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QUANTITATIVE COMPARISON OF GIBBERELLINS 19, 20, 29, 1 AND 8 IN PHOTOPERIODIC ECOTYPES OF *BETULA PENDULA* ROTH.

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ABSTRACT: The homeostasis of endogenous gibberellins (GAs) of plants is maintained by the GAs themselves, via feedback or feedforward mechanisms, and/or by environmental factors, of which the most important is photoperiod. To study the effect of photoperiod on quantities of GAs of latitudinal ecotypes of trees, the levels of gibberellins A_{19}, A_{20}, A_{29}, A_1, and A_8 were determined in three ecotypes of *Betula pendula* Roth. grown under different photoperiods. Generally, all ecotypes, when grown in long days (LD), had similar GA levels. The levels of the quantified GAs from any one of the ecotypes decreased in the following order: $GA_8 > GA_{19} > GA_1 > GA_{29} > GA_{20}$. However, photoperiod influenced the level of GA_1. GA_1 levels in short day (SD)-grown plants were lower than the LD-grown ones, reduction being pronounced in the northern ecotype. Although the differences in GA_1 levels between LD and SD were very small, SD-treated plants were significantly shorter than LD-treated ones in all ecotypes. It seems that LD not only increased the GA_1 level, but also elevated tissue sensitivity to GA_1.The level of GA_1 in LD-grown plants decreased during the night-time and increased during the light period, with its maximum being towards the end of the light period. The rate of stem elongation was higher during the night than during the day period. Enhanced elongation during night could be a reflection of the build up of GA_1 level during the day.

Key words/phrases: Diurnal fluctuation; Endogenous gibberellins; Rhythm.

INTRODUCTION

Plants, throughout their developmental transitions, organ differentiation or responses to environmental conditions, have their growth regulated, at least in part, by changes in levels of endogenous phytohormones. One class of phytohormones, the gibberellins (GAs), are involved in regulating many processes, including seed germination, cell proliferation, stem elongation, flowering, anther development, seed and fruit growth (Hedden and Kamiya, 1997; Huang *et al.*, 1998). Of the 136 GAs that have been identified (Nakayama *et al.*, 2001), GA_1 and GA_4 appear to be the most common of the growth active (growth promoting) 3β-hydroxylated GAs.

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The biosynthesis of the growth active GAs can be controlled by light (photoperiod) and oxidation at carbon number 20 which is a key and early regulation step (Graebe, 1987; Junttila *et al.*, 1997; Hedden and Proebsting, 1999; Ait-Ali *et al.*, 1999). The activity of GA C20-oxidase, which catalyses the metabolism of GA_{53} through GA_{44} and GA_{19} to GA_{20}, increases in light and decreases in dark for at least some plants (Ait-Ali *et. al.*, 1999). For example, transfer of the rosette plant *Silene armeria* L. from short days (SD) to long days (LD) caused GA_1 content to increase several folds by elevating the GA C20-oxidase activity (Talon and Zeevaart, 1990). In addition, LD may also increase the sensitivity of the plant tissue to GA (Talon and Zeevaart, 1990). In contrast, SD induces cessation of shoot growth, bud formation and dormancy, and these responses are correlated with reductions in the levels of 13-hydroxylated GAs (Olsen *et al.*, 1995a; 1995b; 1997a and 1997b). Growth cessation in SD can be prevented by the application of exogenous GAs (Junttila, 1976; 1991; Olsen, 1995; Berhanu Abraha, 2002; Mølmann *et al.*, 2003) indicating a photoperiodic control of GA biosynthesis and / or activity.

In many temperate woody species including *Populus* sp., *Alunus* sp. and *Betula* sp., GA_1 seems to be the major bioactive GA and the 13-hydroxylation pathway is probably the main pathway for GA biosynthesis (Davis *et al.*, 1985; Junttila *et al.*, 1988; 1991; Junttila, 1991; Zanewich and Rood, 1994; Olsen *et al.*, 1994, 1995b). Tree species with a wide geographical distribution have developed photoperiodic ecotypes that are closely adapted to local environmental conditions, mainly daylength and temperature (Junttila and Nilsen, 1993). The photoperiodic control of stem extension growth can readily be related to the control of GA biosynthesis. Hence, it is logical to expect that there might be qualitative and quantitative differences in GA levels among ecotypes of *Betula pendula* Roth. that are adapted to different photoperiods. Although earlier studies indicated an effect of photoperiod on endogenous GAs of woody species (Junttila, 1980; 1991; 1993; Junttila and Jensen, 1988; Rood and Junttila, 1989; Junttila *et al.*, 1992; Olsen *et al.*, 1995a and b), such effects have not been studied comprehensively in various ecotypes with contrasting photoperiodic responses. Thus, we have examined levels of gibberellins A_8, A_1, A_{29}, A_{20}, and A_{19} from three latitudinal ecotypes of *B. pendula* grown under LD and SD conditions. The relationship between levels of GA_1 and the inherent growth potential of each ecotype is also discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

This experiment was conducted in the phytotron at the University of Tromsø. Seedlings of three latitudinal ecotypes of *Betula pendula* Roth were used. Seeds were collected from southern Norway (Hurum, 59° N), the middle of Norway (Sticklestad, 64° N) and northern Norway (Rognan, 67° N). They were stratified in moist sand for about a month at 0.5°C and germinated in continuous light (180-200 μmol $m^{-2}s^{-1}$) from fluorescent tubes (Philips TLD 58/840) and incandescent bulbs at 18°C (Berhanu Abraha, 2002). The quantity and quality of light used was measured using a data loger (Model L_1-1000, Serial No. LDL 3527, USA) with the light sensor, LI-CDR Quantum, Q2012 attached to it. The spectral distribution of the photosynthetically active radiation (PAR) was as shown on Fig. 1.

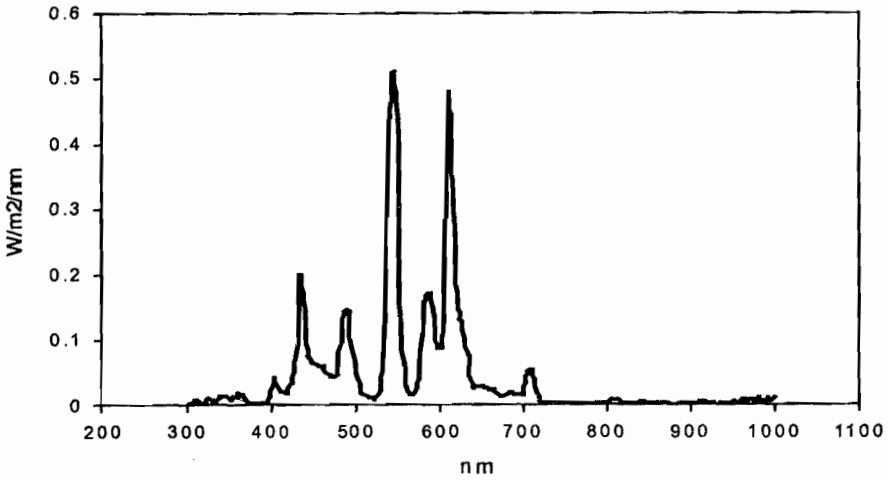

Fig. 1. Spectral photon distribution of the fluorescent light (Phillips TLD 58/840) used in the growth chambers (trolleys).

The stratified seeds were sown on Peat, Perlite, and Sand (6:1:1, V/V) on plastic trays covered with plastic sheets. After 7 days, the plastic covers were removed and seedlings were watered every other day. The seedlings were potted 8 days after germination and grown in 12 h PAR and 12 h light extension with incandescent bulbs. After about 4 weeks of growth (to a height of 5-6 cm), plants were transferred to two daylength treatments (SDs of 12 h and LDs of 24 h). The main light period of 12 h (08:00-20:00 h) had a photon flux density of 188-192 μmol $m^{-2}s^{-1}$ at 400-750 nm from

fluorescent tubes (Philips TLD 58/840) and incandescent bulbs. The length of the day, in LD treatments, was extended beyond 12 h with incandescent bulbs (10 μmol $m^{-2}s^{-1}$, Osram).

The height of 15-16 plants for each ecotype from each daylength treatment was measured throughout the experiments. Samples for GA analyses consisted of stem tips from 15 plants and tissue was harvested in duplicate batches in the middle of the 12 h main light period. To determine whether growth rates of *B. pendula* during the daytime (PAR) and during the night-time (low intensity light extension) differ, plants of the northern ecotype were used. Plants, 2-3 cm tall, were treated with PAR (160 μmol $m^{-2}s^{-1}$) during the day and incandescent light (10 μmol $m^{-2}s^{-1}$) during the night. Stem length was recorded at the beginning of each of the PAR and the light extension periods for 7 days.

Extraction and Purification of GAs

Quantitative analyses of GAs were made as described in Olsen *et al.*, (1994) and Olsen and Junttila (2002). About 1 gram (fresh weight) of shoot apex (excluding young leaves) was homogenised with a Warren blender in 80% methanol and extracted over night at 4°C with continuous shaking. As internal standards, deutrated (17,17-$^2H_2$) gibberellins A_8, A_1, A_{29}, A_{20}, and A_{19} (20 ng for about 1 g fresh weight tissue) were added to the homogenised samples prior to extraction. After filtration, the plant residue was re-extracted at 4°C for about 4 h with continuous shaking. The extracts were reduced to the water phase in a rotavapour at 40°C. The water phase was frozen at –20°C over night and thawed at room temperature. It was filtered, adjusted to pH 2.8 and partitioned three times against ethyl acetate. The ethyl acetate was evaporated to dryness *in vacou* (40°C). The residue was dissolved in about 10 ml water, adjusted to pH 8 and applied to a 10 ml QAE-Sephadex A-25 column (Pharmacia, Sweden). The column was equilibrated and washed with about 40 ml of water at pH 8. The GAs were then eluted with 50 ml 0.2 M formic acid directly on to a pre-equilibrated 0.5 g Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA). The GAs were then eluted from the C_{18} column with 4 ml 80% methanol and the solvent evaporated in a speedvac. The dry samples were methylated using etheral diazo-methane and purified further on 0.1 g Bond Elute Cartridges (aminopropylsilyl, APS) (Varian Associates, Harbour city, CA, USA).

The samples were then dissolved in 50 μl 100% methanol plus 50 μl water and GAs separated by reversed phase HPLC (Waters Associates, Milford, MA, USA) using a NOVAC-PAK C_{18} column (Waters, 100 mm x 8 mm, id.

4 μm). The mobile phase was a linear gradient of 25 min. from 20 to 80% methanol in water (both acidified with 1% aqueous acetic acid) at a flow rate of 2 ml min^{-1} (25°C). Fractions were collected at 1-minute intervals, combined according to the elution of standards and evaporated to dryness.

Combined GC-MS

After HPLC all samples were trimethylsilylated using N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (12 μl) and heated to 100°C for 10 min. They were then injected in GC-MS and quantified by selected ion monitoring (GC-MS-SIM) mode.

RESULTS AND DISCUSSION

GA Levels in LD-grown *Betula pendula* Ecotypes

Although the level of GA_{20} in the intermediate ecotype was less than that found in contrasting ecotypes, there was no significant difference (at 1% significance level) in GA_{19}, GA_{29}, GA_1, and GA_8 between ecotypes (Table 1). Generally, levels of the quantified GAs decreased in following order in all ecotypes: $GA_8 > GA_{19} > GA_1 > GA_{29} > GA_{20}$ (Table 1). This was different from that reported by Zanewich and Rood (1994), where the order decreased in the following manner: $GA_{20} > GA_1 > GA_{19} > GA_8$. Plant age and growth environments might have contributed to such differences. Zanewhich and Rood (1994) used branches from mature outdoor-grown trees, and in this experiment very young seedlings grown in a controlled, relatively low light environment were used.

Table 1 Level of endogenous gibberellins (ng/g FW) in ecotypes of *B. pendula* grown in LDs. Each point is a mean value of 4 replicate tissue samples. Results are mean ± standard deviation.

Ecotype	GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8
Southern	5.4 ± 0.8	1.4 ± 0.6	1.6 ± 0.5	2.2 ± 0.6	6.5 ± 0.7
Intermediate	5.0 ± 0.5	0.8 ± 0.2	2.0 ± 0.7	2.7 ± 1.1	5.0 ± 1.0
Northern	5.9 ± 1.1	1.5 ± 0.6	1.7 ± 0.6	2.4 ± 0.9	6.1 ± 0.9

Effect of Photoperiod on GA Levels in Ecotypes of *B. pendula*

In LDs, the levels of GA_1 in the southern and the intermediate ecotypes were similar, almost constant for about 18 days, while in the northern ecotype there was a slight reduction of GA_1 with an increasing age of the plants (Fig. 2). In SDs, there was an obvious decrease of GA_1 in all ecotypes, with the greatest reduction being in the northern ecotype relative to the southern ecotype. This might be related to differences in their critical daylength (Berhanu Abraha, 2002). A 12 h photoperiod is a more extreme

SD for the northern and the intermediate ecotypes than for the southern ecotype.

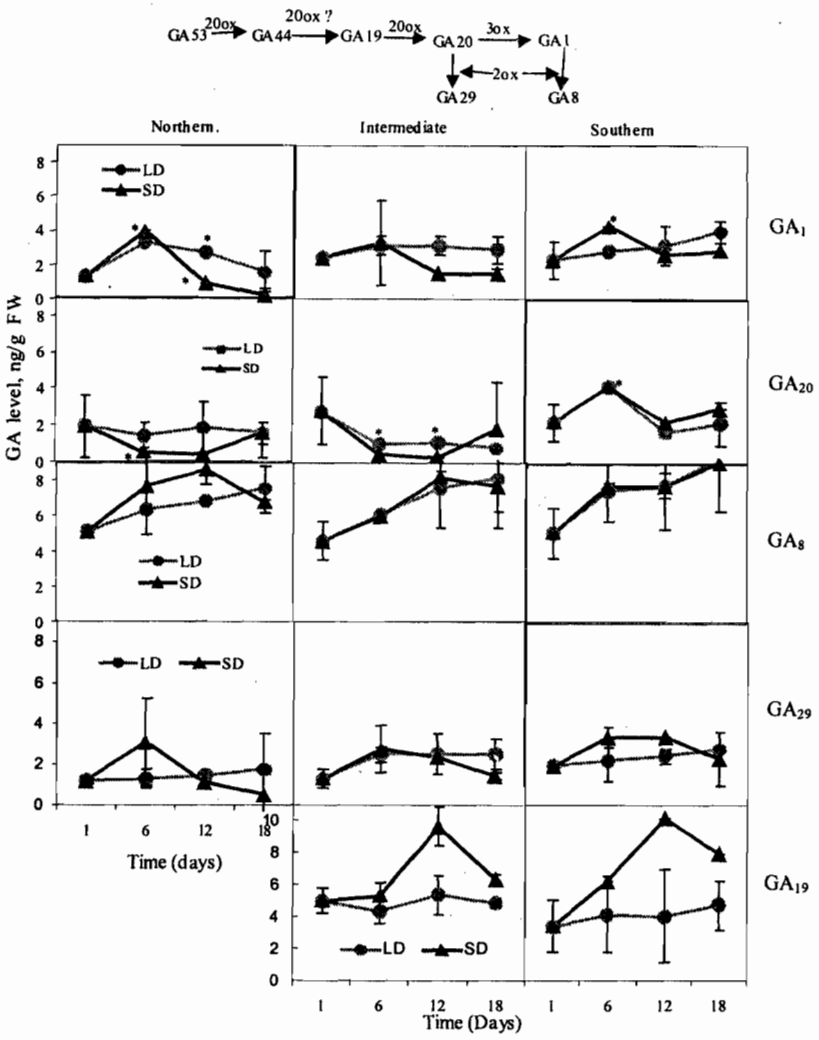

Fig. 2. GA levels in apical stem tissue of three *B. pendula* ecotypes as affected by photoperiod. LD treatments were 12 h PAR (188-192 μmol $m^{-2}s^{-1}$) extended with 12 h incandescent (10 μmol $m^{-2}s^{-1}$), while SD treatments had 12 h PAR only. Apical stems of 15 plants were used for the analyses. Sampling was accomplished at about mid-day and two independent replicate samples were assayed for each time point, except those marked with asterisk (*). Bars show difference between the analyzed replicate samples.

There was no difference in the levels of GA_{20} between LD- and SD-grown plants in any of the ecotypes, except that SD-grown plants of the northern ecotype had lower GA_{20} levels at day 6 and day 12 than LD-grown plants. Similarly, there was no consistent effect of photoperiod on levels of GA_8

and GA_{29} in any of the ecotype. However, there was accumulation of GA_{19} in SDs in the southern and intermediate ecotypes (Fig. 2). This accumulation of GA_{19} clearly showed that photoperiod had an influence on the metabolism of this C20 gibberellin as was shown much earlier for spinach (Talon and Zeevaart, 1990). Such an effect has also been shown from a metabolic study of ^{14}C-labelled GA_{19} in *B. pendula* (Berhanu Abraha, 2002).

Correlation Between Vegetative Growth and Levels of GA_1

While the difference in quantity of the growth active GA_1 in SD- and LD-grown plants was very small in all ecotypes (Fig. 2), differences in growth extension were very large (Fig. 3). Plants treated with 6 SDs stopped growing, while those in LDs continued growing in an almost linear fashion. These results indicate that LD likely promotes growth of the plants by maintaining homeostasis for the required level of the growth active GA_1. However, there is also the issue of tissue sensitivity to GAs. This was addressed by studying the effect of photoperiod on the quantity of endogenous GA_1 vis-à-vis stem elongation in a herbaceous plant, *Silene armeria* (Talon and Zeevaart, 1992). Thus, in *S. armeria*, there was more GA_1 when the apices were kept in the dark and the mature leaves in SD, compared to when the apices were kept in SD and the mature leaves in LD (Talon and Zeevaart, 1992). However, the former treatment did not result in stem elongation while the later one did. The authors suggested that LDs resulted in transmission of signals from the leaves to the apex, which increased the sensitivity of the apical tissue to the available endogenous GAs. Such suggestions had also been made earlier (Cleland and Zeevaart, 1970; Talon and Zeevaart, 1990). Although the site of daylength signal perception in *Betula* might also be in the apical tissues (Wareing, 1954), the suggestions made by the above authors that LD increased sensitivity of elongating tissues to GAs seems meaningful in *B. pendula,* too. That is, LDs might have a dual effect on growth of *B. pendula* ecotypes: not only causing an increase in the amount of active GA content by modulating the activity of enzymes of GA metabolism, but also by enhancing sensitivity of the apical meristem to the growth active GA_1. Generally, the number of LDs and stem length of *B. pendula* (the southern ecotype) correlated strongly ($r = 0.90$) compared to level of endogenous GA_1 versus stem length ($r = 0.75$), and number of LDs versus level of endogenous GA_1 ($r = 0.60$). Correlating stem length with the level of endogenous GA_1 and also number of LDs with level of endogenous GA_1 might not be feasible since plants regulate their GA metabolism to the optimum GA_1 level; that is, as growth continues, the

quantity of GA_1 may not exceed its required level. However, the relationship between number of LDs and stem elongation explains the role played by LDs, not only in driving the process of GA metabolism, but possibly also in enhancing tissue sensitivity to GA_1 (by an as yet unknown mechanism). In SD treated plants, no or very little association was seen between number of SDs, quantity of GA_1 and stem length.

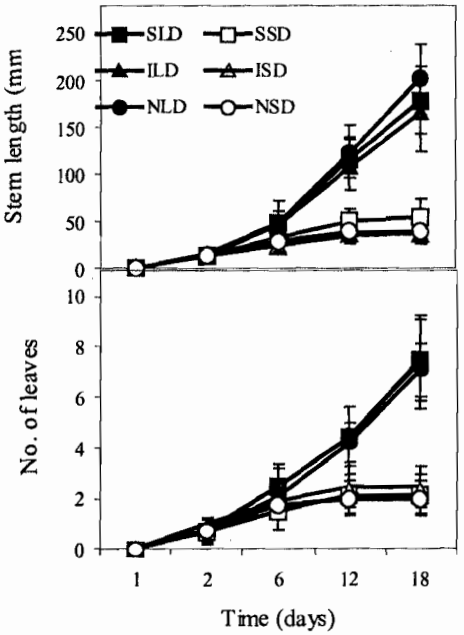

Fig. 3. Effect of daylength on stem elongation (above) and on production of new leaves (below) in three ecotypes of *B. pendula* grown under LD (12 h PAR plus 12 h incandescent light) or SD (12 h PAR) conditions. Each point is a mean of two experiments, each of which had 15-16 plants per ecotype and treatment. Error bars show standard deviations. S: southern, I: intermediate, N: northern ecotype.

Preliminary study on rhythmic accumulation of GA_1 level in *B. pendula* showed that GA_1 increased during the day reaching its maximum towards the end of the PAR period (Berhanu Abraha, 2002). After observing such a trend, the height increments of 20 individual plants grown in LD was measured in the morning (08:00 h) and in the evening (20:00 h) for seven days. In order to get measurable elongation growths per day; plants were grown in LDs. As shown in Fig. 4, elongation rate was faster during the daylength extension (incandescent light) than during the PAR light period. This could be related to the accumulation of GA_1 towards the end of the PAR period, or it could be a low light induced etiolating response.

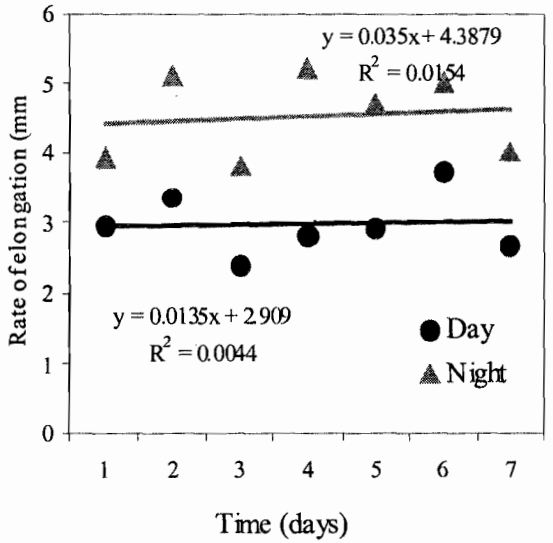

Fig. 4. Rate of stem elongation of *B. pendula* grown in LDs (12 h PAR plus 12 h incandescent light). Twenty seedlings were used in each treatment. Measurement of elongation rate was started when the plants reached the size of 2 to 3 cm and continued for 7 days. Rates of stem elongation during daylength extension (night-time, 20:00 h-08:00 h) and during the day-time (08:00-20:00 h) were measured using digital millimetre scale (Jocal, C.E. Johansson, Sweden).

CONCLUSION

Generally, the three ecotypes of *B. pendula* had similar levels of GA_{19}, GA_{29}, GA_1 and GA_8, although the intermediate ecotype had slightly reduced levels of GA_{20}. In all ecotypes, quantities of the analysed GAs in these very young seedlings decreased in the following order: $GA_8 > GA_{19} > GA_1 > GA_{29} > GA_{20}$. There was little difference in the levels of GA_{20}, GA_{29} and GA_8 between LD- and SD-treated plants. However, there was an accumulation of GA_{19} in SDs compared to LDs. This indicates that the metabolism of the C20 GA, GA_{19}, to the C19 GA, GA_{20}, is under the control of photoperiod in *B. pendula*. Moreover, day-length has a profound role on stem elongation by increasing the quantity of GA_1 and/or by increasing the sensitivity of the tissues to GA_1 by yet unknown mechanism.

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