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ORIGINAL PAPER

Shooting control in *Eucalyptus grandis* × *E. urophylla* hybrid: Comparative effects of 28-homocastasterone and a 5α-monofluoro derivative

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Abstract 28-Homocastasterone (28-HCTS), a brassinosteroid, was used to treat in vitro-grown shoots of a hybrid between *Eucalyptus grandis* and *E. urophylla*. Treated shoots showed enhanced elongation and formation of new main shoots (the shoots originating directly from the initial explant) at low doses. Coincidently, there was reduced elongation and formation of primary lateral shoots (shoots originating from the main shoot). However, a 5α-monofluoro derivative of 28-HCTS (5F-HCTS) was unable to either stimulate elongation or formation of new main shoots, although it did stimulate elongation of primary lateral shoots. In conclusion, it is quite apparent that exogenously supplied brassinosteroids are able to change shooting patterns in *Eucalyptus*. These findings have practical biotechnological applications, for example on the improvement of micropropagation techniques for clonal propagation of woody angiosperms.

Keywords 5F-HCTS · 28-HCTS · 28-homocastasterone · Apical dominance · Shoot elongation

Abbreviations

5F-HCTS	5fluoro-28-homocastasterone or (22R, 23R)-2α, 3α, 22, 23-tetrahydroxy-5α-fluorostigmastan-6-one
28-HCTS	28-homocastasterone or (22R, 23R)-2α, 3α, 22, 23-tetrahydroxy-5α-fluorostigmastan-6-one
BRs	brassinosteroids
MS	Murashige and Skoog (1962)

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Introduction

Brassinosteroids (BRs) comprise a class of polyhydroxylated steroids found throughout the plant kingdom. Analyses of mutants defective in BR synthesis or signaling have revealed a key role for BRs in plant growth and development. A broad spectrum of physiological responses can be elicited by exogenous application of BRs to intact plants and excised organs (Kauschmann et al.

1996), including increased cell elongation and division, bending, enhanced reproductive development, membrane polarization and proton pumping, a change in source/sink relationships, the modulation of stress (Clouse 1996; Clouse and Sasse 1998), modification of cell wall properties and control of aquaporin activities (Mussig 2005). Among this, stimulation of longitudinal growth of young tissues via cell elongation and cell division is considered to be the major biological effect of BRs (Clouse 1996). Additionally, BRs will stimulate vascular differentiation as the stem elongates (Clouse and Zurek 1991; Oda et al. 2005).

In the search to develop higher activity analogs of BRs, and elucidate biosynthetic and signal transduction pathways, several research groups have introduced structural modifications through various means such as alkylation, alkoxylation or halogenation (Saito et al. 1998; Back and Pharis 2003). Substitution of a hydrogen atom by fluorine in what was originally a carbon–hydrogen bond, causes only a small increase in size of the BR molecule, but it significantly increase electronegativity and hydrogen bonding potential (Kirk and Cohen 1971). Thus, fluorination of biologically active compounds often changes biological activity of the parent compound (Jones 1976; Todoroki et al. 1995). Because of this, it was expected that fluorination in the 5α-position of the steroidal structure would change the bioactivity of BRs, especially since previous reports indicated that substitutions such as hydroxylation at the 5α-position lowers bioactivity (Brosa et al. 1996; Brosa 1999; Ramirez et al. 2000). Although substantial efforts towards the development of methods to introduce fluorine groups in bioactive BRs have been carried out (see review in Back and Pharis 2003; Pereira-Netto et al. 2003), only a limited number of studies describing their biological activity have been reported.

Herein, we report on the effect of 28-homocastasterone (28-HCTS) on the formation of main shoots, and the reduced elongation and formation of primary lateral shoots in shoots of a hybrid *Eucalyptus grandis* × *E. urophylla* grown in vitro as shoot explants. Additionally, we show that the 5α-monofluoro derivative of 28-HCTS (5F-HCTS) induces responses remarkably different from those induced by the parent compound, 28-HCTS.

Materials and methods

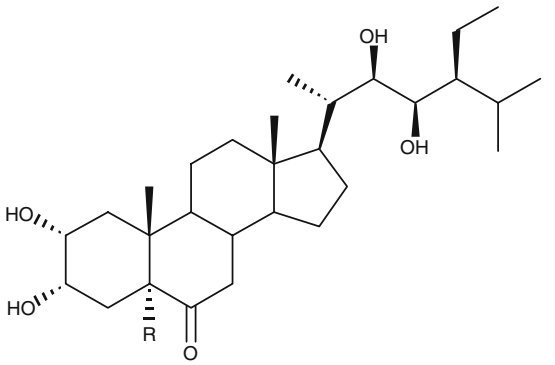
Shoot microculture

Seeds from a hybrid *Eucalyptus grandis* × *E. urophylla* were surface sterilized in a sterile-flow hood by immersion in 80% (v/v) ethanol for 2 min, followed by immersion in 0.5% (v/v) sodium hypochlorite for 5 min and three rinses in sterilized distilled water for 5 min. The seeds were then placed on 40 ml of MS (Murashige and Skoog 1962) basal medium supplemented with (μM): 555 myo-inositol, 4.06 nicotinic acid, 2.43 pyridoxine-HCl, 26.64 glycine, 6.25 thiamine-HCl, and also with 3% (w/v) sucrose and 0.6% (w/v) agar. The pH was adjusted to 5.7 prior to autoclaving.

Plant material to be employed in the brassinosteroid experiments was prepared from single node microcuttings measuring between 15 mm and 20 mm in length. These cuttings were taken from a 30-day-old aseptically grown shoot of a plantlet originated from seed as above described. The cuttings were subcultured and grown on 40 ml of MS basal medium, supplemented as described above, with an additional enrichment of 1 μM N^6-Benzyladenine and 6 μM α-naphthalene acetic acid.

Application of 28-homocastasterone and 5F-28-homocastasterone

28-Homocastasterone (28-HCTS, Fig. 1) and 5F-28-homocastasterone (5F-HCTS) were synthe-



1 R= H 28-homocastasterone

2 R= F 5α-fluoro-28-homocastasterone

Fig. 1 Chemical structure of 28-homocastasterone (28-HCTS) and 5F-HCTS

sized from stigmasterol acetate, as previously described (Ramirez et al. 2000). Fifteen-day-old shoots originated from single node microcuttings (see above) were dipped once into 70% (v/v) aques acetone containing known amounts of 28-HCTS or 5F-HCTS and left to dry over filter paper. Once the acetone had evaporated the shoots were returned to the culture medium. Control shoots were also dipped into 70% acetone.

Culture conditions

Cultures were maintained in a completely randomized design under continuous light provided by cool-white fluorescent tubes giving a photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1} at the culture level. Relative humidity was kept at 70 ± 5%. Air temperature around the cultures was 27.0 ± 1.0°C.

Statistical analyses

Each treatment consisted of five replicates (1 replicate = 1 culture vessel) with four explants per culture vessel. The data were analyzed using the SAS-JMP 5 software package (SAS Institute, Inc., Cary, NC, USA). If the ANOVA was significant, the differences between means for treatments were analyzed by Student–Neuman–Keuls pairwise comparison test. Each experiment was repeated at least two times.

For the purpose of this study, ''main shoots'' were defined as new shoots originating directly from the initial explant, and ''primary lateral shoots'' were defined as new shoots originating from the main shoots.

Results

Main shoots

A significant ($P = 0.05$) stimulation of main shoot (the shoots originating directly from the initial explant) elongation was found for shoots treated with the 10 mg l^{-1} solution of 28-HCTS (Fig. 2). For shoots treated with the 5-fluoro derivative of 28-HCTS, no significant change in main shoot elongation was found.

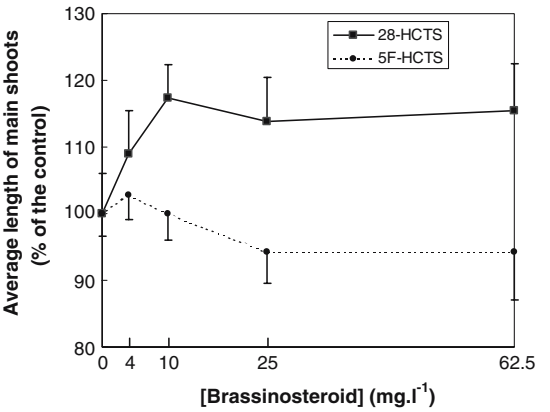


Fig. 2 Effect of 28-HCTS and 5α-fluoro-HCTS on the average length of newly formed main shoots. Averages were statistically significant ($P = 0.05$) only for the 10 mg l^{-1} concentration of 28-HCTS. Vertical bars indicate ± standard error (shown just in one direction for clarity). Average length of main shoots was 13.3 and 14.3 mm, respectively, for 28-HCTS and 5α-fluoro-HCTS controls (0 mg l^{-1})

Interestingly, treatment of shoots with 4 and 10 mg l^{-1} 28-HCTS concentrations led to an increase in the number of main shoots formed (Fig. 3), although the effect was statistically significant ($P = 0.05$) only for the 10 mg l^{-1} concentration. At very high concentrations of 28-HCTS (62.5 mg l^{-1}) a reduction in the number

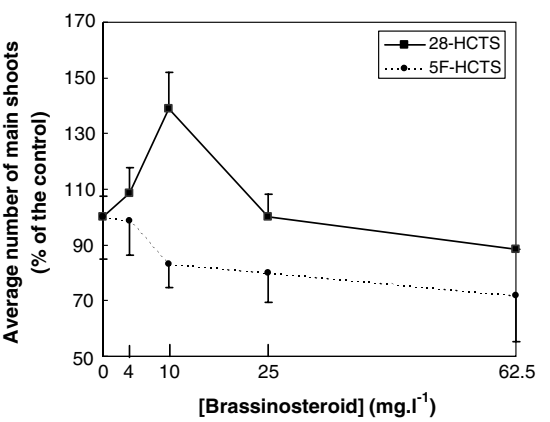


Fig. 3 Effect of 28-HCTS and 5α-fluoro-HCTS on the average number of newly formed main shoots. Averages were statistically significant ($P = 0.05$) only for the 10 mg l^{-1} concentration of 28-HCTS. Vertical bars indicate ± standard error (shown just in one direction for clarity). Average number of main shoots was 2.3 and 2.9, respectively, for 28-HCTS and 5α-fluoro-HCTS controls (0 mg l^{-1})

of main shoots formed occurred, relative to the controls (shoots treated with acetone, only). In fact, a progressive reduction in the average number of main shoots useful for micropropagation (minimum length of 15 mm) was seen for shoots treated with the higher concentrations of 5F-HCTS, although this reduction was not statistically significant.

Primary lateral shoots

In contrast to what we saw for main shoots, treatment with the higher concentrations of 28-HCTS led to inhibition of primary lateral shoots (shoots originating from the main shoot) elongation. This inhibitory effect was statistically significant ($P = 0.05$), however, only for the 25 mg l^{-1} concentration (Fig. 4). Surprisingly, a progressive enhancement in the average length of primary lateral shoots was seen for shoots treated with increasing concentrations of 5F-HCTS, up to 25 mg l^{-1} (significant at $P = 0.05$ only for the 25 mg l^{-1} concentration).

Both 28-HCTS and 5F-HCTS negatively affected formation of primary lateral shoots (Fig. 5). Increased concentrations of 28-HCTS and 5F-HCTS thus led to a progressive reduction in the number of primary lateral shoots formed during the culture cycle. However, at the 25 mg l^{-1} concentration, 28-HCTS was significantly ($P = 0.05$) more effective in inhibiting primary lateral shoots formation than 5F-HCTS.

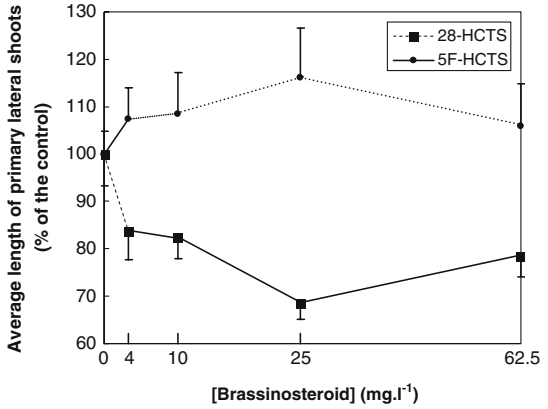


Fig. 4 Effect of 28-HCTS and 5α-fluoro-HCTS on the average length of newly formed primary lateral shoots. Averages were statistically significant ($P = 0.05$) only for the 25 mg l^{-1} concentration of 28-HCTS and 5F-HCTS. Vertical bars indicate ± standard error (shown just in one direction for clarity). Average length of primary lateral shoots was 10.7 and 8.5 mm, respectively, for 28-HCTS and 5α-fluoro-HCTS controls (0 mg l^{-1})

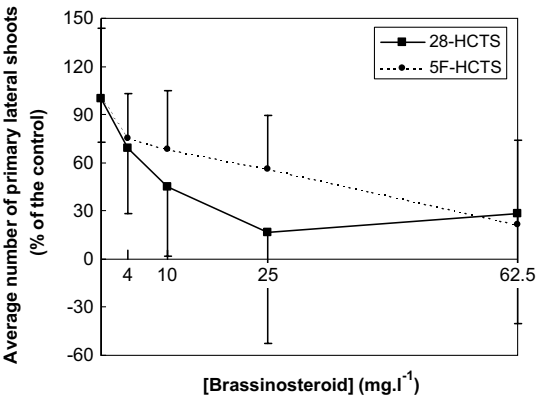


Fig. 5 Effect of 28-HCTS and 5α-fluoro-HCTS on the average number of newly formed primary lateral shoots. Averages were statistically significant ($P = 0.05$) only for the 25 mg l^{-1} concentration of 28-HCTS. Vertical bars indicate ± standard error (shown just in one direction for clarity). Average number of primary lateral shoots was 0.8 for both, 28-HCTS and 5α-fluoro-HCTS controls (0 mg l^{-1})

Discussion

Both 28-HCTS and its 5α-monofluoro derivative were shown to change shooting patterns in in vitro-grown shoots of a hybrid *E. grandis* × *E. urophylla*.

The biological properties of fluorinated derivatives of another class of hormones known to regulate stem elongation, gibberellins, are dependent upon the degree of fluorination, the concentration, and the type of bioassay used to access the biological activity (Jones 1976). Because of their high electronegativity, monofluoro analogues occasionally bind enzymes irreversibly, which might have a fatal effect on the organism (Welch 1987). Only few reports on the effects of fluoroBRs have been published. For example, substitution of a 5α-hydroxy group by a 5α-fluoro group has been reported to enhance cytotoxicity of (22S,23S)-5α-3β,22,23-trihydroxystigmastan-6-one against measles virus in a virus-yield reduction assay (Wachsman et al. 2002). In another example, 5α-fluorotyphasterol has been

found to be more active than typhasterol in the rice lamina inclination test (Ramirez et al. 2000). In the present study, our finding that 28-HCTS was able to stimulate elongation and formation of main shoots in the *Eucalyptus* hybrid, prompted us to test the 5α-fluoro derivative. However, 5F-HCTS was unable to either stimulate elongation or formation of new main shoots, although it did stimulate elongation of primary lateral shoots. Thus, 28-HCTS and 5F-HCTS induced contrasting responses. A reduced ability of the 5F derivative of 28-HCTS to bind to the BR receptor might explain the inability of 5F-HCTS to stimulate elongation and formation of main shoots in our system. However, that does not explain how 5F-HCTS stimulated the elongation of primary lateral shoots. Perhaps there are more than a single receptor site for BRs (Clouse 2002) and these mediate different responses to BRs? Although BRs are known to stimulate elongation of young tissues (reviewed in Clouse 1996), they have also been reported to inhibit shoot elongation, i.e. brassinolide and 24-epibrassinolide inhibit shoot (tiller) elongation in species such as rice (*Oryza sativa*) (Chon et al. 2000) and pea (*Pisum sativum*) (Kohout et al. 1991), respectively. BRs have also been known to stimulate ethylene biosynthesis in various systems (Arteca et al. 1991). Since the threshold ethylene concentrations affecting the release of axillary bud from inhibition and subsequent bud elongation may differ among species (Yeang and Hillman 1984), a possible way 5F-HCTS or 28-HCTS inhibit main or primary lateral shoot elongation, and perhaps also inhibit formation of primary lateral shoots, would be through a stimulation of ethylene production. However, this hypothesis still needs to be tested.

We would speculate that the effects of BRs and their analogues on shoot formation and elongation depend on the extent to which these molecules satisfy the structural requirements of BR receptors, or influence BR biosynthetic enzymes. In our system, the differential responses seen for 28-HCTS and its 5α-fluoro substituent on shoots of *Eucalyptus* described in this paper suggest either different BR biosynthetic routes, differential chemical stability or perhaps different receptor sites for each 28-HCTS and 5F-HCTS in our system.

The tendency for 5α-fluoro HCTS to be inhibitory while 28-HCTS is promotive towards main shoot growth and most interestingly, the promotive effect of 5α-fluoro HCTS on elongation of primary lateral shoots requires some comment. Fluorine has the smallest van der Waal's radius after hydrogen, and the largest electronegativity. It may thus act as a hydrogen mimic with regard to size and as a hydroxyl mimic with regard to electronegativity (Saito et al. 1998). In addition, the C–F bond is physicochemically similar to the C–OH bond, rather than the C–H bond (Todoroki et al. 1995; Welch 1987; Penglis 1981). Thus, fluorine could be considered as being equivalent to the oxygen of the hydroxyl group. Brosa (1999) have reported reduced bioactivity for 5α-OH substituted analogs, relative to the parent brassinosteroid in the rice lamina inclination test. Those authors suggested that an H-bonding between the 3α and the 5α-hydroxyl groups could cause a reduced ability of the hydroxylated compound to bind to the active site of the BR receptor through its C-3 hydroxyl group. Our molecular modeling calculations predict a very close contact of 1.97(between the hydrogen of the 3α-hydroxyl group and the fluorine atom in C-5α, forming a favorable and stable conformation of a six membered ring (Pereira-Netto et al. 2003; Ramírez 2003) and this conclusion is consistent with a typical hydrogen bond involving fluorine (Howard et al. 1996; O'Hagan and Rzepa 1997; Dunitz and Taylor 1997). This is also a plausible explanation for interpreting the general reduction in bioactivity shown by increasing concentrations of 5F-HCTS. Further studies should be done in this regard. However, the intriguing almost reciprocal effect of 5α-fluoro HCTS (promotive) and 28-HCTS (inhibitory) on elongation of primary lateral shoots is not easily explained. Perhaps here the reduction in lateral shoot elongation caused by 28-HCTS is a secondary effect of its promotion of main shoot elongation and in a similar way the promotion of lateral shoot elongation by the 5α-fluoro derivative is a secondary effect of its tendency to inhibit main shoot elongation, i.e. an ''apical dominance effect'' in overall shoot morphology.

Apical dominance is a classical developmental event thought to be controlled by cross-talk between auxin and cytokinin. For many decades,

repressed outgrowth of axillary buds has been associated with auxins, while promoted outgrowth of these buds has been related to cytokinin. However, although considerable progress towards the understanding of the mechanisms behind the apical dominance has recently been made (McSteen and Leyser 2005), little is known about the underlying molecular mechanisms. In the past few years, auxin has been shown to negatively regulate cytokinin biosynthesis. For example, auxin represses the expression of both *Arabidopsis* CYP735As, a gene that catalyzes hydroxylation in the last step of trans-zeatin biosynthesis in *Arabidopsis* roots (Takei et al. 2004), and pea (*Pisum sativum* L.) gene adenosine phosphate-isopentenyltransferase (PsIPT) (Tanaka et al. 2006). Although recent evidence suggests that auxin and brassinosteroid signaling pathways are overlapping and interdependent (Hu and Ma 2006), the possible role of brassinosteroids on the establishment of the apical dominance has not been addressed. In our model, although an auxin and a cytokinin were used in the culture medium, it is quite apparent that exogenously supplied brassinosteroids are able to change shooting patterns (apical dominance) in *Eucalyptus*, and it seems likely that shooting in *Eucalyptus* will be influenced by the endogenous pool of bioactive brassinosteroids. Additionally, our findings have practical applications in forestry, for example on the improvement of micropropagation techniques for clonal propagation of woody angiosperms and the possibility of using applied brassinosteroids to promote (main) shooting in seed orchards. Along with these immediate applications, brassinosteroids can also be seen as possible controlling factors in the allocation of growth from main shoots to primary lateral shoots, especially in forest and horticultural species.

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