole-P on the formation of tension wood.

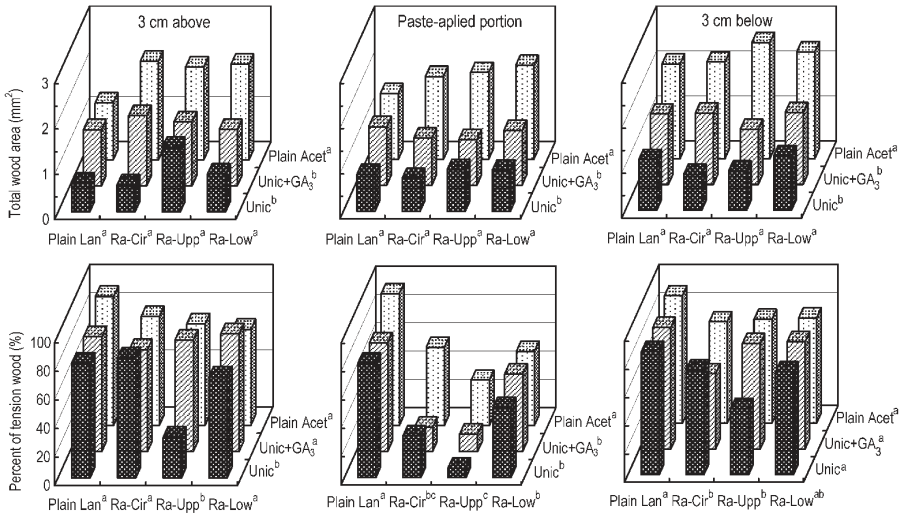


Fig. 3. Xylem area and the percentage of tension wood in it on the upper side of the tilted seedlings treated with raphanusanin in combination with uniconazole-P or GA₃. The seedlings were applied either with plain lanolin or with raphanusanin lanolin pastes to the circumference (Ra-Cir), upper side (Ra-Upp), or lower side (Ra-Low), in combination with the treatments of solutions containing uniconazole-P, uniconazole-P + GA₃ or plain acetone, separately.

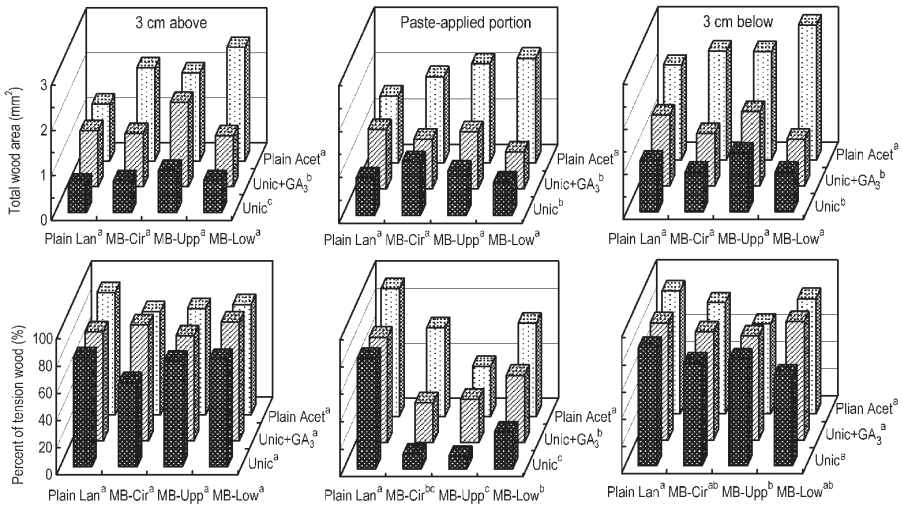


Fig. 4. Xylem area and the percentage of tension wood in it on the upper side of the tilted seedlings treated with MBOA (MB in figures) in combination with uniconazole-P or GA₃. The treatments were carried out as described in Figure 3.

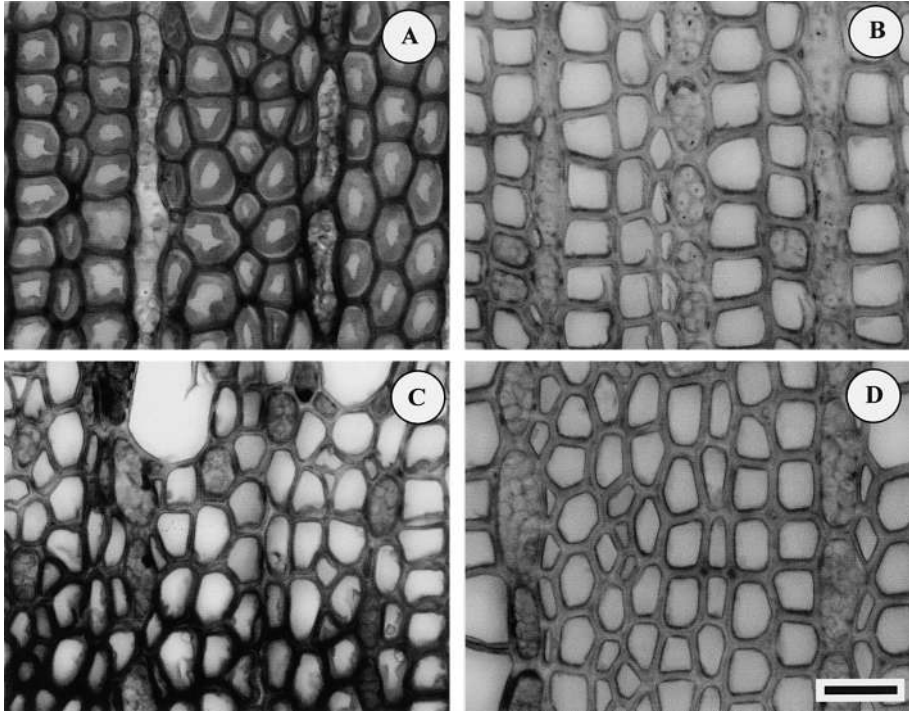


Fig. 5. Transverse sections taken at the stem site where lanolin pastes were applied. Sections A and B were from the upper and lower sides of tiled stems ringed with plain lanolin, respectively. Sections C and D were from the upper side of tiled stems ringed with a NPA lanolin paste in combination with applications of plain acetone (C) and uniconazole-P (D), respectively. Note a large amount of typical gelatinous fibers appearing in A. — Scale bar = 20 μm .

In both experiments, tension wood fibers were much fewer in the uniconazole-P-applied seedlings than in those treated with plain lanolin solution. The application of GA₃ together with uniconazole-P partially negated this inhibitory effect caused by uniconazole-P alone. As a result of the inhibition of total xylem growth by uniconazole-P, its effect on the percentage of tension wood area was not statistically significant in some of the sections such as those taken at 3 cm above or below the paste application site.

In Experiment 2, the application of raphanusanin or MBOA resulted in decreases in the percentage of tension wood area at the treated portion (Fig. 3 & 4, middle). This effect was smaller when the pastes were applied to the lower half of the tilted stems, opposite to the side of tension wood formation, than to the upper side or around the entire stem. There was little influence of these applied chemicals at 3 cm above and below the application site, except a decrease in the percentage of tension wood area at 3 cm below the portion of which the paste was applied on the upper side or the entire circumference (Fig. 3 & 4, right).

DISCUSSION

The decrease in the quantity of total wood formation resulted from uniconazole-P applications logically can be attributed to a deficiency of GAs, as it is widely documented that, in addition to auxin, GA is involved in cambial activity of woody plants (Aloni 1979; Little & Pharis 1995). From the experiment with horizontally positioned seedlings of *Fraxinus mandshurica* var. *japonica*, Jiang et al. (1998b) revealed that both GAs and uniconazole-P, when applied alone, did not affect tension wood formation. In the present experiments, except the NPA-applied portion of uniconazole-P-dripped seedlings, certain amounts of tension wood fibers formed in most of the stem samples under each treatment. These results again imply that the factors that regulate tension wood formation are extremely complicated. Nevertheless, the inhibitory effect of uniconazole-P quantitatively on tension wood formation and the compensatory effect of GA₃ support the hypothesis that GAs are involved in tension wood formation (Baba et al. 1995).

According to a general hypothesis regarding roles of auxins in reaction wood formation (Timell 1986), the decrease in tension wood formation at and above the NPA-treated portion could be attributed to a postulated accumulation of IAA. However, a similar decrease in tension wood formation at 3 cm below the NPA-applied portion was an unexpected result, as the IAA content should be much lower in this portion (Fig. 2, right). In *Pinus sylvestris* shoots, Sundberg et al. (1994) found a lack of IAA accumulation above the application site of either of the two IAA transport inhibitors NPA and morphactin CF (methyl-2-chloro-9-hydroxyfluorene-9-carboxylic acid). They attributed the differentiation of compression wood formation above the NPA or CF ring to an interaction between NPA receptor and acropetally transported NPA or CF. Therefore, in the present experiments, the possibility cannot be excluded that NPA directly inhibited tension wood formation *per se*, rather than by regulating endogenous IAA content.

Similarly, there has been evidence that phototropism is regulated by unequal distribution of growth inhibitors of auxin, such as raphanusanin and MBOA, rather than a lateral auxin gradient (Bruinsma & Hasegawa 1990; Hasegawa et al. 2000). Lateral applications of those substances to several herbaceous plants caused differential growth resulting in bending toward the applied side (Noguchi et al. 1986). The decrease in tension wood formation resulted from raphanusanin or MBOA application also was not expected according to the former hypothesis. In contrast, it can be hypothesized that a certain auxin content not subjected to inhibitory influences is required for differentiating tension wood fibers. Therefore, under a severe condition of auxin deficiency or lowered auxin activity, tension wood formation would be expected to be reduced.

It deserves to be mentioned that a higher evolution rate of ethylene also was detected in compression- and tension-wood-differentiating stem halves in tilted *Abies balsamea* (Little & Eklund 1999), *Metasequoia glyptostroboides* (Du & Yamamoto 2002) and *Aesculus turbinata* (Du & Yamamoto 2002) seedlings. These phenomena may also be associated with auxin, as auxin and ethylene could regulate each other's activities and levels (Abeles et al. 1992).

The interaction of auxin and GA was considered to be of paramount importance for understanding the hormonal regulation of plant growth, even though much of our knowledge on auxins and GAs has come from studying them independently (Ross et al. 2002). It was proposed in the early literature that GA might increase auxin levels (Kuraishi & Muir 1962; Law & Hamilton 1984; Law & Davies 1990). Recent reports from researches on pea and tobacco emphasized that auxin is necessary for normal GA biosynthesis in stems (Ross et al. 2000; Wolbang & Ross 2001; Ross & O'Neill 2001; Ross et al. 2002). Nakamura et al. (1995) also suggested that IAA may play some role together with GA in the tension wood formation. These findings about auxin-GA correlations probably provided us a clue to the explanation of synergistic inhibitory effects of uniconazole-P on tension wood formation when applied together with raphanusanin, MBOA or NPA. Thus, it can be concluded that both auxin and GAs regulate the quantitative production of tension wood fibers in *Aesculus turbinata* seedlings, and both hormones are indispensable to tension wood formation.

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