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Quantitation of Gibberellins A_1, A_3, A_4, A_9 and a Putative A_9-Conjugate in Grafts of Sitka Spruce (*Picea sitchensis*) during the Period of Shoot Elongation

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ABSTRACT

The levels of endogenous gibberellin A_1 (GA_1), GA_3, GA_4, GA_9, and a cellulase hydrolyzable GA_9 conjugate in needles and shoot stems of mature grafts of Sitka spruce (*Picea sitchensis* [Bong.] Carr.) grown under environmental conditions that were either inductive, hot, and dry, or noninductive, cool, and wet, for flowering, were estimated by combined gas chromatography-mass spectrometry selected ion monitoring using deuterated $[^2H_2]GA_1$, GA_3, GA_4, and GA_9 as internal standards. The samples were taken when the shoots had elongated about 30, 70, and 95% of the final shoot length and 17 days after elongation had terminated. The concentration of putative GA_9-conjugate, estimated by GC-SIM of GA_9 after cellulase hydrolysis of the highly water soluble fraction, was 33 nanograms per gram fresh weight in the needles of both heat and drought- and cool and wet-treated plants sampled just after bud burst. The concentration gradually decreased to a final value of 13 nanograms per gram fresh weight in the heat and drought-treated grafts and 6 nanograms per gram fresh weight in the cool and wet-treated grafts. The stems contained no detectable putative GA_9 conjugate. Free GA_9 was highest in heat and drought-treated material. For plants subjected to this treatment, GA_9 increased from 22 to 32 nanograms per gram fresh weight in needles and from 1 to 22 nanograms per gram fresh weight in stems during the rapid stem elongation phase. By day 17, after cessation of shoot elongation, GA_9 had decreased to 12 nanograms per gram fresh weight in needles and 9 nanograms per gram fresh weight in the shoot stems. The cool and wet-treated material also showed an increase in GA_9 concentration during shoot elongation. However, the concentration was not as high and was also delayed compared with heat and drought-treated material. By day 17, after cessation of shoot elongation, GA_9 concentration was 9 nanograms per gram fresh weight in needles and 5 nanograms per gram fresh weight in stems for cool and wet treatment plants. The concentration of GA_4 was very low in tissue from both treatments. Fluctuation in concentration of the more polar gibberellins, GA_1 and GA_3, showed the same pattern as fluctuations in the content of GA_9. However, the heat and drought-treated material had lower amounts of GA_1 and GA_3 during the later phases of shoot elongation, than the cool and wet-treated material. These results imply differential metabolism between clones treated with conditions inductive and noninductive for flowering. Higher concentrations of putative GA_9 conjugate and free GA_9 in the hot and dry treatment indicate a higher capacity of synthesizing, for flowering, the physiologically important GA_4 in the heat and drought-treated material. This synthesis does not, however, result in a buildup of the GA_4 pool, probably because of a high turnover rate of GA_4. The cool and wet-treated material had higher amounts of GA_1 and GA_3, indicating that the differentiation was preferentially directed toward vegetative growth.

Flowering stimulation by GA[1] application and culture treatments, *e.g.* heat and drought, is today often used in the genetic improvement of Pinaceae conifers (27). A general rule is that flowering is stimulated by hot and dry conditions and that an even stronger effect can be achieved if GA_4, GA_7, or a mixture ($GA_{4/7}$) of them is applied (1, 20). Cool temperatures and a wet soil are considered as noninductive conditions for flowering (21, 22). The timing of these treatments determines the success of the work. Therefore, knowledge about the phenology of bud differentiation in the species under study is very important.

The phenology of predetermined shoot- and bud-growth of Sitka spruce (*Picea sitchensis* [Bong.] Carr.) have been studied in detail by Owens and Molder (17). The growth rate during the elongation period follows a sigmoid curve, starting with a period of slow growth, followed by a phase of rapid elongation, and finally a period of slow growth until the shoot elongation eventually ceases (2, 11). The formation of next year's apical and lateral buds, including cone buds, starts at bud break with the formation of bud scales. At the time of shoot growth cessation, the differentiation to bud type, either vegetative, male, or female types, starts. The best period for influencing cone bud differentiation is, therefore, slightly before or at the time of bud type determination (25, 26). In addition, it is also possible to force a transition of latent or vegetative buds into reproductive buds later during the season (31).

The ability to promote flowering in Pinaceae conifers by exogenous application of less polar GAs, *e.g.* GA_4 and GA_7, has raised questions about the physiological role of GAs in flower bud differentiation (1, 4, 18, 19, 20, 21). It has been proposed that GAs are preferentially used in vigorosly growing juvenile trees for vegetative growth. As the tree matures, vegetative growth is, for a variety of reasons, reduced and as a consequence the GA concentration increases up to a certain

[1] Abbreviations: GA_n, gibberellin A_n; EtOAc, ethyl acetate; MeOH, methanol; PVPP, poly-*N*-vinylpolypyrrolidone; SIM, selected ion monitoring.

threshold, large enough for reproductive growth to occur (18, 28). Data supporting this hypothesis are mainly based on exogenously applied GAs. Shoot growth of Douglas-fir (*Pseudotsuga menziesii*) seedlings responded positively to exogenously applied GAs (24, 29). However, with increased maturity the effect on shoot growth decreased and the effect on flowering increased. Webber *et al.* (33) found a positive effect of $GA_{4/7}$ on shoot growth and no effect on flowering of Douglas-fir families with a poor flowering history. Conversely, families with a history of good flowering responded to $GA_{4/7}$ with an increased flowering (29).

Reliable information on endogenous GAs in relation to flower bud differentiation is almost completely absent. Lorenzi *et al.* (11) showed that the amounts of an endogenous nonpolar GA, later identified as an isomer of GA_9 (12), increased in Sitka spruce shoots during the period of shoot elongation. Moritz *et al.* (15) found that needles and shoot stems of clones of Sitka spruce with a good-flowering history contained higher levels of a cellulase hydrolyzable conjugate of GA_9 and free GA_9, compared to poor-flowering clones, when shoot elongation was 95% complete. In addition, the needles and shoot stems of the poor-flowering clones contained relatively large amounts of GA_1 together with small amounts of GA_3, whereas the good-flowering clones contained no detectable amounts of these GAs. GA_1 and GA_3 are considered to be the 'effector' GAs controlling stem elongation in maize (*Zea mays*) (5) and GA_1 is considered to be the 'effector' GA in pea (*Pisum sativum*) (5, 8, 23).

In previous studies, the major endogenous bioactive GAs of Sitka spruce have been identified (13) and the metabolism of some of these GAs has also been studied in Sitka spruce (14). In this study we report the quantitation of endogenous GA_1, GA_3, GA_4, GA_9 and a putative GA_9 conjugate (based on hydrolysis and release of free GA_9) during shoot elongation and shortly thereafter for grafted propagules growing under flower-inductive (hot, dry) and nonflower-inductive (cool, wet) conditions.

MATERIALS AND METHODS

Plant Material

Twenty-four 6-year-old grafted propagules, representing three clones, of mature Sitka spruce (*Picea sitchensis* [Bong.] Carr.) ortets were grown in 20 L pots. On May 5, prior to budbreak, half of the trees were placed in a polyethylene greenhouse and treated with heat and drought. The remaining trees were placed outside the greenhouse and watered daily, *i.e.* cool and wet treatment. Leaf water potentials (Ψ) of plants in the greenhouse were measured once or twice a week, and they were watered when Ψ fell below -2 MPa. Shoot length was measured one to two times a week. Shoots were collected on four occasions: when the shoots had elongated about 30% of their final length (May 14, hot, dry; May 21, cool, wet), when the shoots had elongated about 70% of their final length (June 1, hot, dry; June 8, cool, wet), when the shoots had elongated about 95% of their final length (June 15, hot, dry; June 26, cool, wet), and finally 17 d after the elongation growth had ceased (July 2, hot, dry; July 13, cool, wet). The shoots were immediately frozen in liquid N_2 and stored at -20°C until analyzed.

Extraction and Purification

Current year shoot samples were divided into needles and shoot stems. The samples, processed in triplicates of about 5 g fresh weight each, were extracted in 20 mL g^{-1} ¢reshweight of refrigerated MeOH containing 0.02% (w/v) diethyldithiocarbamic acid as an antioxidant. As internal standards, 25 ng 17,17-[^{2}H]GA_1, 25 ng 17,17-[^{2}H]GA_3, 25 ng 17,17-[^{2}H]GA_4, and 80 ng 17,17-[^{2}H]GA_9 (purchased from Prof. L. N. Mander, Research School of Chemistry, Australian national University, Canberra, Australia) were added. After extraction for 2 h at 4°C, the MeOH was filtered and the residue was washed with 50 mL MeOH. The combined MeOH was evaporated under reduced pressure at 35°C. The aqueous residue was adjusted to 5 mL with 0.05 M sodium phosphate (pH 8.0), and slurried with 0.2 g insoluble PVPP. The slurry was centrifuged at 2000*g* for 5 min, and the buffer phase was decanted from the PVPP which was washed with another 5 mL of buffer and centrifuged again. The buffer phases were combined and adjusted to pH 8.0 and extracted one time with 5 mL EtOAc in a centrifugation vial. The EtOAc phase, containing the putative GA_9-conjugate, was evaporated under N_2 and enzymatically hydrolyzed with cellulase as described below. One mL 0.5 M sodium phosphate was added to the neutral buffer phase, which contained the major part of free GAs, and the pH was adjusted to 2.8 with 6 M HCl. The buffer phase was then extracted three times with 5 mL EtOAc. The combined EtOAc phase was washed once with 5 mL of water, acidified with 6 M HCl to pH 3.0, and then evaporated under a stream of N_2 and further purified by HPLC as described below.

Enzymatic Hydrolysis

The neutral EtOAc phase containing the putative GA_9-conjugate was enzymatically hydrolyzed with cellulase (EC 3.2.1.4; Sigma) at 37°C in 1 mL 0.2 M sodium acetate (pH 4.5) for 16 h. After hydrolysis, 9 mL of 0.1 M sodium phosphate and, as internal standard, 80 ng 17,17-[^{2}H]GA_9 were added. The pH was adjusted to 2.8 and the buffer phase was extracted three times with 5 mL EtOAc. The combined EtOAc phase was washed one time with 5 mL of water, acidified with 6 M HCl to pH 3.0, and then evaporated under a stream of N_2 and further purified by HPLC as described below.

HPLC

The acidic, EtOAc-soluble fractions and the hydrolysed putative GA_9-conjugate fractions were purified by normal phase HPLC (16). The system consisted of two Waters M501 pumps connected to a 200 × 4.6 mm i.d. column packed with 5 μm Nucleosil NO_2, via a Waters U6K injector. The mobile phase was a linear gradient from 75% *n*-heptane, half-saturated with 0.5 M formic acid in water, and 25% EtOAc:water:formic acid (98.5:1:0.5, v/v), to 100% EtOAc:water:formic acid (98.5:1:0.5, v/v). The gradient sweep time was 35 min, and the flow rate was 2 mL min^{-1}. The

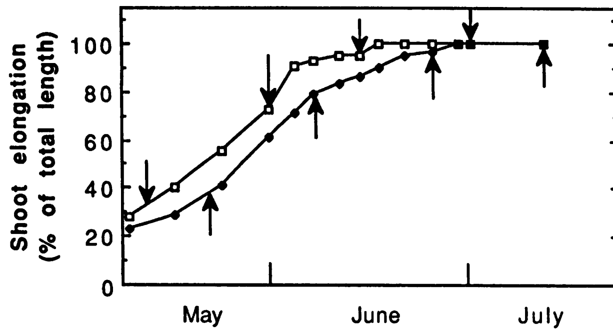

Figure 1. Shoot elongation growth of grafts of Sitka spruce treated with hot and dry conditions (□) and cool and wet conditions (◆). The arrows indicate sampling times.

fractions corresponding to the elution volume of GA_1, 48 to 54 mL; GA_3, 48 to 54 mL; GA_4, 28 to 34 mL; GA_9, 10 to 16 mL, were collected and analyzed by GC-MS as described below. For purification of the hydrolysed GA_9-conjugate the mobile phase was 80% *n*-heptane, half-saturated with 0.5 M formic acid in water, and 20% EtOAc:water:formic acid (98.5:1:0.5, v/v). The column was isocratically eluted and the fraction corresponding to GA_9, 14 to 20 mL, was collected and analyzed by GC-MS as described below.

GC-MS

The fractions containing the GAs were methylated with ethereal diazomethane and, except GA_9-Me, after evaporation, silylated by adding 50 μL of N,O-bis(trimethylsilyl)-trifluoroacetamide with 1% (v/v) trimethylchlorosilane and heating to 80°C for 20 min. The samples were then evaporated in a Speed-Vac concentrator, dissolved in *n*-heptane, and injected splitless into a Hewlett Packard 5890 GC equipped with a fused silica glass capillary column, SE-30 chemical bonded phase 0.25 μm, 25 m long, i.d. 0.25 mm (Quadrex). The injector temperature was 230°C and the column temperature was 80°C for 2 min. The temperature was then increased by 20°C min^{-1} to 200°C and by 4°C min^{-1} to 250°C. The column effluent was led to the ion source of a Hewlett Packard 5970 mass selective detector. The interface temperature was 275°C and the electron energy was 70 eV. Each sample was chromatographed two times in SIM mode and data were processed by a Hewlett Packard 9133 data system. For each GA, two characteristic ions and the deuterated analogs were recorded, GA_1 m/z: 491, 493, 506, 508; GA_3, m/z: 489, 491, 504, 506; GA_4, m/z: 284, 286, 418, 420; GA_9, m/z: 270, 272, 298, 300. A calibration curve of the response ratio to amount ratio of the nondeuterated to deuterated GA was constructed for each GA in the same way. The dwell time was 100 ms.

RESULTS AND DISCUSSION

Sampling times for the cool, wet- and hot, dry-treated grafts were separated in time due to different rates of elongation (Fig. 1). The two different materials were, however, in the same phenological stage of development when the samples were taken. The most important periods for flowering stimulation are represented by the third and fourth sampling times: slightly before bud type determination starts, in this case when the shoots had elongated about 95%, and during the bud type determination period, in this case 17 d later (17). The second sampling time, when the shoots had elongated about 70% of the final length, corresponds to the period of most rapid elongation.

The cellulase hydrolyzable putative GA_9-conjugate was quantitated using [^{2}H]GA_9 as an internal standard, added after separation of the conjugate from the free GAs according to Hedden (7). Even though the recovery corrections did not cover the whole purification procedure, the precision of the triplicate analyses was acceptable, 7–18%, and therefore the data are considered reliable. The conjugate was only detected in the needles (Fig. 2) as was the case in a study quantitating GAs in needles and shoot stems of good- and poor-flowering clones of Sitka spruce (15). The amounts were highest in samples collected when the shoots had elongated about 30% of the final length, about 33 ng g^{-1} fresh weight in materials from both treatments. As shoot elongation proceeded, the amounts decreased, and samples from the last sampling time, 17 d after the elongation had ceased (fourth sampling time), contain 13 and 6 ng g^{-1} fresh weight for the hot, dry- and cool, wet-treated materials, respectively. The results, indicating higher amounts of putative GA_9-conjugate during later periods of shoot elongation in grafts grown under flower inductive condition, are in agreement with previous results showing higher amounts of the putative GA_9-conjugate in needles of good-flowering clones than in needles of poor-flowering clones when the samples were taken at about 95% shoot extension (15). The decrease in amounts during shoot elongation and the absence of detectable amounts of GA_9-conjugate in the shoot stems, strongly imply that the conjugate is a storage form of GA_9 that can be hydrolysed yielding free GA_9 (30).

The concentration of GA_9 in the hot, dry-treated material

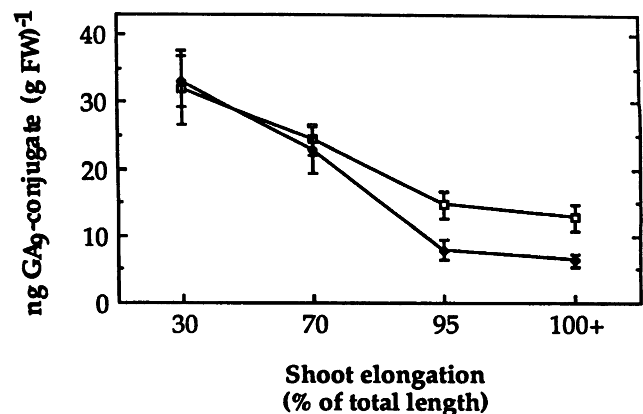

Figure 2. Quantitation of cellulase hydrolyzable GA_9-conjugate in needles of hot and dry (□) and cool and wet (◆) treated grafts of Sitka spruce, at four different occasions during the shoot elongation period. Samples denoted 100+ were collected 17 d after cessation of shoot elongation.

shows a similar pattern of changes during the shoot elongation in both needles and stems (Fig. 3). On sampling date 1 the needles contained 22 ng GA_9 g^{-1} fresh weight; the stems, 1 ng g^{-1} fresh weight. The concentrations had increased at the phase of rapid elongation to 32 ng g^{-1} fresh weight in the needles and 22 ng g^{-1} fresh weight in the shoot stems. When the shoots had elongated about 95% of the final length the concentrations had decreased to 11 ng g^{-1} fresh weight and 9 ng g^{-1} fresh weight, in needles and stems, respectively. In the cool, wet-treated material the highest concentrations of GA_9 were estimated in samples from the third sampling occasion, *i.e.* later than in the hot, dry-treated material. Compared with the hot, dry-treated material, the concentration of GA_9 was significantly lower in the cool, wet-treated material, except in samples from the first sampling occasion, where equal amounts of GA_9 in both materials was found. The positive correlation between the amount of GA_9 and flowering stimulatory treatment or flowering was also found in another study where the content of GA_9 was analyzed in good- and poor-flowering clones of Sitka spruce during the period of flower bud differentiation (15).

GA_4 was only detected in the needles (Fig. 4). The concentration in the hot, dry-treated material decreased from 1.3 ng g^{-1} fresh weight at the first sampling time to 0.5 ng g^{-1} fresh weight in the final sample. The amount in the cool, wet-treated material was significantly lower in the sample from the first sampling occasion, 0.8 ng g^{-1} fresh weight, but no differences between the two treatments were observed during the later phases of shoot elongation, when the flower bud

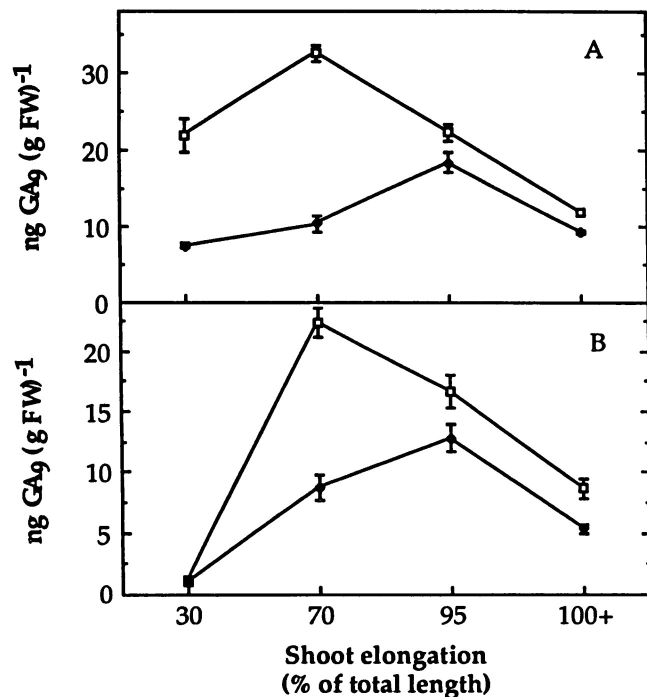

Figure 3. Quantitation of GA_9 in needles (A) and stems (B) of hot and dry (□) and cool and wet (◆) treated grafts of Sitka spruce, at four different occasions during the shoot elongation period. Samples of 100+ were collected 17 d after cessation of shoot elongation.

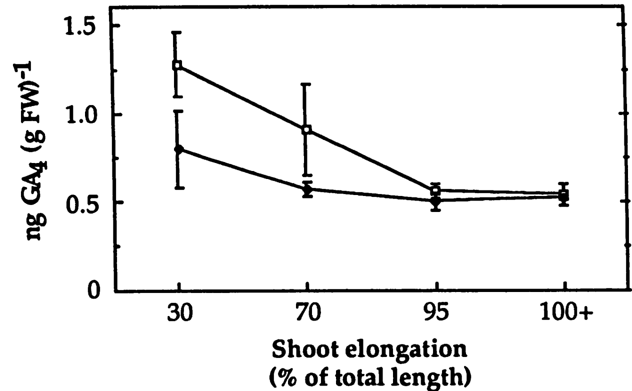

Figure 4. Quantitation of GA_4 in needles of hot and dry (□) and cool and wet (◆) treated grafts of Sitka spruce, at four different occasions during the shoot elongation period. Samples denoted 100+ were collected 17 d after cessation of shoot elongation.

differentiation is thought to occur (17). In another study comparing good- and poor-flowering clones, a positive correlation between GA_4 concentration slightly before the time of bud differentiation and flowering could not be found either (15). The amounts of GA_4 generally were small, possibly due to a high turnover rate.

The polar GAs, GA_3, and GA_1 could not be detected in the samples collected when the shoots had elongated about 30% of the final length (Figs. 5 and 6). The concentration of GA_1 (Fig. 5) in the needles increased during the time of rapid elongation to about 6 ng g^{-1} fresh weight in the hot, dry-treated material and to 4 ng g^{-1} fresh weight in the cool, wet-treated material, as did GA_1 concentration in stems, although without differences between the two treatments. Samples from the last two collection dates contained significantly lower concentrations of GA_1 in both needles and stems of hot, dry-treated material, than for cool, wet-treated material. The concentration of GA_3 (Fig. 6) increased to about 8 ng g^{-1} fresh weight in the needles of the materials from both treatments taken during the rapid elongation phase (70% of the final length), but thereafter decreased to below detection limits in the final samples, the decrease being more rapid in the hot, dry-treated material. The concentrations of GA_3 in the stems were lower in the needles, but showed the same fluctuation pattern as in the needles. The positive correlation between the high concentrations of GA_3 slightly before the start of bud differentiation and poor flowering was also found when GA-concentrations of good- and poor-flowering clones of Sitka spruce were compared (15).

This study indicates that there are differences in the concentrations of GAs in shoots of Sitka spruce subjected to flower inductive and nonflower inductive treatments. The results are in many ways consistent with previous results (15), where good-flowering clones contained higher amounts of GA_9 and a GA_9-conjugate slightly before bud determination starts, than poor flowering clones and, as a consequence, had a higher capability to synthezise the physiologically important GA_4. Moritz *et al.* (14) showed that exogenously applied GA_9 was metabolized to GA_4 to a higher degree in grafts subjected

to a flower inductive treatment than in grafts subjected to a nonflower inductive treatment. Gibberellin A_4 and GA_7 are the most successfully used GAs for stimulating flowering (18). Since no correlation between the amounts of GA_4 or GA_7, which could not be detected at all, and flowering could be found we conclude that the turnover rate of GA_4 is of great importance. In order to study the turnover rate, it is necessary to estimate levels of the biologically inactive GAs as well (6). In several studies it has been shown that a 2β-hydroxylation of GA_4 to GA_{34} occurs in conifers (3, 10, 14, 32) and it has been suggested that under nonflower-inductive conditions the formation of GA_{34} from GA_4 is higher than under flower-inductive conditions (20).

In summary, it can be concluded that the highest concentration of GA_9, GA_1, and GA_3 is closely correlated with the time of most rapid shoot elongation. This correlation might indicate that the GAs at this time are preferentially used for controlling shoot extension (33). If the conditions are favorable for vegetative growth or if the material is of a poor flowering clone, bud differentiation may be directed preferentially toward vegetative buds due to a more rapid conversion of the less polar GAs, GA_9 and GA_4, to the more polar GAs, GA_1 and GA_3, the latter GAs being the known 'effectors' of vegetative growth in maize, and probably in many higher plants (23). If the vegetative growth is restricted in any way, the conversion of less polar GAs to polar GAs is blocked, therefore resulting in a decreased concentration of polar GAs and also an increased availability of the less polar GAs, especially the physiologically important GA_4.

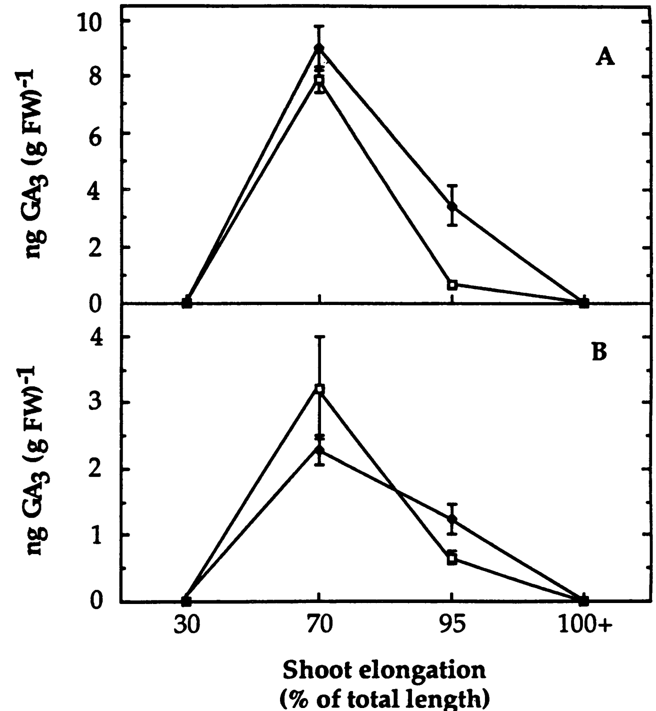

Figure 6. Quantitation of GA_3 in needles (A) and stems (B) of hot and dry (□) and cool and wet (◆) treated grafts of Sitka spruce, at four different occasions during the shoot elongation period. Samples of 100+ were collected 17 d after cessation of shoot elongation.

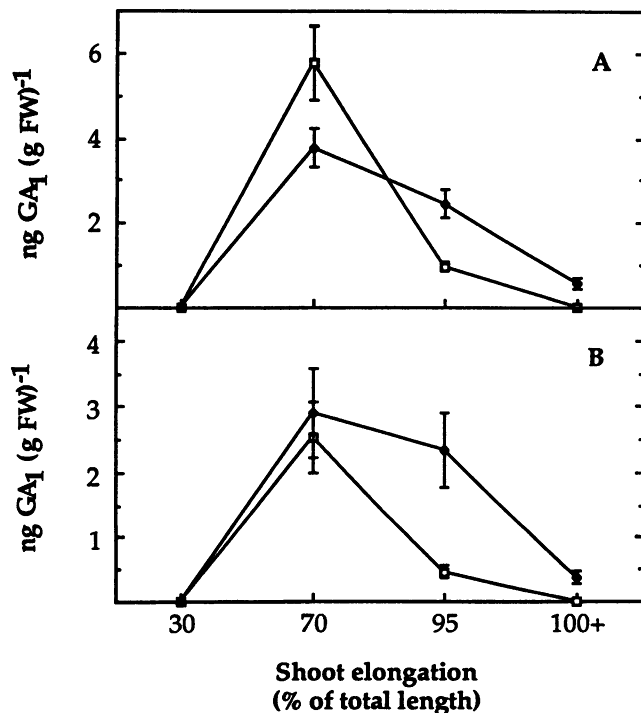

Figure 5. Quantitation of GA_1 in needles (A) and stems (B) of hot and dry (□) and cool and wet (◆) treated grafts of Sitka spruce, at four different occasions during the shoot elongation period. Samples denoted 100+ were collected 17 d after cessation of shoot elongation.

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