Scientia Horticulturae 181 (2015) 13–17

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Comparative analysis of the effects of homocastasterone and two derivatives on shoot and main root elongation of tomato plantlets

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ARTICLE INFO

Article history:
Received 17 May 2014
Received in revised form 24 October 2014
Accepted 28 October 2014

Keywords:
28-homocastasterone
Brassinosteroids
Lycopersicum esculentum
Rooting

ABSTRACT

Brassinosteroids (BRs) comprise a specific class of low-abundance plant steroids of ubiquitous occurrence in plants. Molecular genetic analysis of mutants defective in BRs biosynthesis or response has demonstrated that the ability to synthesize, perceive and respond to BRs is critical for normal plant growth. In this work, the structure-activity relationships of two synthetic derivatives of 28-homocastasterone (28-HCS) were evaluated on tomato plantlets using the native 28-HCS and epibrassinolide (epi-BL) as control. Progressive enhancement in the main root elongation was found for plantlets treated with increased doses of 5α-fluoro-28-homocastasterone (5F-HCS) within the 0.5–4 μg shoot^{-1} range. When compared to epi-BL, to the parental 28-HCS and to a mono 5-hydroxy HCS derivative (5OH-HCS), no difference between 5F-HCS and the other BRs, towards stimulation of main root elongation, was found when these BRs were applied at a dose of 2.0 μg per plantlet. Both, 5F-HCS and 5OH-HCS significantly enhanced shoot elongation, though significantly less than HCS and epi-BL did. Besides provinding an insight into the morphological responses of tomato plantlets to application of 28-HCS derivatives, these results also demonstrate that the 5F-HCS-induced stimulation of main root elongation might be useful to improve the plants's nutrient and especially water uptake, particularly under water deficit, besides improving anchorage.

1. Introduction

Similar to the animal steroidal hormones, the structures of brassinosteroids (BRs) consist of a cholesterol skeleton containing various hydroxyl substitutions and attached functional groups. BRs comprise a specific class of low-abundance plant steroids of ubiquitous occurrence in plants (Fujioka, 1999; Belkhadir and Chory, 2006). BRs can induce a broad spectrum of responses, including stimulation of longitudinal growth of young tissues via cell elongation and cell division in a wide range of plant species (Zurek et al., 1994; Oh and Clouse, 1998; Hu et al., 2000). In fact, mutant analysis has clearly shown that the ability to synthesize, perceive and respond to BRs is essential to normal plant growth and development (Haubrick and Assmann, 2006). Exogenous applications of BRs are known to stimulate cell elongation and division, and vascular differentiation (Mandava, 1988; Clouse and Sasse, 1998; Sasse, 1999; Clouse, 2002), all important to allow shoot/root elongation.

Experiments carried out in various countries demonstrated the beneficial effects of BRs, the newest discovered class of plant hormones, and consequently their several potential practical applications, for example, in agriculture and promotion of human health (Pereira-Netto, 2012). However, the high production cost of these natural plant steroids has hampered initiatives for their wide commercial use. But, the increasing availability of affordable commercial formulations produced in Belarus, China, India, Japan and Russia has opened new perspectives for the wider commercial use of BRs. In fact, BRs are now officially registered and have been commercially used, in extremely low doses, to enhance crop yield and quality (Pereira-Netto, 2012). BRs present a positive safety profile, once no treatment-related adverse effect was observed for orally provided doses up to 4000 mg/kg for epibrassinolide (epi-BL) (Kuzmitsky and Mizulo, 1991) or homobrassinolide (HBL) (Murkunde and Murthy, 2010).

Although the structural requirements for bioactivity in plants are known, various compounds bearing minor to major structural modifications have been shown to present potent plant growth promoter activity (Romero-Avila et al., 2007; Esposito et al., 2011), being continuously under scrutinization. BL precursors such as

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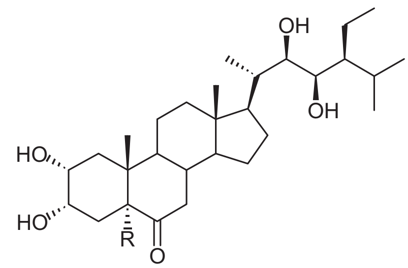
http://dx.doi.org/10.1016/j.scienta.2014.10.048
0304-4238/© 2014 Published by Elsevier B.V.

28-homocastasterone (28-HCS) has been widely employed in field trials because of its greater synthetic accessibility compared to typically more active BRs, such as BL (Mandava, 1988; Fujioka and Sakurai, 1997; Baron et al., 1998). 28-HCS and 28-HBL are the most active naturally occurring C_{29} BRs (Yokota, 1999; Bajguz and Tretyn, 2003). Since it is well established that changes on the functional groups often alter the magnitude of response elicited by a given compound (Brosa, 1999; Back and Pharis, 2003), we synthesized 28-HCS and two derivatives in order to enlarge studies on the effects of BRs analogues on bioactivity, more specifically on shoot and root elongation. In this paper, we report on the differential effects of synthetic derivatives of 28-HCS, containing a fluoro or a hydroxy group at C-5 position, on tomato main root elongation and its potential physiological consequences. The structure–activity relationships of the two analogs were evaluated using the native 28-HCS and epi-BL as control.



1 R=H 28-homocastasterone (28-HCS)

2 R=F 5 α-fluoro-28-homocastasterone (5F-HCS)

3 R=OH 5 α-hydroxy-28-homocastasterone (5OH-HCS)

Fig. 1. Structural formulae of 28-homocastasterone and its fluro and hydroxy derivatives used in this trial.

2. Materials and methods

2.1. Plant material

Seeds from the Santa Cruz Kada Gigante Paulista variety of tomato (*Lycopersicum esculentum*) (Feltrin Sementes, Farrupilha, RS, Brazil) were sown in a mixture (1:2) of grounded coco nut husks (Nutriplan, Cascavel, PR, Brazil) and a commercial substrate consisted of *Eucalyptus* bark, turf, sugar cane bagasse and sawdust in 1.5 l polyethylene containers. About 100 mL of water per plantlet was provided twice a week.

2.2. Growth conditions

Plantlets were maintained in a Conviron reach-in MTR30 growth chamber (www.conviron.com, Controlled Environments Limited, Winnipeg, Manitoba, Canada), using a completely randomized design. Light was provided by 10 cool-white fluorescent tube lamps (Phillips F72T8/TL841/HO, Andover, Massachusetts, U.S.A.), supplemented by six incandescent bulbs (GE A19, Fairfiled, California, U.S.A.) providing a photosynthetic photon flux density (PPFD) at canopy height according to Table 1.

2.3. Synthesis of the fluoro and hydroxy derivatives of homocastasterone

28-homocastasterone (Systematic name (22R,23R)-2α,3α,22,23-Tetrahydroxy-5α-stigmastan-6-one, 28-HCS) and 5α-monofluoro and 5α-monohydroxyl analogs of 28-HCS, 5F-HCS ((22R, 23R)-2α, 3α, 22, 23-Tetrahydroxy-5α-fluorostigmastan-6-one) and 5OH-HCS ((22R, 23R)-2α, 3α, 22, 23-Tetrahydroxy-5α-hydroxystigmastan-6-one), respectively, were synthesized from stigmasteryl acetate, through (22E)-5-Fluoro-3β-hydroxy-5α-stigmast-22-en-6-one, as previously described (Ramirez et al., 2000) (Fig. 1).

Table 1
Average light intensity on the top of the plantlets, and temperature and relative humidity around plantlets.

Time	Light intensity ($\mu mol\, m^{-2}\, s^{-1}$)	Temperature (°C)	Relative humidity (%)
8–11 a.m.	328 ± 2	22.0 ± 0.03	73.3 ± 0.8
11 a.m.–1 p.m.	440 ± 2	24.1 ± 0.30	71.1 ± 0.6
1 p.m.–6 p.m.	322 ± 3	24.0 ± 0.03	71.3 ± 0.9
6 p.m.–8 a.m.	–	20.0 ± 0.40	73.0 ± 0.7

2.4. Effect of different doses of 5F-homocastasterone on tomato main root elongation

Two acetone (P.A., Vetec Química fina, Rio de Janeiro, RJ, Brazil) microdrops (5 μL, each) containing known amounts of 5F-HCS (please see synthesis description above) were pipetted, with the help of an adjustable, single channel, manual, 0–20 μL micropipettor (Gilson, Inc., Middleton, WI, U.S.A) onto the main vein of the cotiledonary leaves (one microdrop per leaf). The cotiledonary leaves were at least 2 mm wide, and between 15 and 20 mm in length, of eight day-old plantlets originating from seeds as described above. Only single applications were used and control plantlets were treated with 5 μL acetone microdrops, only.

2.5. Comparative effect of 28-HCS, 5F-HCS, 5OH-HCS and epi-BL on tomato main root elongation

Two acetone microdrops (5 μL) containing 1 μg of 28-HCS, 5F-HCS, 5OH-HCS and epi-BL (22R,23R,24R-2α,3α,22,23-Tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one), each, were pipetted onto the cotyledonary leaves, as above described. Control shoots were treated with 5 μL 95% (v/v) acetone microdrops. Epi-BL was purchased from Sigma-Aldrich Co. (St. Louis, USA).

2.6. Experimental design and statistical analysis

Each treatment, i.e., dose of 28-HCS, 5F-HCS, 5OH-HCS or epi-BL, consisted of 22 replicates (one replicate = one plantlet). Each experiment was repeated at least twice, with similar results. After a significant analysis of variance, the differences between means for treatments were analyzed by Student's *t*-test ($p = 0.05$).

3. Results and discussion

3.1. Effect of different doses of 5F-homocastasterone on tomato main root elongation

Since we have previously found that 5F-HCS was highly effective towards shooting in *in vitro*-grown Marubakaido apple rootstock (Schaefer et al., 2002), we decided to investigate if this response to 5F-HCS would be either widespread in plants or restricted to specific systems like *in vitro*-grown Marubakaido apple rootstock. Progressive enhancement in the main root elongation was found for tomato plantlets treated with increased doses of 5F-HCS within the 0.5–4 μg $shoot^{-1}$ range (Fig. 2). Application of 5F-HCS at 2.0 μg per plantlet resulted in a statistically significant 50% increase in main root elongation, compared to plantlets treated with 5 μL acetone (control).

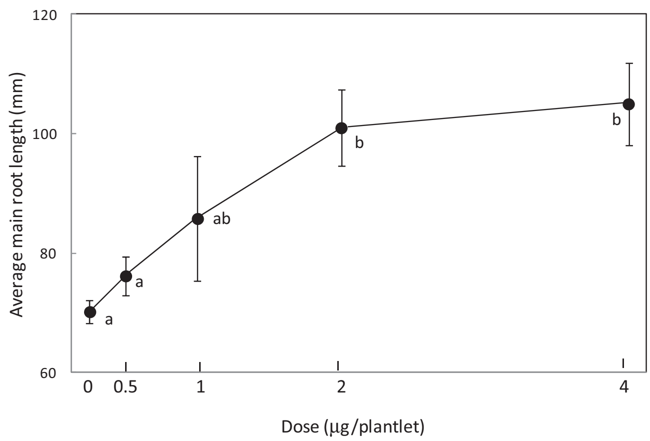


Fig. 2. Effect of different doses of 5F-HCTS on the average main root length. Vertical bars indicate ± standard error. Values followed by the same letter are not significantly different at $p = 0.05$ (Student's t test), within the treatment (5F-HCTS).

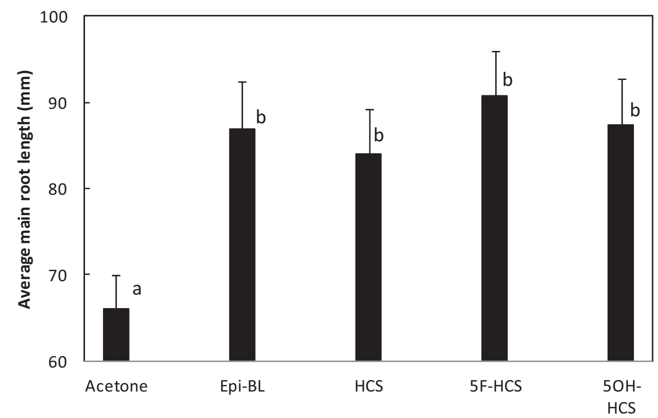


Fig. 3. Effect of 2 μg of 28-homocastasterone and its derivatives, and epibrassinolide on the average main root length. Vertical bars indicate ± standard error. Values followed by the same letter are not significantly different at $p = 0.05$ (Student's t test), within the treatment.

Architecture of the root system is critical for the proper anchorage of the plant and to the plant's ability to compete effectively for water and nutrients as well (Lynch, 1995; Dastidar et al., 2012). The root system architecture is determined by the pattern of root branching and by the rate and trajectory of growth of individual roots. This plasticity relies not only on direct genetic control but also on the permanent integration of environmental stimuli and internal developmental programs controlling root branching (Schiefelbein and Benfey, 1991; Robinson, 1994; Dastidar et al., 2012). The internal components of plant signaling are typically mediated by chemical growth regulators (Azpiroz et al., 1998), and most multicellular organisms rely on steroids as signaling molecules for the regulation of physiological and developmental programs (Wang et al., 2001). We have previously demonstrated that exogenous BRs at doses at the microgram per shoot range are able to significantly change shoot arquitecture, stimulating or inhibiting shoot formation and elongation of *in vitro*-grown trees such as a hybrid between *Eucalyptus grandis* and *Eucalyptus urophylla* (Pereira-Netto et al., 2006a) and the Marubakaido rootstock (Schaefer et al., 2002; Pereira-Netto et al., 2006b), depending on the kind and dose of the BR used and/or the kind of shoot considered. Analysis of the changes on the average shoot and main root length in tomato plantlets (this work) clearly show that 5F-HCS more effectively stimulates main root elongation compared to shoot elongation. These findings undoubtedly show that 5F-HCS affects differentially the morphogenetic potential of shoots and especially of the main roots, and, consequently the whole plantlet growth/development pattern.

Molecular and genetic approaches have indicated that balanced BR signaling is a requirement for the optimal root growth and that, BRs are involved in the control of cell cycle progression and differentiation in the root meristem of *Arabidopsis thaliana* (González-García et al., 2011). In addition, semi-quantitative reverse-transcriptase PCR analysis of the effects of different plant hormones on *DWF4* transcription, which encodes a C-22 hydroxylase, crucial for BR biosynthesis and for the feedback control of endogenous BR levels in *Arabidopsis* has suggested that BRs act positively on root elongation (Yoshimitsu et al., 2011). However, earlier work carried out on *in vitro*-grown tomato excised roots demonstrated that no growth alterations were observed for roots treated with brassinolide, 24-epibrassinolide, 22,23,24-trisepibrassinolide and 28-homobrassinolide at 10^{-11} M or less, though treatment with those BRs resulted in some root growth inhibition when the BRs were used at one or more of the higher concentrations (10^{-6}–10^{-10} M) (Roddick, 1994). On the other hand, analysis of the data available on the effect of BRs on root growth indicate that exogenous application of BRs to BR-deficient and BR-insensitive mutants, which display a short-root phenotype, seem to promote root growth at low concentrations, to inhibit it at high concentrations (reviewed in González-García et al., 2011). Thus, BR effects on root growth seem to be significantly dependent on the kind and concentration of the used BR. It is possible that the fluorinated HCS used in this trial shows biological activity by itself towards the stimulation of main root and shoot elongation in tomato plantlets, although other possibilities like that enzymes of the BR biosynthetic pathway might be able to convert 5F-HCS into brassinolide, a more bioactive form of BR (Bancos et al., 2006) cannot be ruled out.

3.2. Comparative effect of homocastasterone (HCS), 5F-HCS, 5OH-HCS and epibrassinolide on tomato main root elongation

In order to help to elucidate the mechanism through which the monofluoro derivative of HCS (5F-HCS) used in this trial was able to stimulate shoot elongation and especially main root elongation in tomato plantlets, we synthesized a 28-homocastasterone analog in which a 5α-H group was introduced at C-5 (Ramirez et al., 2000). Afterwards, we tested the effectiveness of the parental compound 28-HCS, its mono hydroxyl derivative 5OH-HCS and the widely commercially available epi-BL on the modification of the morphogenetic ability of tomato plantlets. When compared to the commercially most widely used and biologically active BR epi-BL, to the parental 28-HCS and to a mono 5-hydroxy HCS derivative, no difference between 5F-HCS and the other BRs, towards stimulation of main root elongation, was found when these BRs were applied at a dose of 2.0 μg per plantlet ($p = 0.05$; Fig. 3). However, all of the BRs used in this trial significantly enhanced main root elongation when compared to acetone-treated plantlets.

3.3. Comparative effect of homocastasterone (HCS), 5F-HCS, 5OH-HCS and epibrassinolide on tomato shoot elongation

In addition to the evaluation of the effects of the mono 5-hydroxy and 5-fluoro HCS derivatives, the parental 28-HCS and epi-BL on main root elongation, we assessed the effect of these BRs on tomato shoot elongation, as well. Both, 5F-HCS and 5OH-HCS significantly enhanced shoot elongation in tomato plantlets, though this effect was significantly reduced when compared to the 28-HCS and epi-BL-induced stimulation of shoot elongation (Fig. 4). No significant difference was found between 28-HCS and epi-BL towards stimulation of shoot elongation.

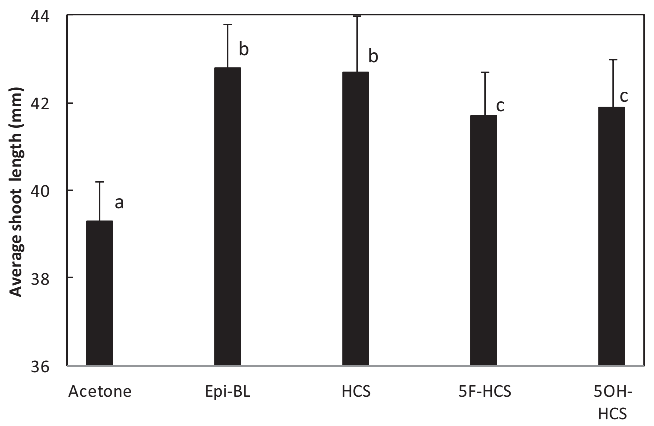


Fig. 4. Effect of 2 μg of 28-homocastasterone and its derivatives, and epibrassinolide on the average shoot length. Vertical bars indicate ± standard error. Values followed by the same letter are not significantly different at $p = 0.05$ (Student's t test), within the treatment.

Although being also able to stimulate shoot elongation in this study (Fig. 4), the maximum incremental effect of 5F-HCS on shoot elongation was about 10-fold less when compared to the effect of 5F-HCS on the increment of main root elongation (Figs. 2 and 3).

Mutants deficient in BR biosynthesis or response have significantly helped to enhance knowledge about BRs and their actions (Clouse and Sasse, 1998; Li and Chory, 1999; Bishop and Yokota, 2001; Clouse, 2002; Yun et al., 2009). However, the use of BR derivatives is an alternative way for the analysis of the BRs functions. In the search to develop higher activity analogs of BRs, several research groups have introduced structural modifications through various means such as alkylation, alkoxylation or halogenation (Saito et al., 1998; Back et al., 2000; Back and Pharis, 2003; Eignerová et al., 2009; Litvinovskaya et al., 2009). Replacement of a hydrogen atom by fluorine in what was originally a carbon—hydrogen bond is unique in effecting the least increase in size but, simultaneously, markedly increasing electronegativity and hydrogen bonding potential (Kirk and Cohen, 1971), besides having significant potential effects on the electron distribution and dipole moments within the substituent (Welch, 1987). 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). The first fluorinated BR derivative was synthesized back in 1993 through the cleavage of the side chain of hydeoxycholic acid (Jin et al., 1993). Since then, several new fluoro BR derivatives were prepared. Although substantial efforts towards the development of methods to introduce fluorine groups in bioactive BRs have been carried out (see reviews in Pereira-Netto et al., 2003; Back and Pharis, 2003; Acebedo et al., 2009, 2011; Esposito et al., 2011), only a limited number of studies describing their biological activity have been reported, most of these reports being on animal cells. For example, 5α-fluorotyphasterol has been found to be more active than typhasterol in the rice lamina inclination test (Ramirez et al., 2000), while 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, introduction of a fluorine at C-3 in the A ring led to increased cytotoxicity, as measured by MTT assay, against murine fibroblast cell line NIH-3T3, as compared to the original HBL and 28-HCS (Esposito et al., 2011). Pioneer work concerning the biological activities of C-5 heteroatom substituents on synthetic 28-homoBR derivatives demonstrated that a hydroxyl substitution resulted in reduced biological activity in the rice lamina inclination test, although no significant difference was found for a fluorine substitution, when compared to the parent compound (HCS) (Ramirez et al., 2000). In this study, comparative analysis of the effect of the 5α-monofluoro derivative of 28-HCS, a compound highly effective towards shooting in the Marubakaido apple rootstock (Schaefer et al., 2002), 5OH-HCS and the parent compound 28-HCS, along with epi-BL on shoot elongation demonstrated that shoots are more responsive to 28-HCS, compared to both of the 28-HCS derivatives used in this trial. When seen together, results from the pioneer work (Ramirez et al., 2000), together with previously reported data from our laboratories for shoots of *Eucalyptus* (Pereira-Netto et al., 2006a) and the Marubakaido apple rootstock (Pereira-Netto et al., 2003, 2006b) treated with fluoro and hydroxy derivatives of 28-HCS, and 28-HCS itself, along with results from this study provide some interesting information concerning biochemical action of these compounds at a molecular level. These findings, clearly show that fluoro and hydroxy substitutions on carbon 5 of 28-HCS, change the ability of the parental 28-HCS to stimulate or inhibit plant's responses, for example root and shoot elongation, depending on the chosen system, i.e. plant species and/or growth conditions (*in vivo* or *in vitro*). That is not unexpected, once it is well established that the presence of a substituent at C-5 with the ability to form an hydrogen bonding with the substituent of C-3 is able to change the biological activity of a given compound (Brosa et al., 1997). Possible explanations for the differential biological activity of 28-HCS and its 5 fluoro and hydroxy derivatives, along with epi-BL, found in this study might rely on the extent to which these molecules influence BR biosynthetic enzymes, present chemical stability, or, eventually, satisfy the structural requirements of BR receptors. In addition, the differential responses seen for BRs described in this paper might suggest the existence of different BR biosynthetic routes or perhaps that there are more than a single receptor site for each 28-HCS and its fluoro and hydroxy derivatives, and epi-BL in our system mediating different responses to BRs.

In most of BRs, an S-oriented alkyl (methyl or ethyl) group is present at C-24 of the side chain. Nevertheless, there are five exceptions among BRs which have R-oriented alkyl group on the side chain of the steroid nucleus, and 24-epiBL or 24-epiCS are examples of this (Bajguz and Tretyn, 2003). It has been pointed out that the attachment of epi-BL to its receptor at the plasma membrane results in more distorted three dimensional conformational states when compared to homo-BL (HBL). This new, thermodynamically acquired, stable state of epi-BL seems to be more effective at triggering BR signal cascades than HBL (Hayat et al., 2011). Thus, it was somewhat surprisingly to find that no significant difference towards shoot and root elongation was found for plantlets treated with either epi-BL or 28-HCS in this trial. The reason(s) for that remain unclear.

4. Conclusions

The results reported here provide an insight into the morphological responses of tomato plantlets to application of 28-HCS and a 5 fluoro and hydroxy derivatives, along with epi-BL. These results demonstrate that stimulation of main root elongation can be achieved at the biochemical/physiological level in tomato plantlets through application of 5F-HCS, as effectively as epi-BL, the most widely used BR. The 5F-HCS-induced stimulation of main root elongation reported here might be used, among others, in agriculture and forestry, to improve the plants's nutrient and water uptake, which might be especially useful when soil water supply is limited, besides improving anchorage. Along with this practical immediate application, 5F-HCS can also be seen as a possible controlling factor in the allocation of growth between shoots and roots.

Acknowledgments

The authors thank CNPq-Brazil and Conicet-Argentina for financial support.

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