Trees
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SHORT COMMUNICATION

Alpha monofluoro substitution at C5 in homotyphasterol enhances shoot production and multiplication rate of in vitro-grown marubakaido apple rootstock shoots

Adaucto B. Pereira-Netto[1] · Javier A. Ramírez[2,3] · Lydia R. Galagovsky[2]

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Abstract

Key message **5 fluoro-typhasterol is more effective than the most potent natural brassinosteroid, brassinolide, towards stimulation of shoot production in the marubakaido apple rootstock.**

Abstract Brassinosteroids (BRs) comprise a class of low-abundance plant steroids required for normal plant growth. Here we demonstrate that treatment of in vitro-grown shoots of the marubakaido apple rootstock with 2.5 µg per shoot of 5α-monofluoro homotyphasterol (5F-HTY), a synthetic derivative of the natural BR homotyphasterol (HTY), resulted in significant enhancement in the formation of primary lateral shoots. This treatment also resulted in increased length of primary lateral shoots and main shoots. This growth-stimulatory effect led to a significant increase in the multiplication rate for the rootstock. In contrast to what was found for 5F-HTY, neither HTY nor the synthetic derivative 3α-monofluoro homotyphasterol (3F-HTY) were able to significantly stimulate shoot formation. HTY and 3F-HTY were not able to stimulate primary lateral shoot elongation as well. However, both HTY and 3F-HTY were able to significantly stimulate main shoot elongation. The 5F-HTY-driven enhancement of the multiplication rate described here demonstrates the potential of this compound to improve micropropagation techniques, not only for the marubakaido apple rootstock.

Keywords Brassinolide · Brassinosteroid · *Malus prunifolia* · Micropropagation · Stigmasterol

Introduction

Brassinosteroids (BRs) comprise a specific class of low-abundance plant steroids widely distributed in the plant kingdom (Kanwar et al. 2017). The capacity to synthesize, perceive and respond to BRs is a requirement for normal plant growth and development (Lei et al. 2017). Biologically active BRs exhibit their effects at very low concentration, inducing a broad range of responses, including stimulation of shoot elongation (Lei et al. 2017; Liu et al. 2017).

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 (Kanwar et al. 2017). All natural bioactive BRs, like brassinolide (BL), castasterone (CS) and typhasterol (TY) present a vicinal 22*R*, 23*R* diol structural functionality, which is 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 network 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 broadly considered to present higher biological activity

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✉ Adaucto B. Pereira-Netto
apereira@ufpr.br

[1] Department of Botany-SCB, Centro Politecnico, Parana Federal University, C.P. 19031, Curitiba, PR 81531-970, Brazil

[2] Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, C1428EGA Buenos Aires, Argentina

[3] CONICET, Universidad de Buenos Aires, Unidad de Microanálisis y Métodos Físicos Aplicados a Química Orgánica (UMYMFOR), Ciudad Universitaria, C1428EGA Buenos Aires, Argentina

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when compared to other natural BRs (Lei et al. 2017). 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 constraint for a broader commercial use of natural BRs, such as BL, in commercial 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 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 rings 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, significantly increases electro negativity and receptor-binding potential (Ferrer-Pertuz et al. 2017). Thus, fluorination of BRs can change their ability to bind to BRI1, the BR receptor, changing consequently the biological activity of the parent compound. In an attempt to enlarge studies on the effects of BR analogs on bioactivity we synthesized the naturally occurring BR homotyphasterol (HTY) and two derivatives in which the 5α-H group of HTY was replaced by a 5α-F group or the 3α-OH group was replaced by a 3α-F group (Fig. 1). In this paper, we first describe growth-promotive effects of the 5α-monofluoro derivative of HTY on in vitro-grown shoots of the marubakaido apple rootstock and consequent changes in the multiplication rate. Afterwards, we describe the effects of the parent HTY and the 3α-monofluoro derivative, tested side by side against the 5F-HTY, on the shoot morphology of the apple rootstock.

Materials and methods

Synthesis of HTY, 3F-HTY, and 5F-HTY

Homotyphasterol was synthesized from the low-cost stigmasterol as described by Takatsuto and Ikekawa (1984).

3F-homotyphasterol was synthesized by the introduction of a 3α-F group on the C-3 of the steroidal carbon skeleton. Briefly, the hydrolytic cleavage of a 3α,5-cyclo-6-keto stigmastane (Galagovsky et al. 2001) yielded a compound that had a 3β-hydroxy group at C-3. Afterwards, this compound was fluorinated with diethyl amino sulfur trifluoride (DAST), yielding an intermediate where the 3β-hydroxyl group was substituted by a fluorine, with a concomitant inversion of the configuration. The resulting 3α-fluoro steroid was dihydroxylated at the side chain following the standard Sharpless's procedure (Sharpless et al. 1992). The resulting compound, 3F-homotyphasterol, presented a 9% total yield from stigmasterol. For details please see Galagovsky et al. (2001).

5F-homotyphasterol was obtained by the introduction of a 5α-F moiety into the homotyphasterol structure. Treatment of 5β,6β-epoxy stigmastane with boron trifluoride, with the 3β-hydroxyl group of the stigmastane properly protected, resulted in both the opening of the oxyrane ring and the simultaneous introduction of the fluorine atom at C-5. The resulting intermediate, after oxidation of the OH group at C-6, inversion of the configuration of the OH group at C-3, and dihydroxylation of the Δ^{22} double bond at the side chain, yielded the desired product 5F-homotyphasterol. For details please see Ramírez et al. (2000).

Plant material

Nodal segments, 10–20 mm in length, containing a single node, were obtained from 30 day-old aseptically grown shoots (subcultured from shoots cultured in vitro for about 5 years) of a clone of the marubakaido apple rootstock [*Malus prunifolia* (Willd.) Borkh var. Marubakaido]. These nodal segments were used as explants in this study. Shoots

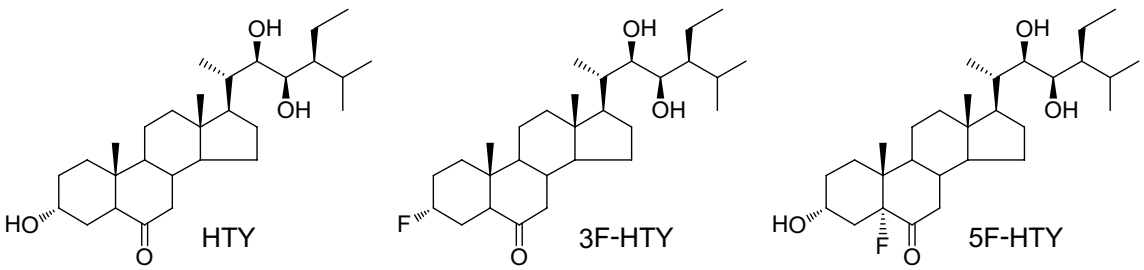

Fig. 1 Structural formulae of homotyphasterol (HTY), 3α-fluoro-homotyphasterol (3F-HTY) and 5α-fluoro-homotyphasterol (5F-HTY)

used as explant sources and explants used in this study were all grown in 230 mL glass vessels. The glass vessels were closed with semitransparent screw-on polypropylene lids. Glass vessels contained 50 mL of MS (Murashige and Skoog 1962) basal medium supplemented with (μM): 555 myo-inositol, 26.64 glycine, 6.25 thiamine.HCl, 4.06 nicotinic acid, 2.43 pyridoxine.HCl, 2.2 N^6-Benzyladenine, and 3% (w/v) sucrose and 0.6% (w/v) agar. The pH was adjusted to 5.7 before autoclaving.

Culture conditions

Cultures were kept in a culture room, using a completely randomized design. A photoperiod of 16/8 (light/dark) hours with a photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}, at the culture level, was provided by cool-white fluorescent tubes. Relative humidity and air temperature surrounding cultures vessels were, respectively, 38 ± 3% and 27 ± 1.0 °C.

Application of homotyphasterol and fluoro derivatives

Single acetone micro drops [5 μL, 95% (v/v)] containing known amounts of homotyphasterol (HTY), 5F-HTY or 3F-HTY (0.1, 0.5, 2.5 and 5.0 μg) were pipetted onto the uppermost leaf, measuring at least 3 mm wide, of shoots originated from nodal segments, 15 days after subculturing, as above described. Control shoots received 5 μL acetone micro drops.

Statistics

Each treatment, i.e., dose of HTY or their fluoro derivatives used in this trial, consisted of five replicates (five culture vessels) with five explants per replication. After ANOVA, differences among averages for treatments were tested using Tukey's test ($p = 0.05$). Regression and correlation analyses were carried out and resulting fitted curves and correlation coefficients (r^2) are shown in Figs. 4, 5, and 6.

For this study, "multiplication rate" was defined as the number of newly formed shoots, at least 15 mm in length, the minimum length appropriate for micropropagation purposes, 30 days following the treatment. "Main shoot" was defined as shoots, at least 15 mm in length, originated from the axillary buds of the explanted nodal segment (shoot treated with homotyphasterol or homotyphasterol derivative) while "primary lateral shoot" was defined as shoots, at least 15 mm in length, originated directly from main shoots (Fig. 2).

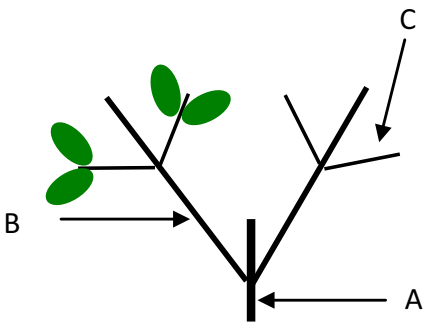

Fig. 2 Diagram showing the origin of the different types of plant material mentioned in this study. *A* original explant (nodal segment), *B* main shoot, *C* primary lateral shoot

Results

Each experiment was repeated three times, yielding similar results. Plots in the graphs represent the means of five replications (five explants per replication) of a single experiment.

Effects of the 5α-monofluoro derivative of typhasterol (5F-HTY) on shoot production by the marubakaido apple rootstock

Statistically significant ($p = 0.05$) enhancement in the number of neo-formed primary lateral shoots (shoots originating directly from the main branches) for in vitro-grown marubakaido was observed for shoots treated with 0.1, 0.5, and 2.5 μg of 5F-HTY per shoot (Figs. 3, 4), compared to shoots treated with acetone only (control shoots). The use of 2.5 μg of 5F-HTY per shoot resulted in an enhancement of 142% in the number of neo-formed primary lateral shoots, compared to control shoots. However, no significant ($p = 0.05$) change in the number of neo-formed main shoots (shoots originated directly from the explanted nodal segment, i.e., shoot treated with 5F-HTY) was observed for shoots treated with any of the doses of 5F-HTY used in this study (Fig. 4). Correlation analysis showed a higher correlation coefficient between the number of neo-formed shoots and dose of 5F-HTY for primary lateral shoots compared to main shoots.

Significant ($p = 0.05$) increase on the average length was found for primary lateral shoots treated with 5F-HTY at the doses of 2.5 and 5.0 μg per shoot. For the main shoots, enhanced shoot elongation was restricted to shoots treated with 2.5 μg per shoot of 5F-HTY, only (Fig. 5). Correlation analysis showed similar correlation coefficient between the average length of neo-formed shoots and dose of 5F-HTY for primary lateral shoots and main shoots.

Statistically significant ($p = 0.05$) enhancement in the multiplication rate (MR) was observed for shoots treated with 0.1, 0.5, and 2.5 μg of 5F-HTY per shoot (Fig. 6), compared to shoots treated with acetone, only (control

Fig. 3 Morphological characteristics of 3α-fluoro-homotyphasterol (5F-HTY)-treated (right side) and untreated (left side) shoots of *M. prunifolia*, 30 days after treatment

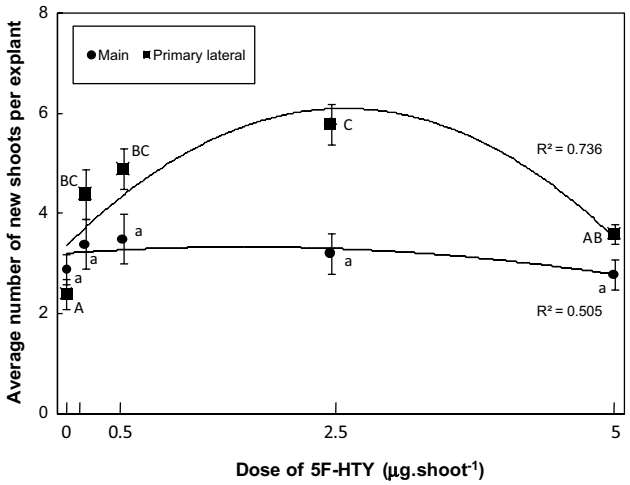

Fig. 4 Effect of 5α-monofluoro homotyphasterol (5F-HTY), at 0.1, 0.5, 2.5, and 5.0 µg per shoot, in the average number of newly formed main and primary lateral shoots suitable for micropropagation (measuring at least 15 mm in length). Vertical bars indicate ± standard error. Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Values followed by the same letter are not significantly different at $p = 0.05$ (Tukey's test). Upper case letters show statistical differences for primary lateral shoots while lower-case letters show statistical differences for main shoots

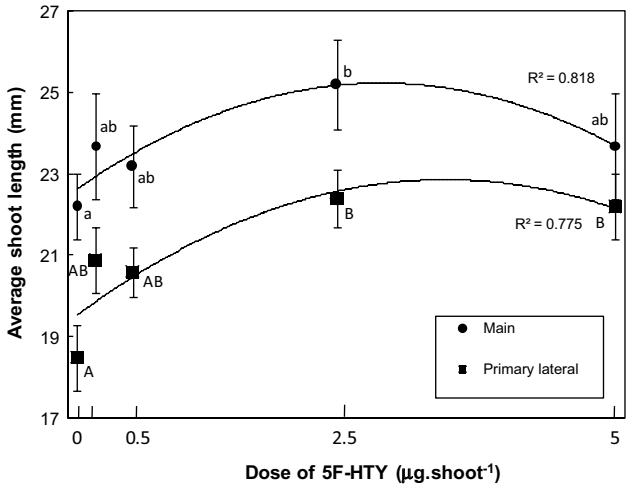

Fig. 5 Effect of 5α-monofluoro homotyphasterol (5F-HTY), at 0.1, 0.5, 2.5, and 5.0 µg per shoot, in the average shoot length of newly formed main and primary lateral shoots. Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Values followed by the same letter are not significantly different at $p = 0.05$ (Tukey's test). Upper case letters show statistical differences for primary lateral shoots while lower-case letters show statistical differences for main shoots. Vertical bars indicate ± standard error

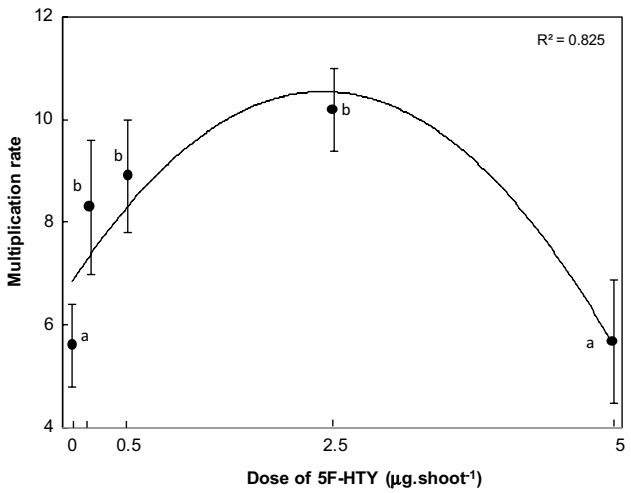

Fig. 6 Effect of 5α-monofluoro homotyphasterol (5F-HTY), at 0.1, 0.5, 2.5, and 5.0 µg per shoot, in the multiplication rate. Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Values followed by the same letter are not significantly different at $p = 0.05$ (Tukey's test). Vertical bars indicate ± standard error

shoots). The use of 2.5 µg per shoot of 5F-HTY resulted in an enhancement of 82% in the MR of 5F-HTY-treated shoots (10.2), compared to shoots treated with 5 µL acetone, only (MR of 5.6). This 5F-HTY-driven enhancement on MR was essentially a consequence of the enhancement on primary lateral shoot formation. Correlation analysis showed a high correlation coefficient between multiplication rate and dose of 5F-HTY.

Comparative effects of the 3α and 5α-monofluoro derivative of typhasterol (5F-HTY) and the parent compound on shoot production by the marubakaido apple rootstock

Mutants deficient in BR biosynthesis or response have significantly contributed to increased knowledge about BRs and their actions (Wei and Li 2016; Cheng et al. 2017). However, the use of precursors of the BRs biosynthetic pathway and their derivatives is an alternative way for the understanding of physiological functions of BRs especially for trees, as no BR-deficient mutant has been identified in trees until now. And, although there is a wealth of information available, it is very difficult to compare the biological activity of the numerous derivatives reported by different research groups, as frequently different testing conditions were used to evaluate their biological potency. Thus, since 5F-HTY was effective to promote new shoot formation and further elongation in the marubakaido apple rootstock (this work), we decided to probe the potential effects of the 3α-monofluoro analog of typhasterol (3F-HTY), and the parent compound (HTY), against the formerly tested 5α-monofluoro derivative of typhasterol (5F-HTY) in our testing system, the marubakaido rootstock. In contrast to what was found for 5F-HTY, neither 3F-HTY nor HTY were able to significantly stimulate new shoot formation, at the dose used (2.5 µg per shoot, the most effective dose of 5F-HTY), regardless of the kind of shoot, i.e., main or primary lateral shoot (Fig. 7). However, similar to what was observed for 5F-HTY, both, HTY and 3F-HTY were able to significantly stimulate main shoot elongation, although, in contrast to 5F-HTY, HTY, and 3F-HTY were not able to stimulate primary lateral shoot elongation (Fig. 8). Since neither 3F-HTY nor HTY were able to stimulate new shoot formation, it was not surprising to find that neither 3F-HTY nor the parent compound were able to enhance the multiplication rate of the in vitro-grown marubakaido rootstock (Fig. 9).

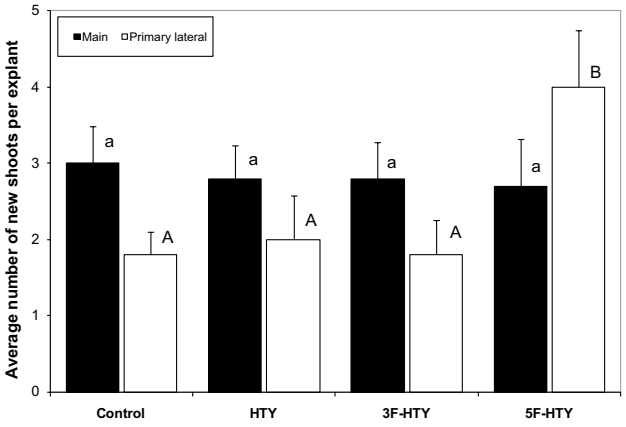

Fig. 7 Comparative effect of homotyphasterol (HTY), 3α-monofluoro homotyphasterol (3F-HTY) and 5α-monofluoro homotyphasterol (5F-HTY), at 2.5 µg per shoot, in the average number of newly formed main and primary lateral shoots suitable for micropropagation (measuring at least 15 mm in length). Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Columns topped by the same lower-case letters indicate average number of newly formed main shoots which do not differ at $p=0.05$ (Tukey's test). Columns topped by the same upper case letters indicate average number of newly formed primary lateral shoots which do not differ at $p=0.05$ (Tukey's test). Vertical bars indicate ± standard error

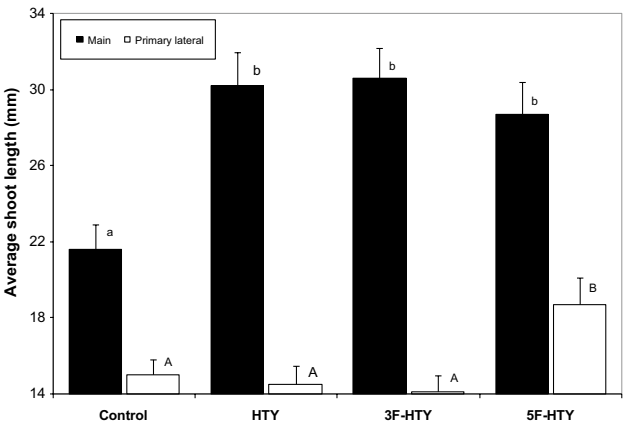

Fig. 8 Comparative effect of homotyphasterol (HTY), 3α-monofluoro homotyphasterol (3F-HTY) and 5α-monofluoro homotyphasterol (5F-HTY), at 2.5 µg per shoot, in the average shoot length of newly formed main and primary lateral shoots. Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Columns topped by the same lower-case letters indicate average number of newly formed main shoots which do not differ at $p=0.05$ (Tukey's test). Columns topped by the same upper case letters indicate average number of newly formed primary lateral shoots which do not differ at $p=0.05$ (Tukey's test). Vertical bars indicate ± standard error

Discussion

Effects of the 5α-monofluoro derivative of typhasterol (5F-HTY) on shoot production by the marubakaido apple rootstock

BRs act interactively with other plant hormones in the regulation of plant elongation, i.e., additive effects with gibberellins and synergistic effects with indoleacetic acid on stem segment elongation (Tong et al. 2014; Unterholzner et al. 2015). BRs have particularly strong growth-promoting effects in stems of seedlings and young plants, mainly via promotion of expression of genes involved in cell elongation and wall extensibility (Horvath et al. 2003; Yang et al. 2011). In this study, analysis of the effect of the synthetic BR 5F-HTY on the shoot production in our testing system, the

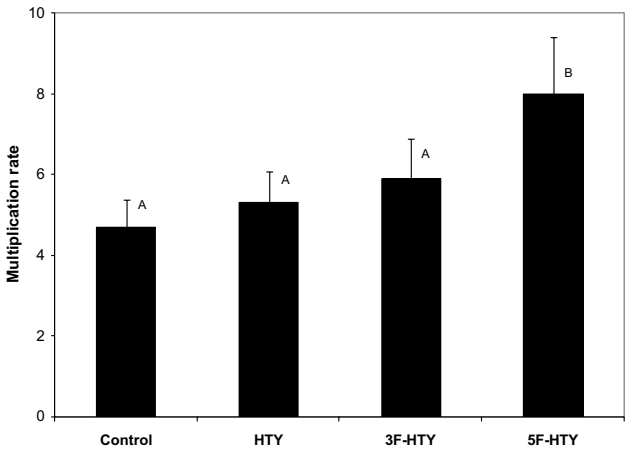

Fig. 9 Comparative effect of homotyphasterol (HTY), 3α-monofluoro homotyphasterol (3F-HTY) and 5α-monofluoro homotyphasterol (5F-HTY), at 2.5 µg per shoot, in the multiplication rate. Values represent averages of five replicates (five culture vessels) with five explants per replication, of a single experiment. Columns topped by the same letters indicate averages which do not differ at $p=0.05$ (Tukey's test). Vertical bars indicate ± standard error

marubakaido apple rootstock, demonstrated that the fluoro-BR-stimulated shoot elongation, although it had a greater effect on the elongation of primary lateral shoots than on elongation of main shoots. The high correlation coefficients found for the average main shoot and primary lateral shoot lengths, and dose of 5F-HTY, indicate that changes in the average length of these shoots are very closely related to the different doses of 5F-HTY applied to the marubakaido shoots. In addition, analysis of the effect of the synthetic BR 5F-HTY on the shoot production in the marubakaido showed that 5F-HTY significantly stimulated formation of primary lateral shoots. However, this stimulation of primary shoot formation was not paralleled by any statistically significant effect on the formation of main shoots. The higher correlation coefficient found for the number of neo-formed primary lateral shoots and dose of 5F-HTY, compared to correlation coefficient found for the number of neo-formed main shoots and dose of 5F-HTY indicates that formation of primary lateral shoots is more closely related to the dose of applied 5F-HTY, compared to the formation of main shoots.

We have previously shown that BL, the most potent natural BR (Lei et al. 2017), induced a maximum 46% increase in the formation of new primary lateral shoots, which was found when shoots were treated with 0.50 µg of BL (Pereira-Netto et al. 2009). Thus, it was somewhat surprising to find that in this study, the 5α-monofluoro derivative of homo-TY (5F-HTY) was much more effective for stimulation of primary shoot formation, compared to BL, inducing a 142% increase in the number of newly formed primary lateral shoots in the marubakaido apple rootstock. However, this maximum stimulation of primary lateral shoot formation was only achieved when shoots were treated with 2.5 µg of 5F-HTY. Although BL was not tested in this work and consequently a direct comparison cannot be made, findings described in this work, along findings previously reported on the effects of BL, indicate that 5F-HTY is more effective at stimulating maximum formation of primary lateral shoots, when compared to BL. If that is in fact the case, it becomes especially relevant when we consider that: (1) homoBRs, like HBL, typically show similar or reduced biological activity when compared to their counterparts, like BL (McMorris et al. 1994); (2) 7-oxalactone BRs, such as BL and HBL, generally present stronger biological activity when compared to 6-oxo BRs, such as HTY (Bajguz and Tretyn 2003). The reason(s) why BL was less effective at stimulating primary lateral shoot formation, compared to 5F-HTY, is(are) not clear. It is possible that these differential effects might depend on the extent to which these different BRs satisfy the structural requirements of BR receptors. For example, an increased affinity of the 5F derivative of HTY for the receptor or an enhanced binding time of the derivative 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 5F-HTY did not stimulate formation of main shoots, compared to BL. The higher effectiveness of 5F-HTY towards stimulation of the formation of primary lateral shoots, compared to BL, might also be due to an eventually higher ability of 5F-HTY to stimulate BR biosynthetic enzymes, compared to BL. However, other possibilities such as an eventually higher susceptibility of the natural BL to inactivation by BAS1, a BR-inactivating gene (Roh et al. 2012), compared to 5F-HTY, a synthetic BR, cannot be ruled out. Besides the stimulatory 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). In this study, 5F-HTY significantly stimulated both main and primary lateral shoot elongation, like BL did. And again, similar to what we have previously shown for BL, the 5F-HTY growth-promotive effects were more effective for primary lateral shoots compared to main shoots. Thus, when put together, data from this study and data that we have previously shown for shoots of *Eucalyptus* (Pereira-Netto et al. 2006a) and marubakaido rootstock (Pereira-Netto et al. 2006b) treated with 28-HCS and 5F-HCS, respectively, clearly demonstrate that 5F-HTY and other BRs, affect differentially the ability to stimulate main and primary lateral shoot formation.

The closer the intermediate in the pathway to BL, the greater is its activity, and bioactivities for homoBRs present the same trend (Reviewed in Ramirez and Galagovsky

2008). TY, one of the two immediate precursors of CS in the BL biosynthetic pathway, is converted to CS by the cytochrome P450 CYP90C1, which is considered an activation step in the BL pathway (Kim et al. 2005). Thus, it was somewhat surprising to find that in contrast to our previously reported effects of homocastasterone (HCS) on in vitro-grown marubakaido rootstock (Pereira-Netto et al. 2003), 5F-HTY (this study) effectively stimulated shoot production. However, since we previously have shown that 5F-HCS also significantly stimulated shoot production in in vitro-grown marubakaido (Schaefer et al. 2002) it is reasonable to consider that fluorination at C5 prevails over position of the compound on the BR biosynthetic pathway, regarding stimulation of shoot production in our system. In addition, HTY is not considered to present high biological activity per se, 5F-HTY presents similar activity towards stimulation of primary lateral shoot elongation, when compared to BL, and 5F-HTY is more effective than BL towards stimulation of primary lateral shoot formation. Thus, the results reported in this work and in previous reports from our group suggest that 5F-HTY might be biologically active per se, at least in our system, without requiring its conversion to other forms of BRs that are downstream in the BRs biosynthetic pathway to present high biological activity.

Repressed outgrowth of axillary buds has been associated with auxins while cytokinins have been associated with promoting outgrowth of these buds. Similarly to cytokinins, although more recently, BRs have also been reported to control shoot production. For example, application of BL and CS to the dumpy (dpy) mutant of tomato, a mutant presenting reduced axillary branching, is known to rescued the dpy phenotype (Koka et al. 2000). Although the cytokinin benzyladenine was used in our culture medium, it is apparent that exogenously applied BRs can change patterns of shoot production, since BR concentration-dependent responses were found in this study as well as in our previously reported work (Schaefer et al. 2002; Pereira-Netto et al. 2006b, 2009). BRs have also been reported to increase endogenous cytokinin levels in various plant species. For example, treatment with 5 µM epiBL or homobrassinolide resulted in increased trans-zeatin riboside content in *Phaseolus vulgaris* (Upreti and Murti 2004). Thus, the 5F-HTY-driven stimulation of primary shoot production observed in our testing system, might also be due to an eventual 5F-HTY-driven enhancement in cytokinin content. In addition, since transport of BRs over long distances is not known and their homeostasis is tightly regulated to ensure that their cellular levels are adequate to induce a response (Peng et al. 2015), the 5F-HTY-induced stimulation of shoot production observed in this work likely relies on mechanism(s) yet to be identified, that may not be restricted to potential changes in endogenous levels of BRs at the BR application sites.

The 5F-HTY-driven stimulation of primary lateral shoot formation and further elongation reported in this study resulted in significantly enhanced multiplication rate (MR) for the marubakaido apple rootstock. The high correlation coefficient found for multiplication rate and dose of 5F-HTY, indicates that changes in MR observed in this study are very closely related to the different doses of 5F-HTY applied to the marubakaido shoots. In vitro multiplication rates reported for this rootstock before we treated them with BRs (Schaefer et al. 2002; Pereira-Netto et al. 2006b) were in the 4–5 new shoots per explant range (Nunes et al. 1999), which resulted in an in vitro propagation technique for this rootstock barely feasible for commercial purposes. Thus, the 5F-HTY-driven enhancement of MR reported in this study 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 constraint for efficient micropropagation protocols.

Comparative effects of the 3α and 5α-monofluoro derivative of typhasterol (5F-HTY) and the parent compound on shoot production by the marubakaido apple rootstock

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 well known that alteration of the functional groups in the carbon skeleton affects the degree of response elicited by a given compound (Wendeborn et al. 2017). The carbon–fluorine bond is physicochemically similar to the C–OH bond, rather than the C–H bond (Penglis 1981; Welch 1987; Todoroki et al. 1995). Thus, fluorine could be considered as being equivalent to the oxygen of the hydroxyl group. In the present study, our finding that 5F-HTY was able to stimulate formation and further elongation of primary lateral shoots and elongation of main shoots in the marubakaido rootstock prompted us to test the potential effect of the parent compound (HTY) and a 3α-fluoro derivative as a way to investigate if the presence of the fluoro atom at C5 was or was not a requirement for the homotyphasterol derivative to present strong biological activity. HomoTY (HTY), like all natural brassinosteroids, occurs in various plant species, including monocots and dicots, a gymnosperm and an alga (reviewed in Joo et al. 2015). In the rice lamina inclination bioassay, HTY has been previously shown to present much less biological activity when compared to 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, we have previously observed

that HCS was not able to stimulate new shoot formation (Pereira-Netto et al. 2012) or shoot elongation (unpublished data). Thus, surprisingly, in this study we have found that besides 5F-HTY, HTY was also able to significantly stimulate main shoot elongation, although only 5F-HTY was able to stimulate primary lateral shoot elongation. Neither HTY nor 5F-HTY was able to significantly stimulate main shoot formation. Thus, the BR-induced stimulation of main shoot elongation reported in this study might not rely on C2 α-hydroxylation of HTY.

HTY, as all natural bioactive BRs, possesses a vicinal 22*R*, 23*R* diol structural functionality, which seems to be essential for high biological activity (Duran et al. 2017). 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 this study, 5F-HTY presented 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 this study also demonstrated that HTY presented similar activity, towards stimulation of main shoot elongation, compared to our previously reported data on the effect of BL on main shoot elongation (Pereira-Netto et al. 2009). However, in contrast to BL, HTY presented no activity towards main or primary lateral shoot formation or towards primary shoot elongation in our system. As mentioned before in this report, 5F-HTY presented higher biological activity towards primary shoot formation, compared to both HTY and our previously reported data on the effect of BL on shoot production in the marubakaido rootstock. Since BL usually shows higher activity when compared to HBL (Khripach et al. 2000), all of these data, seen together, provide further support to the idea that 5F-HTY might be active per se towards primary shoot formation in our system. It is noteworthy that 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 results from this study (stimulation of primary shoot 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 HTY (this study), BL (Pereira-Netto et al. 2009), HCS (Pereira-Netto et al. 2006b, 2012) and F-derivatives of these homoBRs provide support to the idea that biological activities of BRs cannot be generalized from a single bioassay system.

It is well known that a fluorine atom can be introduced into bioactive molecules without significantly changing their geometry and shape (Liu et al. 2017). However, the metabolic stability of a C–F bond (Slavikova et al. 2008) 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. In fact, when used at 5 and 50 ng per plant, the biological activity of 3F-HTY was about half of the biological activity of HTY in the rice lamina inclination test (Galagovsky et al. 2001). This reduced activity is in agreement with the fact that 3F-HTY is not hydroxylated at C2–CS, the immediate precursor of HBL (Galagovsky et al. 2001). In this study, when used at 2.5 µg per shoot, 3F-HTY was 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 was enhanced by HTY and their two monofluoro derivatives used in this study. However, length of primary lateral shoots was enhanced by 5F-HTY and unaffected by HTY and 3F-HTY. It might imply that these BRs might have different activity 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.

Conclusions

In this publication, we report on the evaluation of the biological activity of 5F-HTY, a synthetic BR, and compare its effectiveness against the parent HTY and 3F-HTY towards stimulation of shoot production in the marubakaido apple rootstock. The results reported here provide an insight into the morphological responses of in vitro-grown marubakaido shoots to fluoro substitutions, in alpha configuration, on the sterol structure of exogenously supplied HTY. The biological activity of the two fluorinated derivatives of HTY used in this study is clearly dependent on the position of the substitution. 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. Furthermore, data on the effects of exogenous HTY and their 5C and 3C fluoro derivatives used in this trial on both, shoot elongation and formation, demonstrate that modification of the allocation of growth among the various types of shoots can be effectively achieved at the biochemical/physiological level in marubakaido through applications of 5F-HTY.

Beside being capable of effectively enhancing shoot production and also being non-toxic, BRs are environmentally friendly which bring vast perspectives for the application of compounds like 5F-HTY in agriculture, forestry

and horticulture. In horticulture, for example, practical applications for our findings include the 5F-HTY-driven enhancement of the multiplication rate for in vitro-grown marubakaido. In addition, the 5F-HTY-stimulated shoot production reported here is potentially useful to improve micropropagation techniques for clonal propagation of other plant species as well, especially woody species, in which the impossibility of effective stimulation of shoot production is often a limitation for commercial in vitro propagation. In production orchards, potential benefits include promotion of shoot formation, 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 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.

Author contribution statement ABP-N designed the study, carried out the experiments, interpreted the results and drafted the manuscript. JAR and LRG synthesized the brassinosteroid derivatives used in the study and reviewed the draft.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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