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S. Schaefer · S.A. Medeiro · J.A. Ramírez
L.R. Galagovsky · A.B. Pereira-Netto

Brassinosteroid-driven enhancement of the in vitro multiplication rate for the marubakaido apple rootstock [*Malus prunifolia* (Willd.) Borkh]

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Abstract Treatment of shoot apices of the marubakaido apple rootstock with a fluoro derivative of 28-homoethylcastasterone (5F-HCTS) in the range 100–10,000 ng per shoot increased the apple rootstock's multiplication rate (MR). A dose of 500 ng 5F-HCTS in 5 µl ethanol, per shoot, resulted in a significant (P=0.05) 112% increase in MR compared to shoots treated with 5 µl of 95% ethanol when the shoots were grown in culture medium enriched with 2.2 µ*M* N^6-benzyladenine. This increase in MR was due to: (1) a 15% increase in the number of main branches; (2) a 238% increase in the number of primary lateral branches; (3) a 250% increase in the number of secondary lateral branches. These results show that shoot proliferation induced by the fluoro derivative of 28-homocastasterone is an effective method to enhance the in vitro multiplication rate in *Malus prunifolia*.

Keywords 28-Homoethylcastasterone · Shoot proliferation · Brassinosteroid derivative

Introduction

Brassinosteroids (BRs) comprise a group of polyhydroxysteroids that show close structural similarity to steroid hormones from arthropods and mammals (Mussig and Altmann 1999). They are naturally found at low levels in pollen, seeds and young vegetative tissues throughout the plant kingdom (Clouse and Sasse 1998) and are able to induce a large range of responses, such as stem elongation, pollen-tube growth, leaf bending and epinasty, induction of ethylene biosynthesis, proton-pump activation, xylem differentiation and regulation of gene expression (Mandava 1988; Clouse and Sasse 1998). Useful applications have been found for BRs in agriculture, such as increasing yield and improving quality and stress resistance in several major crop plants (Sakurai et al. 1999; Khripach et al. 2000).

BRs are now accepted as a new class of phytohormones due to their ubiquitous occurrence in plants, their highly effective elicitation of various responses and the identification of mutants defective in BR biosynthesis or BR response (Mussig and Altmann 1999). However, BRs can be distinguished from other growth regulators, especially with respect to their promotion of young vegetative growth (Janas 1998).

Brassinolide, the first reported BR (Grove et al. 1979), displays activity in some species at doses as low as 1 ng per individual plant. Castasterone, the B-ring ketone analogue of brassinolide, is also intrinsically bioactive in some plant species, e.g. mung bean (*Vigna radiata*) (Suzuki et al. 1993a), although it serves as the immediate biosynthetic precursor of brassinolide in other species, such as *Catharanthus roseus* (Suzuki et al. 1993b), tomato (*Lycopersicon esculentum*) (Bishop et al. 1999) and *Arabidopsis* (Noguchi et al. 2000). The structures of these two compounds are presented in Fig. 1.

In vitro multiplication rates reported for the marubakaido apple rootstock [*Malus prunifolia* (Willd.)] are in the range of four to five new shoots per explant (Nunes et al. 1999), which make the micropropagation techniques available for this rootstock barely feasible for commercial purposes. Since BRs are known to stimulate young vegetative growth (Janas 1998) – more specifically stem elongation (Mandava 1988; Clouse and Sasse 1998) – in various plant species, we decided to investigate the potential vegetative growth-stimulatory effect of a fluoro derivative of 28-homoethylcastasterone – (22R, 23R)-2α, 3α, 22, 23-tetrahydroxy-5α-fluorostigmastan-6-one (see Fig. 1 for structure) – on the in vitro-grown

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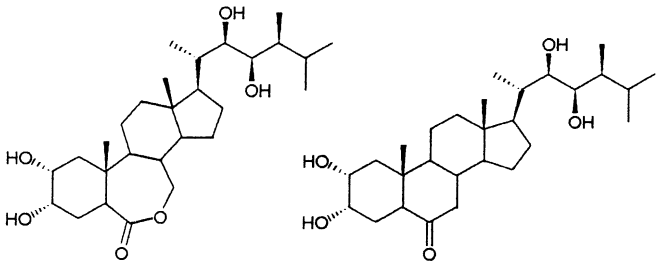
S. Schaefer · S.A. Medeiro · A.B. de Pereira-Netto (✉)
Departamento de Botanica-SCB, Centro Politecnico-UFPR,
CP 19031 Curitiba, PR, Brazil
e-mail: apereira@bio.ufpr.br
Tel.: +55-41-3611631, Fax: +55-41-2662042

J.A. Ramírez · L.R. Galagovsky
Departamento de Química Orgánica,
Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires, Ciudad Universitaria,
Pabellón II, (1428) Buenos Aires, Argentina



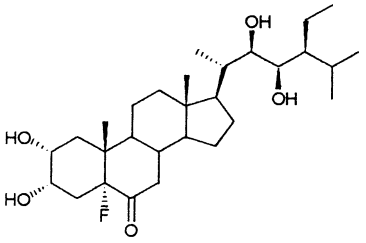


Fig. 1 Structural formulae of brassinosteroids mentioned: *1* brassinolide, *2* castasterone, *3* 5F-28-homocastasterone (5F-HCTS) – (22R, 23R)-2α, 3α, 22, 23-tetrahydroxy-5α-fluorostigmastan-6-one

marubakaido apple rootstock with the purpose of increasing its in vitro multiplication rate. In this paper, we demonstrate that the 5F-28-homoethylcastasterone (5F-HCTS) can be effectively used to increase in vitro multiplication rate of marubakaido apple rootstock.

Materials and methods

Plant material

Nodal segments measuring between 10 mm and 20 mm in length were taken from 30-day-old aseptically-grown shoots of a clone of *Malus prunifolia* (Willd.) Borkh var. Marubakaido and used as explant sources in the experiments. Explants were grown on 40 ml of MS basal medium (Murashige and Skoog 1962) supplemented with: 555 µ*M* myo-inositol, 4.06 µ*M* nicotinic acid, 2.43 µ*M* pyridoxine-HCl, 26.64 µ*M* glycine, 6.25 µ*M* thiamine-HCl, 2.2 µ*M* N^6-benzyladenine, 3% (w/v) sucrose and 0.6% (w/v) agar. The pH was adjusted to 5.7 prior to autoclaving.

Culture conditions

Cultures were maintained in a culture room using a completely randomized design. Continuous light was provided by cool-white fluorescent tubes giving a photosynthetic photon flux density 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°±1.0°C.

5F-28-Homoethylcastasterone application

5F-28-Homocastasterone was synthesized from stigmasteryl acetate, as previously described (Ramirez et al. 2000a). Microdrops (5 µl) of 95% (v/v) ethanol containing known amounts of 5F-HCTS were pipetted onto the uppermost leaf, which was at least 3 mm wide, of 15-day-old shoots originating from nodal segments, as above described. Only single applications were used, and control shoots were treated with 5-µl microdrops of only 95% (v/v) ethanol. Single microdrops were used for each leaf.

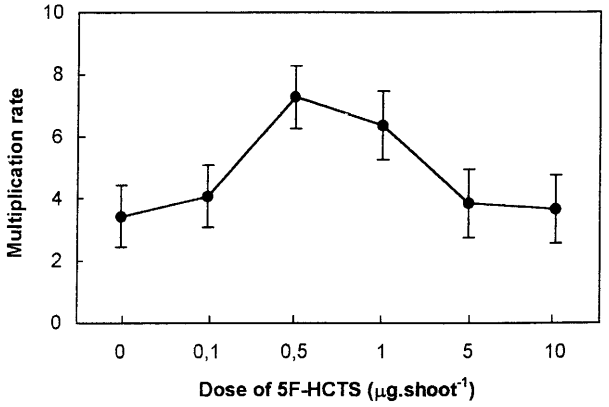


Fig. 2 Effect of 5F-HCTS on the in vitro multiplication rate of *Malus prunifolia*. *Vertical bars* Standard error

Statistical analyses

Each treatment consisted of eight replicates (one replication = one culture vessel) with four explants per replication. A sub-routine (lsmeans/pdiff stderr) of the General Linear Model Procedure in SAS (Statistical package SAS, SAS Institute, Cary, N.C.) was used for the analysis of data. The experiment was repeated twice. The entire dataset obtained in the experiments was used for data analysis.

For the purpose of this study, "multiplication rate" was defined as the number of neoformed branches longer than 15 mm – the minimum length suitable for propagation purposes – 30 days after the treatment.

Results

An increase in the MR for in vitro-grown *Malus prunifolia* shoots was associated with leaf application of the hydrophobic 5F-HCTS (compound 3, Fig. 1) in the range 100–10,000 ng per shoot (Fig. 2). However, 500 ng per shoot was the most effective dose for the enhancement of MR; this amount resulted in a statistically (P=0.05%) significant 112% increase in MR compared to shoots treated with 5 µl 95% ethanol when the explants were grown in culture medium enriched with 2.2 µ*M* N^6-benzyladenine. When used at doses over 1 µg per shoot, as in the case of the main branches (branches originating directly from the initial explant; Fig. 3), or over 500 ng per shoot, as in the case of the primary (branches originating from the main branches; Fig. 4) and secondary lateral branches (branches originating from the primary lateral branches; Fig. 5), 5F-HCTS inhibited stem elongation relative to the effect of the 500 ng per shoot treatment. The 112% increase in MR observed for shoots treated with 500 ng per shoot 5F-HCTS was due to a statistically (P=0.05%) significant increase of: (1) 15% with respect to the number of main branches; (2) 238%

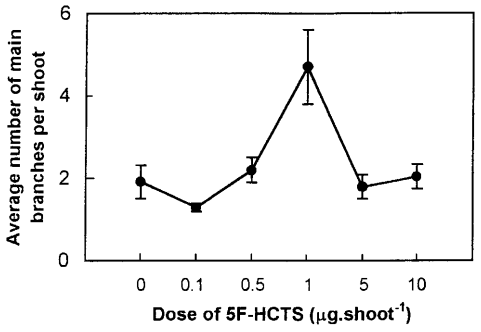


Fig. 3 Effect of 5F-HCTS on the number of main branches of *M. prunifolia* measuring at least 15 mm in length. *Vertical bars* Standard error

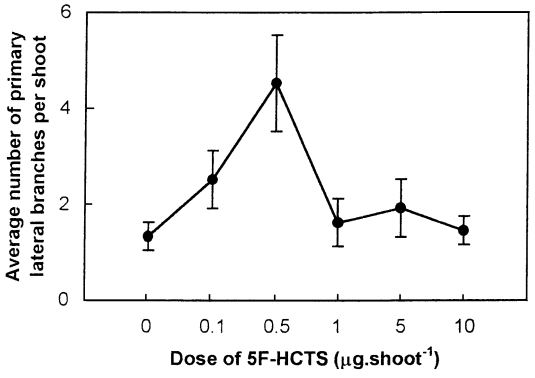


Fig. 4 Effect of 5F-HCTS on the number of primary lateral branches of *M. prunifolia* measuring at least 15 mm in length. *Vertical bars* Standard error

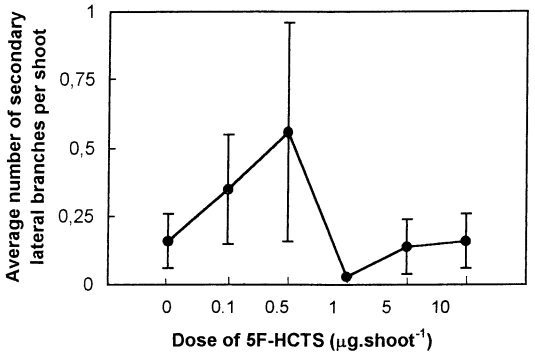


Fig. 5 Effect of 5F-HCTS on the number of secondary lateral branches of *M. prunifolia* measuring at least 15 mm in length. *Vertical bars* Standard error

with respect to the number of primary lateral branches; (3) 250% with respect to the number of secondary lateral branches – all measuring at least 15 mm in length.

Discussion

28-Homoethylcastasterone has been widely employed in field trials because of its greater synthetic accessibility compared to the brassinolide (BL). In an attempt to enlarge studies of the effects of brassinosteroid analogues on bioactivity (Ramirez et al. 2000b), we synthesized a new compound by introducing a fluoro group at the C-5 position of 28-homoethylcastasterone (see compound 3, Fig. 1). In the present work, we found that this compound stimulated shoot proliferation, through stem elongation but especially through an increase in lateral branching, which resulted in an enhanced multiplication rate for the marubakaido apple rootstock.

BRs are known to promote stem elongation in a wide range of plants species (Zurek et al. 1994; Oh and Clouse 1998). Although this specific class of low-abundance plant steroids is capable of eliciting strong growth responses and a variety of physiological changes following exogenous application to plants (Altmann 1998), little is known about the mechanism of action of these plant growth regulators. However, a number of physiological and molecular parameters associated with brassinosteroid-enhanced stem elongation have been investigated. A major role in the positive regulation of cell expansion has been attributed to BRs (Mussig and Altmann 1999). Continuous growth recordings of soyabean (*Glycine max*) cv. Williams 82 epicotyls demonstrated that there was a 45-min lag before 0.1 μ*M* BL exerted a detectable effect on elongation. BL caused a marked increase in Instron-measured plastic extensibility, suggesting that it may promote elongation in part by altering the mechanical properties of the cell wall (wall loosening) (Zurek et al. 1994).

Zurek and co-workers (1994) investigated auxin-BL interactions using small-auxin-up-RNA (SAUR) gene probes and the auxin-insensitive diageotropica (dgt) mutant of tomato (*Lycopersicon esculentum*). They found that in the wild-type tomato, which elongated in response to exogenous auxin, a transcript identical in size to the soyabean SAUR 15A was strongly induced within 1 h by 50 μ*M* 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid (IAA), while in the dgt mutant, which did not elongate in response to auxin, no transcript was expressed. BL stimulated, equally, elongation of the hypocotyls in both wild-type and dgt tomatoes but did not rapidly induce the SAUR 15A homologue in either genotype. In addition, BL did not cause a fast induction of SAUR 6B in elongating soyabean epicotyls but did lead to enhanced expression after 18 h. This late BL activation of SAUR 6B was controlled, at least in part, at the transcriptional level and was not accompanied by an enhancement of free IAA in the tissue. The authors concluded that although both BL and auxin affected wall relaxation processes, BL-promoted elongation in soyabean and tomato stems acted via a mechanism that most likely did not proceed through the auxin signal transduction pathway. Furthermore, in an earlier study involving a side chain diasteromer of brassinolide (compound 1, Fig. 1), hypocotyl elongation in pakchoi (*Brassica chinensis* cv. Lei-Choi) seedlings was stimulated (Wang et al. 1993); this effect was attributed to an acceleration of biochemical processes that cause wall relaxation with-

out induction of a large change in wall mechanical properties. In addition to its effects on the mechanical properties of the cell wall, brassinosteroids cause, at least temporarily, hyperpolarization of the electrical potential difference across the plasmalemma and promote solute uptake (Dahse et al. 1991), which might contribute to the BL-induced cell expansion.

Cytokinins are known to stimulate lateral branching in several plant species. Transformed plants bearing the agrobacterial isopentenyltransferase (ipt) gene, an enhancer of endogenous levels of cytokinins such as zeatin, zeatin riboside and isopentenyladenosine, typically display lateral branching stimulation (Schwartzenberg et al. 1994). Like cytokinins, BRs have also been reported to be involved in branching responses. Application of brassinolide and castasterone (compounds 1 and 2, respectively, in Fig. 1) to the dumpy (dpy) mutant of tomato, a mutant presenting reduced axillary branching, rescued the dpy phenotype, as did C-23-hydroxylated, 6-deoxo intermediates of brassinolide biosynthesis. The brassinolide precursors campesterol, campestanol and 6-deoxocathasterone failed to rescue the dpy phenotype, suggesting that dpy may be affected in the conversion of 6-deoxocathasterone to 6-deoxoteasterone (Koka et al. 2000). BRs have also been demonstrated to change endogenous cytokinin levels in various plant species. When added to a culture medium containing growth-limiting amounts of auxin, 24-epibrassinolide increased the levels of the endogenous predominant cytokinins *N*-6-(Δ-2-isopentenyl) adenine and *trans*-zeatin in tobacco (*Nicotiana tabacum*) callus tissue (Gaudinova et al. 1995). Thus, the 5F-HCTS-driven branching stimulation observed in our system might be due to either a stimulation of lateral branching by the 5F-HCTS itself or to an eventual 5F-HCTS-driven stimulation of cytokinin biosynthesis.

When 5F-HCTS was used at doses over 1 μg per shoot, as in the case of the main branches, or over 500 ng per shoot, as in the case of the primary and secondary branches, it inhibited stem elongation relative to the effect of the 500 ng per shoot treatment. BRs such as brassinolide and 24-epibrassinolide have previously been reported to inhibit stem 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 for a long time to stimulate 1-amino cyclopropane-1-carboxylic acid (ACC) and ethylene biosynthesis in various systems (Arteca et al. 1991). Furthermore, ethylene is known to inhibit stem elongation in various plant species. Consequently, one possible way that 5F-HCTS might inhibit stem elongation in our system, when used at higher doses, would be through a stimulation of ethylene production. In addition to a possible inhibitory effect of the ethylene itself on stem elongation, cyanide, a by-product of ethylene biosynthesis when ethylene is produced from ACC (Chon et al. 2000), might be, at least in part, responsible for the BR-induced inhibition of stem elongation. In conclusion, the results presented in this paper show that the shoot proliferation induced by the C-5 fluoro derivative of 28-homocastasterone is an effective method to enhance the in vitro multiplication rate in *M. prunifolia*.

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