Chapter 4
The Importance of Fluoro and Hydroxyl Substitutions in Brassinosteroids for Shooting-Control: The Use of *In Vitro*-Grown Shoots as Test Systems

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Abstract Biologically active brassinosteroids (BRs) induce a broad spectrum of responses, including stimulation of longitudinal growth of tissues via cell elongation and division, besides stimulation of vascular differentiation, the last one a developmental process critical for shoot elongation. We have been using *in vitro*-grown plants, especially the marubakaido apple rootstock, as test systems to probe into the ability of BRs, mainly new synthetic analogs, to control shooting. Replacement of 5α-H or 3α-OH groups of the steroidal structure of BRs by 5α-F, 3α-F or 5α-OH groups, respectively, has led to significant changes in the abilities of parent compounds such as homocastasterone to control shoot formation and their further elongation, being the effect species and organ-specific, besides being also dependent on the type, i.e., hydroxy or fluoro, position of the substitution. In this chapter, it will also be discussed how treatment of *in vitro*-grown shoots with new synthetic BR analogs has helped to: (1) Enhance our understanding about the relevance of selected functional groups for the BRs's action mechanism(s); (2) Get an insight into the morphological responses of shoots, grown *in vitro*, to the application of BRs and synthetic analogs; (3) Improve micropropagation techniques for clonal propagation, especially of woody species, in which new shoot formation and its further elongation is typically a constrain for efficient micropropagation; (4) Guide the development of novel BR analogs for higher activity, at a lower cost.

Keywords *Malus prunifolia* · Marubakaido · Rootstock, · Micropropagation · Brassinosteroid analog

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1 Introduction

Brassinosteroids (BRs), are highly oxygenated, low-abundance, plant steroids of ubiquitous occurrence in plants. Molecular genetic analysis has demonstrated that the ability to synthesize, perceive and respond to BRs is essential for normal plant growth and development. Biologically active BRs are known to play critical roles in a broad range of physiological processes, when exogenously supplied at very low concentration, at the nanomolar to micromolar levels. These processes include stimulation of longitudinal growth via cell elongation and cell division, and enhancement of phloem and xylem differentiation, all required for shoot elongation, especially of young tissues. A large number of reports have shown that BRs can improve yield and quality of crops, especially under stress conditions, besides being environmentally friendly, for example, by ameliorating toxic effects derived from heavy metals, including aluminum, copper, nickel and plumb. In addition, BRs are known to reduce the need for fertilizers and to accelerate metabolism of herbicides, fungicides and insecticides, and consequently reducing their residual levels in crops. Because of this BR-driven reduction of the risks for human health and environment, BRs have sparked great interest in green agricultural uses.

Similar to steroid hormones in animals, the structures of BRs consist of a cholesterol skeleton with various hydroxyl substitutions and attached functional groups. Sixty two chemical structures of naturally occurring BRs have been confirmed so far. All natural bioactive BRs, like brassinolide (BL, Fig. 4.1), castasterone (CS) and typhasterol (TY) present a vicinal 22*R*, 23*R* diol structural functionality, which are essential for high biological activity. The elucidation of the co-crystal structure of BL bound to BRI1, the leucine-rich repeat receptor kinase that is involved in perception and transduction of BR signaling at the cell membrane, shows that this diol moiety is engaged in a hydrogen-bonding net work within the hydrophobic pocket where the alkyl chain of the hormone fits (Hothorn et al. 2011; She et al. 2011). BL, the end product of the BR biosynthetic pathway, is widely considered to present higher biological activity than any other natural BRs. However, the synthesis of the naturally occurring BL is expensive. In addition, the rapid metabolism of natural BRs in plants and the consequent reduction in their biological activity is a major constrain for a broader commercial use of natural BRs, such as BL, in economical

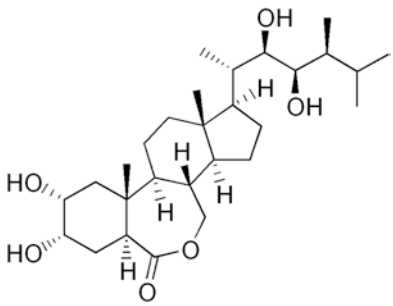
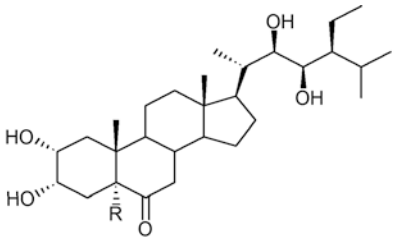


Fig. 4.1 Structural formulae of brassinolide (BL)

activities such as agriculture, horticulture and forestry. The easier to synthesize 24-epibrassinolide (24-epiBL), the stereoisomer of BL, has been the most widely used BR to date. However, 24-epiBL is also expensive, which limits its popularization and practical applications (Lei et al. 2017). Thus, the development of lower cost novel synthetic derivatives, besides enabling studies of structure-activity relationships, biosynthesis and metabolism of BRs, is an effective way to overcome the rapid metabolism of natural BRs in plants once synthetic derivatives have been demonstrated to be more difficult to be metabolized by plants. Such high biological activity new derivatives are expected to allow a broader commercial use of BRs.

Slight structural changes in ring A and B as well as in the side chain of BRs are known to result in moderate to drastic differences in plant growth activity (Liu et al. 2017). 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. Thus, fluorination of BRs can change their ability to bind to BRI1, the BR receptor, changing consequently the biological activity of the parent compound. The degree of response elicited by a given BR depends on the position of functional groups in the carbon skeleton. For example, the presence of C-2α hydroxyl, and especially C-3α hydroxyl, in ring A are needed for enhancement of biological activity. Furthermore, it is known for quite some time that alteration of the functional groups in the carbon skeleton affects the degree of response elicited by a given compound. The carbon-fluorine bond is physic chemically similar to the C–OH bond, rather than the C–H bond. Thus, fluorine could be considered as being equivalent to the oxygen of the hydroxyl group. In an attempt to enlarge studies on the effects of BRs and synthetic analogs on bioactivity, the naturally occurring BRs homocastasterone (HCS) and homotyphasterol (HTY), along with derivatives in which the 5α-H group of HCS and HTY was replaced by a 5α-F and/or a 5α-OH group, or the 3α-OH group has been replaced by a 3α-F group (Figs. 4.2 and 4.3) were applied to *in vitro*-grown shoots of the marubakaido apple rootstock or a clone of a hybrid between *Eucalyptus grandis* and *E. urophylla*. In this chapter, we describe the effects of these compounds on new shoot formation and further elongation, along with their consequences for the *in vitro* multiplication rate.



R=H 28-homocastasterone (28-HCS)
R=F 5α-fluoro-28-homocastasterone (5F-HCS)
R=OH 5α-hydroxy-28-homocastasterone (5OH-HCS)

Fig. 4.2 Structural formulae of 28-homocastasterone (HCS), 5α-fluoro-28-homocastasterone (5F-HCS) and 5α-hydroxy-28-homocastasterone (5OH-HCS)

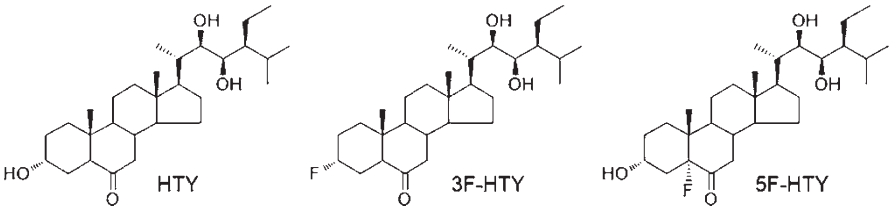


Fig. 4.3 Structural formulae of homotyphasterol (HTY), 3α-fluoro-homotyphasterol (3F-HTY) and 5α-fluoro-homotyphasterol (5F-HTY)

2 Effects of Brassinolide on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Progressive increase in *in vitro*-grown marubakaido shoot length is related to increased doses of BL (Pereira-Netto et al. 2009). A statistically significant increase of 12% and 25%, respectively, for the main (shoots originating directly from the initial shoot segment) and primary lateral (shoots originating from the main shoots) shoot length has been found for shoots treated with 1.25 μg.shoot^{-1} BL, compared to untreated shoots. Shoot treatment with BL also results in enhanced formation of main and primary lateral shoots. Maximum enhancement in the formation of new main (23%) and primary lateral shoots (46%) were found for shoots treated with 0.25 and 0.50 μg.shoot^{-1} BL, respectively.

3 Effects of Homocastasterone and Hydroxy and Fluoro Synthetic Analogs on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Twenty eight-homoethylcastasterone (HCS) has been widely employed in field trials because of its greater synthetic accessibility compared to the BL. However, studies in our laboratory have shown that leaf application (5 μL) of HCS, in which a fluoro group was introduced in alpha configuration at C-5, to *in vitro*-grown marubakido shoots results in enhanced formation of new main shoots, but especially enhanced formation of primary lateral and secondary lateral (shoots originating from the primary lateral shoots) shoots (Schaefer et al. 2002). These enhanced shoot formation is followed by enhanced elongation of main and primary lateral shoots (Pereira-Netto et al. 2006b). This shoot proliferation results in a 112% increase on multiplication rate for *in vitro*-grown marubakaido shoots treated with 500 ng per shoot 5F-HCS. Differently from 5F-HCS, which induced remarkable changes in the arquitecture of *in vitro*-grown marubakaido shoots, 28-HCS and 5OH-HCS applications result in no statistically significant change in formation or elongation of newly formed shoots (Pereira-Netto et al. 2006b).

Since both hydrogen and fluorine atoms are small, univalent and contribute rather little to total molecular polarizabilities, physical properties are less affected by equating fluorine and hydrogen than most of the chemical properties (Liebman 1988). Considering that effects of BRs and analogues on shoot formation and further elongation depend on the extents to which these molecules satisfy the structural requirements of the receptors and/or enzymes, the differential responses found for 28-HCS and its 5α-fluoro substituent-treated shoots of marubakaido described in this paper suggests differences on metabolic routes, higher chemical stability for 5F-HCS or higher affinity and/or binding time of 5F-HCS for the receptor sites of BRI1, the receptor for BRs in this biological system.

Since fluorine and hydroxyl are similar, regarding electronegativity, and the C-F bond is physicochemically similar to the C-OH bond, it was somewhat surprisingly to find that the 5F-HCS effectively promote shooting in the marubakaido apple rootstock, while 5OH-HCS shows no effect. This reduced bioactivity of 5OH-HCS might be due to formation of an H-bonding between the 3α and the 5α-hydroxy groups, an event that might reduce the ability of the hydroxylated compound to bind to the active site of the receptor through its C-3 hydroxyl group.

4 Effects of Homocastasterone and Its 5A-Monofluoro Analog on *In Vitro*-Grown Shoots of a Hybrid Between *E. GRANDIS* and *E. urophylla*

Stimulation of main shoot formation and further elongation is found for shoots of a hybrid between *E. grandis* and *E. urophylla* immersed in solutions of 28-HCS. For shoots treated with the 5-fluoro analog of 28-HCS, no significant change in either main shoot formation or further elongation is observed. Differently from what is seen for main shoots, treatment with 28-HCS lead to inhibition of both primary lateral shoots formation and their further elongation. Conversely, enhancement in the average length of primary lateral branches is found for shoots treated with 5F-HCS, although primary shoot formation is inhibited by 5F-HCS treatment. The extent in which 5F-HCS stimulated primary lateral shoots elongation does not differ significantly from the extent in which 28-HCS stimulats main shoots elongation. Multiplication rate raises significant 34% for shoots treated with 10 $mg.l^{-1}$ 28-HCS, compared to shoots treated with acetone, only (control), being the effect due essentially to the 28-HCS-driven enhancement in the formation of new main shoots. For shoots treated with 5F-HCS, decrease in the multiplication rate is observed as a consequence of the reduced formation of both, main and primary lateral branches. When seen together, these data clearly show that 28-HCTS and its 5α-monofluoro analog differentially change shoot architecture in *in vitro*-grown shoots of the hybrid between *E. grandis* and *E. urophylla* used in our laboratory.

Fluorination-driven changes in biological properties of compounds like gibberellins, tetracyclic diterpenoids that control stem elongation, depend upon the degree

of fluorination and differ according to the type of bioassay used to access the biological activity. Because of their high electronegativity, monofluoro analogues occasionally bind enzymes irreversibly, which might have deleterious effects on the organism. However, monofluoro analogues of gibberellins are shown to present higher biological active in assays such as the lettuce hypocotyls elongation, when compared to their parental counterparts. In our laboratory, the finding that 28-HCTS is able to stimulate elongation and formation of main branches, and consequently to enhance *in vitro* multiplication rate of the *E. grandis* X *E. urophylla* hibrid, prompted us to test the hypothesis that a 5 α-fluoro derivative might be able to amplify the stimulatory effect of 28-HCTS on elongation and formation of main branches. However, 5F-HCTS is unable to either stimulate elongation and formation of new main branches, or enhance multiplication rate, although it stimulates elongation of primary lateral branches. The reason(s) for these differential responses of 28-HCTS and 5F-HCTS does not appear to be straightforward. A possible formation of a hydrogen bond involving fluorine and a consequent reduced ability to bind to the BR receptor might explain the inability of 5F-HCTS to stimulate elongation and formation of main branches in the *E. grandis* X *E. urophylla* hybrid, differently than what is seen for the marubakaido apple rootstock.

Finally, the enhancement in the multiplication rate found for 28-HCTS-treated shoots in this study demonstrate that BRs can be used for the improvement of protocols used for *Eucalyptus* micropropagation (Patent BR 0403642-5). In addition to that, the results presented in this paper indicate that BRs might be useful to manage branching in field-grown *Eucalyptus* trees.

5 Effects of 5F-Homotyphasterol on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Enhancement over a hundred percent on the number of newly formed primary lateral shoots (shoots originating directly from the main branches) is observed for *in vitro*-grown marubakaido shoots treated with 5F-HTY, tough no significant change on the number of newly formed main shoots (shoots originated directly from an original shoot, i.e., shoot treated with 5F-HTY) is observed for shoots treated with 5F-HTY (Pereira-Netto et al. 2019, in press). Enhancement on the average length is also found for both main and primary lateral shoots treated with 5F-HTY. These observed changes in shoot architecture, especially on formation and further elongation of primary lateral shoots, result in significantly higher, i.e. over 80%, multiplication rate (MR) for shoots treated with 5F-HTY.

Because BL is widely considered to present higher biological activity than any other natural BRs, along with its widespread occurrence in the plant kingdom, BL is commonly used as positive control to evaluate the biological activity of BR

analogs. Because of the structural similarity, the closer the intermediate in the pathway to BL, the greater is its activity. For example, the biological activity of typhasterol, which is considered to be an intermediate to CS and BL in the BR biosynthetic pathway, is typically only one tenth of that presented by BL in bioassays. In our laboratory BL has been shown to significantly stimulate elongation of both, main and primary lateral shoots, besides inducing a 46% increase in the formation of new primary lateral shoots in the marubakaido apple rootstock (Pereira-Netto et al. 2009). Since BL is the most potent natural BR, it is somewhat surprisingly to find that the 5α-monofluoro derivative of homo-TY (5F-HTY) is much more effective towards stimulation of primary shoot formation, compared to BL, inducing an over a 100% increase in the number of newly formed primary lateral shoots. Furthermore, 5F-HTY significantly stimulates both, main and primary lateral shoot elongation, tough in a more effectively way, compared to BL. These findings are especially relevant once: 1. homoBRs, like HBL typically show similar or reduced biological activity when compared to their counterparts, like BL; 2. 7-oxalactone BRs such as BL and HBL generally present stronger biological activity when compared to 6-oxo BRs, such as HTY. The reason(s) why BL was less effective towards stimulation of primary lateral shoots formation, compared to 5F-HTY, is (are) not clear. It is possible that these differential effects might result from differences between these different BRs at satisfying the structural requirements of BR receptors. For example, an enhanced affinity of the 5F-HTY for the receptor or an increased binding time of the 5F-HTY to the BR receptor, as a result of an eventually stronger hydrogen-bonding network within the hydrophobic pocket where the alkyl chain of the BR fits, might explain the ability of 5F-HTY to more effectively stimulate formation of primary lateral shoots in our system, compared to BL. However, that does not explain why the fluoro HTY did not stimulate formation of main shoots, compared to BL. Differences in response to the tested BRs might also be due to eventual differences in the way(s) that these BRs might influence BR biosynthetic enzymes. However, other possibilities such as an eventually higher susceptibility of the natural BL to inactivation, compared to 5F-HTY, a synthetic BR, can not be ruled out. Besides the promotive effect of BL on shoot formation in the marubakaido apple rootstock, we have also previously shown that BL significantly stimulated elongation of both, main and primary lateral shoots (Pereira-Netto et al. 2009). Noteworth, 5F-HTY significantly stimulated both, main and primary lateral shoot elongation, very likely as BL did. And again, similarly to what we have previously shown for BL, the 5F-HTY growth-promotive effects are more effective for primary lateral shoots compared to main shoots. Thus, when seen together, data for the effects of 5F-HTY on marubakaido shoots and data for *Eucalyptus* shoots (Pereira-Netto et al. 2006a), along with data for marubakaido shoots (Pereira-Netto et al. 2006b) treated with 28-HCS and 5F-HCS, respectively, clearly demonstrate that 5F-HTY and other BRs, affect differentially the morphogenetic potential of main and primary lateral shoots.

6 Comparative Effects of the 3α and 5α-Monofluoro Derivative of Homotyphasterol and the Parent Compound

Considering that 5F-HTY effectively promotes new shoot formation and further elongation of both main and primary lateral shoots in the marubakaido apple rootstock, the potential effects of the 3α-monofluoro analog of homotyphasterol (3F-HTY) and the parent compound (HTY) were probed in our laboratory against the formerly tested 5α-monofluoro analog of homotyphasterol (5F-HTY) as a way to investigate if the presence of the fluoro atom in α configuration at C5 was or not a requirement for the homotyphasterol to present strong biological activity. Neither 3F-HTY nor HTY are able to significantly stimulate new shoot formation, regardless the kind of shoot, i.e. main or primary lateral shoot. However, both, HTY and 3F-HTY effectively stimulated main shoot elongation, tough neither HTY nor 3F-HTY were effective towards stimulation of primary lateral shoot elongation. Considering that neither 3F-HTY nor HTY are capable to stimulate new shoot formation, it was not unexpected to realize that none of those compounds were able to enhance the multiplication rate of the *in vitro*-grown marubakaido rootstock. So, the presence of a fluoro atom in α configuration at C5 seems to be a requirement for the stimulation of new shoot formation but not shoot elongation in the marubakaido apple rootstock.

In the rice lamina inclination assay, HTY has been shown to present about 1.7 times less activity when compared to TY, which suggests that the activity of 24-ethyl BRs is increased by C-28 demethylation to the 24-methyl BRs (Joo et al. 2015). In our laboratory, 5F-HTY presents activity 2.35 times higher than HTY towards formation of new primary shoots, indicating that fluorination at C5 might mimic, with advantages, C-28 demethylation in HTY regarding stimulation of primary lateral shoot formation in our system. Data from our laboratory also demonstrate that HTY presents similar activity, towards stimulation of main shoots elongation, compared to the effect of BL on main shoot elongation (Pereira-Netto et al. 2009). However, differently from BL, HTY presents no activity towards main or primary lateral shoot formation, or towards primary shoot elongation in our system. As mentioned previously here, 5F-HTY presents higher biological activity towards primary shoot formation, compared to both, HTY and our previously reported data on the effect of BL on shooting in the marubakaido rootstock. Since BL has been shown to usually presents higher activity, when compared to HBL (Khripach et al. 2000), all of these data, seen together, provide support to the idea that 5F-HTY might be active per se towards primary shoot formation in the marubakaido rootstock, not requiring its conversion to other forms of BRs, downstream the BRs biosynthetic pathway, in order to present high biological activity. In addition, fluorination at C-5 of HTY might prevent its enzymatic inactivation which might in turn enhance its chemical stability and consequently prolong its activity, compared to natural BRs, potentially more susceptible to enzymatic inactivation. Noteworth, when probed in the rice lamina inclination (RLI) test, 5F-HTY presented only moderately higher activity, when compared to the parent compound HTY (Ramirez et al. 2000).The results

from RLI test and our results (stimulation of primary shoots formation) are significantly different, demonstrating that a single BR might exhibit different activities, depending on the testing system. Thus, our data on the biological activity of BL (Pereira-Netto et al. 2009), and HTY (Pereira-Netto et al. 2019 in press) and HCS (Pereira-Netto et al. 2006b; Pereira-Netto et al. 2012), and their F-derivatives provide support to the idea that biological activities of BRs can not be discussed in a single bioassay system.

As previously mentioned in this chapter, the closer the intermediate in the BL biosynthetic pathway, the greater is its activity, and bioactivities for homoBRs present the same trend. TY, one of the two immediate precursors of castasterone (CS) in the BL biosynthetic pathway, is converted to CS, an activation step in the BL pathway, in a reaction catalyzed by the cytochrome P450 CYP90C1. As expected, in bioassays such as the rice lamina inclination bioassay, HTY has been shown to present much less biological activity when compared to homoCS (HCS, Joo et al. 2015), which suggested that C2 α-hydroxylation of HTY was important to express a strong BR activity. In our system, i.e., the *in vitro*-grown marubakaido apple-rootstock, HCS is not able to stimulate new shoot formation (Pereira-Netto et al. 2012) or shoot elongation (unpublished data). Thus, surprisingly, differently from HCS (Pereira-Netto et al. 2003), the three compounds HTY, 3F-HTY and 5F-HTY, are all able to significantly stimulate main shoot elongation, tough only 5F-HTY was able to stimulate primary lateral shoot elongation. Neither the parent HTY nor 3F-HTY or 5F-HTY is able to significantly stimulate main shoot formation. Thus, the stimulation of main shoot elongation driven by the parent HTY or its two monofluoro analogs tested might not rely on C2 α-hydroxylation of these compounds by the marubakaido apple rootstock. Furthermore, since HTY is not considered to show high biological activity per se, 5F-HTY present comparable activity towards promotion of primary lateral shoot elongation, compared to BL, and 5F-HTY stimulates primary lateral shoot formation more effectively than BL, it is reasonable to consider that 5F-HTY might be biologically active per se *in vitro*-grown marubakaido shoots.

The metabolic stability of a C-F bond often prevents chemical reactions of the carbon attached to fluorine atom. Thus, one might assume that introduction of a 3α-F or 5α-F group in HTY might reduce the biological activity of HTY due to a reduced conversion of 3F-HTY or 5F-HTY into compounds downstream of the BR biosynthetic pathway, like HBL, once, for example, 3F-HTY has been shown not to be hydroxylated at C2 to CS, the immediate precursor of HBL (Galagovsky et al. 2001). In fact, this predicted reduced activity is actually seen when 3F-HTY is probed against HTY in the rice lamina inclination test (Galagovsky et al. 2001). Somewhat unexpectedly, we have observed that 3F-HTY is as effective as HTY on the stimulation of main shoot elongation in our system, demonstrating that the introduction of the 3α-F group in HTY did not change the biological activity of HTY.

Length of main shoots is enhanced by HTY and their two monofluoro derivatives used in this study. However, length of primary shoots is enhanced by 5F-HTY and unaffected by HTY and 3F-HTY. It might imply that these BRs might have different activities towards stimulation of shoot elongation, depending on the kind of shoot

considered, i.e., main or primary lateral shoot, or that elongation of main and primary lateral shoots might be controlled by different mechanisms.

7 Conclusions

In this chapter, we report on the evaluation of the biological activity of brassinolide (BL), and homocastasterone (HCS) and homotyphasterol (HTY) and synthetic fluoro analogs towards shooting stimulation in the marubakaido apple-rootstock and a hybrid between *E. grandis* and *E. urophylla*. The results reported here provide an insight into the morphological responses of *in vitro*-grown shoots to several natural BRs and fluoro and hydroxyl substitutions, in alpha configuration, in HCS and HTY on the sterol structure of exogenously supplied BRs. The biological activity of the synthetic analogs mentioned in this chapter is clearly dependent on the type, i.e. fluoro or hydroxyl, and position of the substitution. For example, fluorination at C5 but not at C3 significantly increases formation and further elongation of primary lateral shoots of the marubakaido apple rootstock, which results in effective enhancement of its *in vitro* multiplication rate. This BR-driven enhancement on the MR is an effective way to improve the micropropagation technique for the marubakaido rootstock and possibly for other plant systems as well, especially for woody species, in which new shoot formation and elongation is typically a constrain for efficient micropropagation protocols.

The growth promotive effect of fluoro substitution is organ and species specific once eucalyptus shoots respond differently compared to marubakaido shoots to the position of the fluoro substitution. This differences in specificity of the growth promotive effect of the fluoro substitution is an indicative that BR receptors in different plant organ and species might have, at least slight, differences in structural requirements to bind to the ligand BR. Furthermore, the effects of exogenous BRs on both, shoot elongation and formation, mentioned in this chapter demonstrate that modification of the allocation of growth among the various types of shoots can be effectively achieved at the biochemical/physiological level, at least in the *in vitro*-grown shoots mentioned here through applications of BRs.

Besides being capable of effectively enhancing shooting and also being nontoxic, BRs are environmentally friendly which bring vast perspectives for the application of compounds like 5F-HCS and 5F-HTY in agriculture, forestry and horticulture. In horticulture, for example, practical applications for findings described here include the 5F-HTY-driven enhancement of the multiplication rate for *in vitro*-grown marubakaido. In addition, the 5F-HTY-shooting stimulation reported in this chapter is potentially useful to improve micropropagation techniques for clonal propagation of other plant species as well, especially woody species, in which shoot formation and further elongation is typically a constrain for commercial micropropagation. In producing orchards, potential benefits include promotion of shooting, especially diverting allocation of growth from the main to lateral shoots, which is expected to enhance fruit production.

Finally, because of the evident difference in responsiveness of the *in vitro*-grown shoots, especially of the marubakaido rootstock, to fluorinated and non-fluorinated BRs, this *in vitro* system seems to be potentially useful to probe into the biological activity of BRs bearing fluorine atoms, especially at C5 in α configuration.

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