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NO and IAA Key Regulators in the Shoot Growth Promoting Action of Humic Acid in *Cucumis sativus* L.

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Abstract Previous studies have reported that a purified sedimentary humic acid (PHA) was able to increase the concentration of nitric oxide (NO), indole-acetic acid (IAA) and ethylene in cucumber roots. Here, we investigated if these effects are functionally related to the ability of PHA to improve shoot growth. The effect of specific inhibitors of NO, IAA and ethylene functionality and signaling on PHA-induced shoot growth was studied. Likewise, the effect of these inhibitors on the synthesis and activity of the phytoregulators concerned by PHA action in cucumber roots was also explored. The results show that shoot growth promoted by PHA is due to an increase of IAA concentration in the root through both a NO-dependent and a NO-independent pathway. In addition, the increased ethylene production in the root is regulated by an IAA-dependent pathway. Finally, results also showed that the increase of ABA concentration in the root is regulated through both IAA- and ethylene-dependent pathways. In summary, the shoot growth promoting action of PHA involves a complex hormonal network. On one hand, the PHA action is functionally linked to increments in NO and IAA concentration in roots. And on the other hand, PHA action also increases ethylene and ABA root concentration mediated by NO-IAA dependent pathways.

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Introduction

A number of studies have reported the ability of humic substances (HS) from diverse origin to promote the growth of diverse plant species cultivated in soil, inert substrates and hydroponics (Nardi and others 2002; Chen and others 2004; García-Mina and others 2004; Dobbss and others 2007; Zandonadi and others 2007; Canellas and others 2010; Mora and others 2010, 2012; Trevisan and others 2010; Muscolo and others 2013). This beneficial action is usually associated with improvements in plant mineral nutrition (Nardi and others 2002; Chen and others 2004). Indeed, HS increase both the root uptake of several nutrients, such as nitrate (Nardi and others 1991; Pinton and others 1999a; Quaggiotti and others 2004; Mora and others 2010) or iron (Pinton and others 1999b; Chen and others 2004; De Santiago and Delgado 2007; Aguirre and others 2009; Tomasi and others 2009), and the translocation of nutrients from the root to the shoot (Pinton and others 1999a; Mora and others 2010).

In addition, other studies have also reported that HS affect plant metabolism and physiology, at both the molecular and biochemical levels (Canellas and others 2002; Quaggiotti and others 2004; Zandonadi and others 2007; Aguirre and others 2009; Tomasi and others 2009; Trevisan and others 2009; Mora and others 2010, 2012; Muscolo and others 2013). These effects were observed in studies that used (i) HS obtained from compost and other substrates, which contained measurable concentrations of auxin (mainly indole-acetic acid, IAA) (Muscolo and others 1998; Canellas and others 2002; Dobbss and others

2007; Trevisan and others 2009; Jindo and others 2012) or cytokinins (CKs) (Jannin and others 2012; Muscolo and others 2013; Pizzeghello and others 2013), and (ii) purified humic acids with sedimentary origin (PHA), which did not contain measurable concentrations of the main plant phytoregulators including IAA, abscisic acid (ABA), CKs and gibberellins (GA) (Aguirre and others 2009; Mora and others 2010, 2012).

Several authors have reported that the HS-dependent mechanism promoting plant growth involves specific phytoregulators (Nardi and others 2002; Dobbss and others 2007; Trevisan and others 2009; Canellas and others 2010; Mora and others 2010, 2012; Zandonadi and others 2010; Muscolo and others 2013). In contrast, Schmidt and others (2007) hypothesized that HS is not exerting its effects through an auxin-dependent pathway but increasing uptake of essential nutrients. Thus, the hypotheses proposed to explain HS hormonal-mediated effects are diverse, although potentially complementary. This shows that the whole mechanism by which HS directly affects plant metabolism seems to be very complex and, in fact, remains elusive.

On the one hand, the biological action of HS on root growth and lateral root development might partially be explained by the presence of molecules (and/or molecular domains) with marked auxin (IAA-like) activity in HS structure (Canellas and others 2002, 2010; Nardi and others 2002; Dobbss and others 2007; Zandonadi and others 2007; Trevisan and others 2010; Muscolo and others 2013). In this same context, the shoot growth promoting effect of HS migth be explained by the presence of physiologically active CK (Pizzeghello and others 2013). Besides, the specific auxin-mediated effects linked to HS activity, mainly the increase of plasma membrane (PM) H^+-ATPase acitivity in roots, could be associated with (or mediated by) biochemical pathways regulated by nitric oxide (NO) (Mora 2010; Zandonadi and others 2010).

On the other hand, HS of sedimentary origin such as leonardite, without measurable concentrations of the main plant hormones in their structure, affected root development of cucumber plants by increasing the concentration of IAA, NO, and ethylene in the root (Mora and others 2012). Similarly, the shoot growth promoting action of this type of HS was directly associated with an increase in the concentration of some active CKs in shoots, probably mediated by a concomitant increased translocation of nitrate from the root to shoot linked to a PHA-mediated H^+-ATPase activation in the root (Mora and others 2010). These studies showed that the biological action of HS could also be explained by specific HS-structural features, independently of the possible presence of phytoregulators in their structure.

In any case, all these studies suggest that the beneficial effects of HS on plant growth and mineral nutrition are probably related to the ability to increase PM H^+-ATPase activity in the root (Nardi and others 1991; Pinton and others 1999a; Canellas and others 2002; Quaggiotti and others 2004; Zandonadi and others 2007; Mora and others 2010). It has been demostrated that PM H^+-ATPase activity in the root plays a crucial role regulating both mineral root uptake and root-shoot growth (Michelet and Boutry 1995; Sze and others 1999; Morsomme and Boutry 2000; Palmgren 2001; Sondergaard and others 2004). Likewise, it is very well known that PM H^+-ATPase activity is regulated by IAA- (Hager 2003; Rober-Kleber and others 2003), NO- (Zhao and others 2004; Zhang and others 2006) and ethylene- dependent pathways (Lucena and others 2006; Waters and others 2007). All of them are affected in the root by HS as it has been described above. It is therefore possible that the shoot promoting effect is mediated by the action of HS over one or several of those phytoregulators.

To study this hypothesis, we have evaluated the effects of specific inhibitors of the functional action of NO, IAA and ethylene on the increase in both shoot growth and the concentration of concerned phytoregulators, caused by PHA root application. Here we demonstrate that PHA can promote shoot growth through a complex hormonal network involving those PHA-affected phytoregulators in the root.

Materials and Methods

Plant Material and Growth Conditions

Cucumber seeds (*Cucumis sativus* L. cv Ashley) were germinated in darkness on perlite and filter paper moistened with 1 mM of $CaSO_4$ solution. Seven-day-old seedlings were transferred to 8 L plastic recipients in hydroponic solution. Seedlings were irrigated with a pH 6.0, aerated nutrient solution prepared according to Romera and others (1999) with slight modifications: (in mM) 0.50 $Ca(NO_3)_2$; 0.75 K_2SO_4; 0.65 $MgSO_4$; 0.5 KH_2PO_4; (in μM) 50 KCl; 40 H_3BO_3; 4 $MnSO_4$; 2 $CuSO_4$; 2 $ZnSO_4$, 1.4 Na_2MoO_4 and 40 of iron as EDDHA chelate. The nutrient solution was renewed every 2–3 days and pH was set at 6.0. Cucumber plants were grown in a growth chamber at 28/21 °C, 70–75 % relative humidity and with a 15/9 h day/night photoperiod (irradiance: 250 μmol m^{-2} s^{-1}). After 10 days, plants were transferred to renewed nutrient solution and different treatments were supplied.

Extraction, Purification and Characterization of Humic Acid

Humic acid employed in these studies was extracted and purified from a leonardite of Czech origin (PHA) following the International Humic Substances Society (IHSS) procedure as described in the IHSS web page (http://www.ihss.

gatech.edu/soilhafa.html). PHA was characterized using elemental analysis, carbon-^{13} nuclear magnetic resonance spectroscopy (^{13}C NMR) and high performance size exclusion chromatography (HPSEC) according to Mora and others (2012). The carbon concentration of the lyophilized samples was analyzed by a LECO CHN 900 analyzer.

Treatments of Cucumber Plants

Ten-day-old cucumber plants were treated with 2-phenyl-4,4,5,5-tetramethylmidazoline-3-oxide-1-oxyl (PTIO: 200 μM, a NO scavenger), 2-(*p*-chlorophenoxy)-2-methylpropionic acid (PCIB: 50 μM, an inhibitor of IAA action through the stabilization of the Aux/IAA proteins and the interaction with TIR1-related family IAA receptors) (Oono and others 2003) or cobalt nitrate (Co^{2+}: 10 μM, an inhibitor of ethylene biosynthesis through the inhibition of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, which converts the immediate precursor of ethylene, ACC, to ethylene). PTIO was obtained from Tokyo Chemical Industry Co., Ltd. PCIB and Co^{2+} were provided from Sigma-Aldrich. The effective dose of each inhibitor to be used in the experiments was obtained from previous studies described in Mora and others (2012).

Treatments supplied were: a control treatment that only received the NS (nutritive solution), a treatment with NS + PHA as a positive control and the different treatments with NS + inhibitor in the presence or absence of PHA. The NS containing the dose of inhibitors was supplied 16 h before application of PHA treatment. The dose of PHA used in the experiments was 100 mg L^{-1} of organic carbon according to Mora and others (2012).

Plants for IAA, ethylene and ABA analysis were harvested 72 h after application of PHA. We have included the analysis of ABA root concentration with or without PHA in the presence of the above mentioned inhibitors to differentiate inhibitor effects from the possible stress caused by their application. We selected the harvest time for the analysis of each phytoregulator according to previous studies employing inhibitors (Mora and others 2010, 2012).

Material for hormone quantification was immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. The different analytical determinations related to plant growth or hormone analysis were carried out using five replicates and the experiment was carried out twice.

Determination of IAA and ABA Concentrations in Roots

IAA and ABA concentrations were analyzed in root extracts using high performance liquid chromatography-electrospray-mass spectrometry (HPLC–ESI–MS/MS). The extraction and purification of these hormones were carried out using the method described by Dobrev and Kaminek (2002) with some variations according to Bacaicoa and others (2011).

Extraction and Purification Procedures for IAA and ABA

A total of 0.5 g of frozen plant tissue (previously ground to a powder in a mortar with liquid nitrogen) was homogenized with 5 mL of precooled (−20 °C) methanol:water (80:20, v/v) and 2.5 mM Na diethyldithiocarbamate (DDTC). The deuterium labelled internal standards ([$^2H_5$] indol-3-acetic acid, (D-IAA); [$^2H_6$] (+)-*cis*, *trans*-abscisic acid, (D-ABA); from Olchemim, Olomouc, Czech Republic) were added (100 μL of a stock solution of 400 ng mL^{-1} of each standard in methanol) to the extraction medium. After overnight extraction at −20 °C, solids were separated by centrifugation at 12,000×*g* for 10 min at 4 °C using a Centrikon T-124 centrifuge with an A8.24 rotor (Kontron Instruments, Cumbernauld, United Kingdom) and re-extracted for 1 h with an additional 4 mL of extraction mixture. Supernatants were passed through a Strata C18-E cartridge (3 cm^3, 200 mg) (Phenomenex, Torrance, CA; Ref. 8B-S001-FBJ), preconditioned with 4 mL of methanol followed by 2 mL of extraction medium. After evaporation at 40 °C until aqueous phase using a Labconco Vortex Evaporator (Labconco Co., Kansas City, MO), 0.5 mL of 1 M formic acid was added. Then, hormones were extracted successively with two portions of 5 and 4 mL of diethyl ether, and the organic phase was evaporated to dryness. The residue was redissolved in 250 μL of methanol:0.5 % acetic acid (40:60, v/v). Before the injection in the HPLC–ESI–MS/MS system, the solution was centrifuged at 8,000×*g* for 5 min.

Liquid Chromatography-Mass Spectrometry Quantification of IAA and ABA

Hormones were quantified by HPLC–ESI–MS/MS using a HPLC device (2795 Alliance HT; Waters Co., Milford, MA) coupled to a 3200 Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface. A reverse-phase column (Synergi 4 μm Hydro-RP 80A, 150 × 2 mm; Phenomenex, Torrance, CA) was used. A linear gradient of methanol (A) and 0.5 % acetic acid in water (B) was used: 35 % A for 1 min, 35–95 % A in 9 min, 95 % A for 4 min and 95–35 % A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 μL min^{-1}, the injection volume was 40 μL and column and sample temperatures were 30 and 20 °C, respectively. The detection and quantification of IAA and ABA were carried out using multiple reaction monitoring in the negative-ion mode, employing multilevel calibration curves with deuterated hormones as

internal standards. Compound-dependent parameters are described in Bacaicoa and others (2009). The source parameters are: curtain gas: 172.37 kPa, GS1: 310.26 kPa, GS2: 413.69 kPa, ion spray voltage: −4,000 V, and temperature: 600 °C. Data samples were processed using Analyst 1.4.2 Software from Applied Biosystems/MDS Sciex (Ontario, Canada).

Determination of Ethylene Production in Roots

Cucumber plants corresponding to each treatment were separated into shoots and roots. Measurements were performed in root tissue because shoot analyses were below detection limits. Ethylene production was measured according Garnica and others (2009). Fresh root samples (1.5 g) were weighed and transferred into 10 mL syringes with 0.250 mL of deionized water. The syringes were sealed and dark incubated for 2 h at room temperature to allow ethylene production to subside. A 1 mL gas sample was withdrawn with a gas-tight syringe from the 10 mL syringe and analyzed for ethylene using a gas chromatograph (Trace GC 2000 series, ThermoQuest, Milan, Italy) equipped with a GS-Gaspro (J&W Scientific) column (30 m × 0.32 mm) and flame ionization detector. The rate of ethylene evolution was expressed as a function of per unit dry weight.

Statistical Analysis

Five replications, with two plants per replication, were used in the different analytical determinations described above. All data were analyzed statistically by a one-way ANOVA method using the statistical package Statistica 6.0 (StatSoft, Tulsa, USA). Mean comparison of each parameter measured was evaluated by Fisher's post hoc test ($P < 0.05$).

Results

Both NO and IAA are Involved in Promoting Shoot Growth by PHA in Cucumber

Previous studies showed that PHA was able to increase shoot growth (Mora and others 2010) and the concentration in roots of NO, IAA and ethylene in cucumber plants (Mora and others 2012). To further investigate the potential mechanisms by which these phytoregulators promotes shoot growth after addition (treatment) of PHA, we analyzed the effect of specific inhibitors of the synthesis and function of these phytoregulators on both shoot growth and the root concentration. Similarly, taking into account the functional relationships between NO-, IAA- ethylene-, and a possible ABA- functional action, the concentration of ABA in roots was also analyzed in the absence (−PHA) or presence of PHA (+PHA) with or without hormone inhibitors.

The application of PHA (100 mg L^{-1} of organic carbon) significantly increased shoot fresh matter (SFM) production with respect to −PHA plants (Fig. 1 inset). However, the application of a NO scavenger (PTIO), an inhibitor of IAA functionality (PCIB) and an inhibitor of ethylene synthesis (cobalt: Co^{2+}) in −PHA plants did not have any significant effect on SFM at 72 h (Fig. 1a). In contrast, the application of both PTIO and PCIB, to +PHA plants caused a significant decrease in SFM with respect to control plants (Fig. 1b). However, the application of Co^{2+} on +PHA plants did not have any significant effect on SFM compared with +PHA plants without this inhibitor (Fig. 1b).

These results indicate that both NO and IAA, but not ethylene, play an important role in the ability of PHA to promote shoot growth.

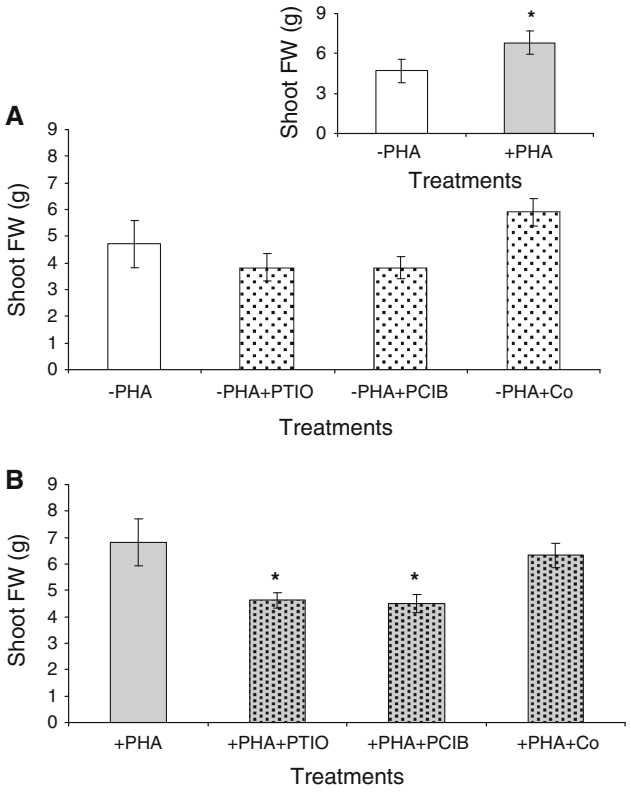

Fig. 1 Effect of PTIO, PCIB and Co on shoot fresh weight (SFW) of cucumber plants treated with 0 (−PHA) (**a**), or 100 mg L^{-1} of PHA (**b**). The *graph inset* shows the increase of SFW due to PHA (+PHA) with respect to its control without PHA (−PHA). Data are means ± SE ($n = 5$); *Asterisks* indicate the presence of significant differences at $P < 0.05$ between treatments and its controls based on one-way ANOVA and Fisher's post hoc test

The Effect of PHA on Root IAA Concentration Involves Both a NO-dependent and NO-independent Pathways

To investigate if the effect of PHA on root IAA concentration is mediated by a previous effect on root NO accumulation; we studied the consequences of PTIO root application on root IAA concentration in +PHA plants. On one hand, when PTIO was applied to −PHA plants, a significant decrease in the IAA root concentration was observed (Fig. 2a). This result indicates that root IAA concentration in cucumber plants is, at least, partially regulated by NO. On the other hand, +PHA significantly increased the IAA concentration in the root with respect to −PHA (Fig. 2 inset). The results also showed that PTIO application to +PHA plants caused a significant decrease in root IAA concentration with respect to +PHA plants without PTIO (Fig. 2b). However, +PHA plants receiving PTIO showed a root IAA concentration that was significantly higher than that of −PHA plants treated with PTIO (68.9 ± 8.8 pmol g^{-1} FW vs. 50.6 ± 9.6 pmol g^{-1} FW, respectively) (Fig. 2a, b). These results indicate that PHA increased the IAA root concentration through both NO-dependent and NO-independent pathways.

The Effect of PHA Increasing Root Ethylene Production is Regulated by IAA

Regarding the effect on root ethylene production, results showed that the presence of PHA was associated with an increase of ethylene in roots (Fig. 3 inset). The application of PCIB in −PHA plants caused a significant decrease in the production of ethylene (Fig. 3a). These data indicate that, in cucumber plants, the concentration of ethylene is, at least, partially regulated by IAA, thus confirming previous results obtained in other plant species (Rahman and others 2001, 2002). In addition, data also showed that PCIB application to +PHA plants caused a significant decrease in the root concentration of IAA with respect to +PHA plants without PCIB (Fig. 3b). This result also indicated that the increase of ethylene production in the root by the

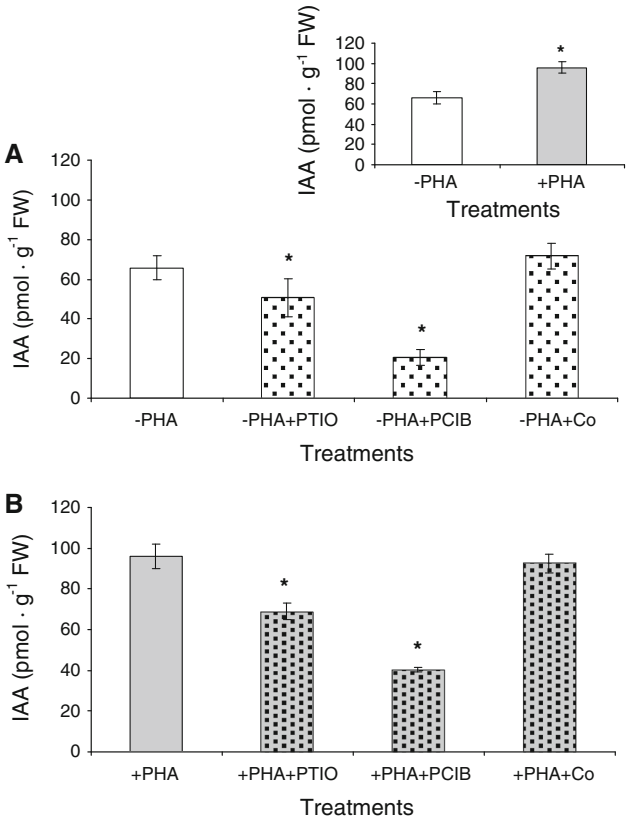

Fig. 2 Effect of PTIO, PCIB and Co on root IAA concentration in cucumber plants treated with 0 (−PHA) (**a**), or 100 mg L^{-1} of PHA (**b**). The *graph inset* shows the increase on root IAA concentration due to PHA (+PHA) with respect to its control without PHA (−PHA). Data are means ± SE ($n = 5$); *Asterisks* indicate the presence of significant differences at $P < 0.05$ between treatments and its controls based on one-way ANOVA and Fisher's post hoc test

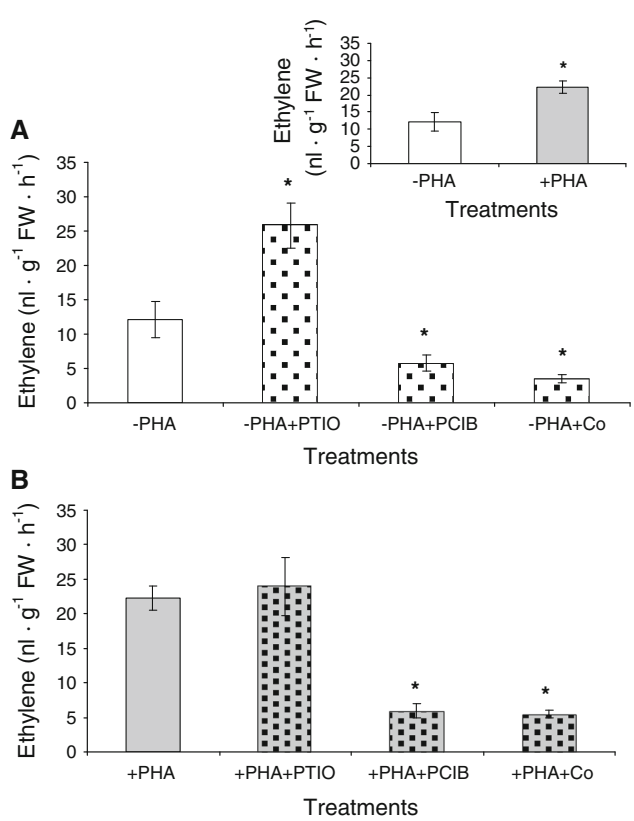

Fig. 3 Effect of PTIO, PCIB and Co on root ethylene production in cucumber plants treated with 0 (−PHA) (**a**), or 100 mg L^{-1} of PHA (**b**). The *graph inset* shows the increase of root ethylene production due to PHA (+PHA) with respect to its control without PHA (−PHA). Data are means ± SE ($n = 5$); *Asterisks* indicate the presence of significant differences at $P < 0.05$ between treatments and its controls based on one-way ANOVA and Fisher's post hoc test

presence of PHA is exerted through an IAA-dependent pathway.

Additionally, the ethylene production in roots of −PHA plants was twofold highest in presence of PTIO than in the absence of PTIO (Fig. 3a). In contrast, when PTIO was applied to +PHA plants any effect on root ethylene concentration was observed (Fig. 3b). This result indicates that NO does not play a determinant role in the effect of PHA on ethylene production in the root. Moreover, a significant decrease in the ethylene production was observed in both −PHA and +PHA plants treated with 10 μM Co^{2+} (Fig. 3a, b). This effect was expected because Co^{2+} is an inhibitor of ethylene production through the inhibition of enzyme ACC oxidase that catalyses the last step of ethylene biosynthesis, converting ACC to ethylene (Lau and Yang 1976; Locke and others 2000). However, the presence of Co^{2+} did not show any effect on the concentration of IAA in −PHA and +PHA plants (Fig. 2a, b). These results support the conclusion that the effect of PHA on root ethylene production is expressed by a previous effect on IAA concentration in the root.

PHA Increases ABA Concentration in Roots Through an IAA-dependent Pathway

Plants treated with PHA have a concentration of ABA in roots significantly higher than plants lacking PHA (Fig 4 inset). To investigate if NO and/or IAA are involved in this PHA action, we investigated the effects of PTIO and PCIB on root ABA concentration.

The application of PTIO to −PHA plants caused a significant increase in ABA concentration in the root (Fig. 4a). This effect might be derived from a stress caused by PTIO in roots (Mora and others 2012). However, in +PHA plants any change in ABA concentration caused by PTIO addition was observed (Fig. 4b). Additionally, the level of ABA in the presence of PTIO in −PHA and +PHA plants was similar (5.0 ± 1.0 vs. 5.0 ± 0.3 pmol g^{-1} FW, respectively). This result suggested that NO is probably not involved in the effect of PHA on ABA concentration in the root.

PCIB- or Co^{2+}- treatments did not show any effect on the concentration of ABA with respect to plants lacking PHA (Fig. 4a). However, the application of both PCIB and Co^{2+} to +PHA plants caused a significant decrease in root ABA concentration in comparison with +PHA plants lacking these inhibitors. The level of ABA for these treatments was similar to that of −PHA plants with or without PCIB or Co^{2+} (Fig. 4a, b). These results indicated that the PHA-mediated action increasing ABA concentrations in roots is IAA- and ethylene- dependent.

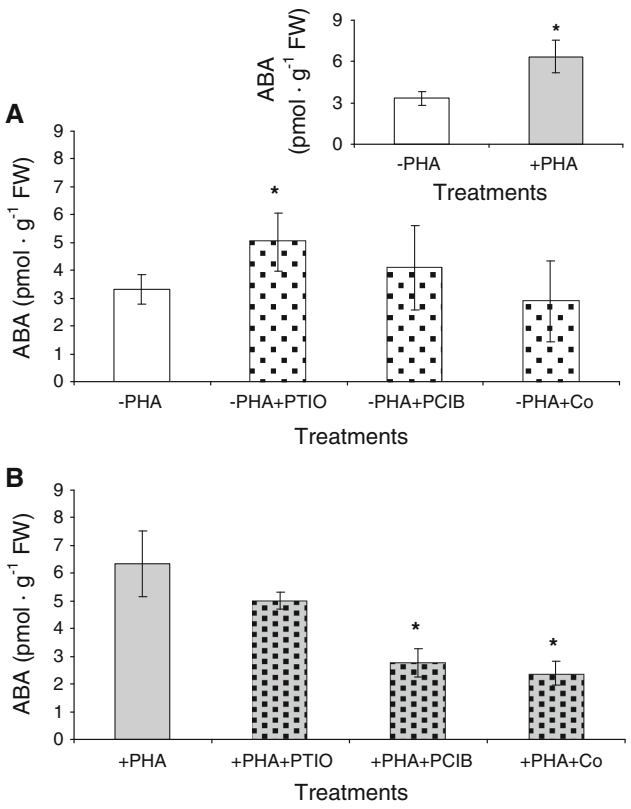

Fig. 4 Effect of PTIO, PCIB and Co on root ABA concentration in cucumber plants treated with 0 (−PHA) (**a**), or 100 mg L^{-1} of PHA (**b**). The *graph inset* shows the increase of root ABA concentration due to PHA (+PHA) with respect to its control without PHA (−PHA). Data are means ± SE ($n = 5$); *Asterisks* indicate the presence of significant differences at $P < 0.05$ between treatments and its controls based on one-way ANOVA and Fisher's post hoc test

Discussion

The effects by HS on morphology and growth of the root have been extensively studied. However, knowledge about the action of HS over the shoot growth is scarce.

In this study, we observed that the shoot growth promoting action of PHA was associated with a significant increase in IAA, ethylene and ABA concentrations in roots. These effects were also associated with an increase in NO root production which was shown by Mora and others (2012). However the functional relationship between these effects of PHA on the root hormonal balance and its ability to enhance shoot growth remains unclear.

Here, we have first investigated the influence of the use of a NO scavenger (PTIO) on the effect of PHA in shoot growth. The results obtained showed that PTIO application significantly decreased the shoot growth promoting effect by PHA up to levels of plants that lacks PHA (Fig. 1b). This result indicated that NO plays a relevant role in the stimulation of shoot growth caused by PHA. Previous

studies have reported that HS are able to increase NO production in roots of diverse plant species (Mora and others 2010, 2012; Zandonadi and others 2010). Moreover, these effects were associated with IAA activity, root PM H^+-ATPase activation and lateral root development (Zandonadi and others 2010).

Secondly, we tested the effect of the PCIB, an inhibitor of IAA action, on PHA-induced shoot growth. The application of this inhibitor to the root abolished the PHA-mediated increase in shoot growth (Fig. 1b). This result indicated that IAA is also involved in the mechanism promoting shoot growth by the action of PHA. The IAA phytoregulator has the ability to affect root PM H^+-ATPase activity, and also plays an important role in meristem biology, particularly in the shoot apical meristem that generates all the aerial parts of the plants (Vernoux and others 2010). These authors showed that auxin transport is not the only factor involved, because the local auxin biosynthesis appears to be essential for meristem function. The increase in shoot growth demonstrated in this study could also implicate an effect of auxin and/or an auxin gradient on leaf development (DeMason and Chawla 2004). The data obtained by these authors using specific inhibitors of auxin action showed that PCIB decreased leaf initiation on the shoot apical meristem.

A number of studies have reported the important functional relationships between NO and IAA (Chen and others 2010; Zandonadi and others 2010; Jin and others 2011; Romera and others 2011). NO is a bioactive free radical which plays important roles in many physiological processes in the plant, for example growth, development and adaptive responses to multiple stresses (Beligni and Lamattina 2001; Zhao and others 2004; Graziano and Lamattina 2005). Pagnussat and others (2002) showed that the existence of a NO and auxin cross-talk during adventitious root formation in *Cucumis sativus*. Other authors have indicated a role for NO in the regulation of lateral root development, operating in the auxin signaling transduction pathway (Correa-Aragunde and others 2004). In previous studies, we demonstrated that the action of PHA increasing root NO concentration is followed by a subsequent increase in the root IAA concentration (Mora and others 2012). In line with this hypothesis, we have observed that the application of PTIO in −PHA plants caused a significant decrease in IAA concentration (Fig. 1a). This result indicated that the concentration of IAA in roots of cucumber plants is, at least, partially regulated by NO. However, although the presence of PTIO in +PHA plants caused a significant decrease on root IAA concentration compared with +PHA plants without this inhibitor, the level of IAA in roots of +PHA plants plus PTIO was significantly higher than that of −PHA plants plus PTIO (Fig. 2a, b). This result suggests that PHA acts on root IAA concentration by at least two mechanisms: one involving NO but also another which is independent of NO.

Similarly, the time-course pattern of PHA effects on the concentration of NO, IAA and ethylene, is also compatible with a potential role of ethylene in the main pathway of events triggered by PHA and responsible for PHA-induced shoot growth. Our results showed that the root application of Co^{2+} on +PHA plants did not affect the enhancement of shoot growth (Fig. 1b). This result indicated that the PHA-action on root ethylene concentration is not essential for the shoot growth promoting action of PHA. Moreover, in our experimental model the application of PTIO on −PHA plants caused a significant increase in root ethylene accumulation (Fig. 3a). This result may be caused by a stress associated with the presence of PTIO, because root development and architecture were very affected by this compound (Mora and others 2012). However, the root application of PCIB on −PHA plants was associated with a significant decrease in root ethylene production (Fig. 3a). These data indicate that in cucumber plants the concentration of ethylene is, at least, partially regulated by IAA. This result was in line with other studies showing the important functional relationships between IAA and ethylene (Rahman and others 2001, 2002). In the case of +PHA plants, the application of PCIB abolished the PHA-mediated increase in root ethylene accumulation (Fig. 3b). This fact indicated that an IAA-dependent pathway, as in the case of −PHA plants, regulates the increase in ethylene production caused by PHA. Arteca and Arteca (2008) demonstrated also that roots of *Arabidopsis* plants produced various levels of ethylene in response to IAA. Additionally, IAA enhances the constitutive expression of the ACS gene family in the root (Tsuchisaka and Theologis 2004).

Finally, that the increase in root ABA concentration caused by PHA compared with −PHA plants was noteworthy. Taking into account that ABA metabolism is also related to the metabolism of the other studied plant hormones, it is possible that the PHA-mediated effect on ABA concentration in roots is inter-connected with the effects of PHA on NO and/or IAA and/or ethylene. Our results partially supported this hypothesis. We observed that the presence of PTIO to −PHA plants caused a significant increase of ABA concentration in the root (Fig. 4a). As in the case of ethylene, this increase is probably due to the stress caused by PTIO application (Mora and others 2012). This conclusion is also supported by the fact that in control plants, the root ABA concentration was not affected by the application of PCIB or Co^{2+} (Fig. 4a). However, the application of PCIB or Co^{2+} on plants treated with PHA abolished the increase in root ABA concentration (Fig. 4b), indicating that this effect is IAA- and ethylene- dependent. ABA is generally regarded as an inhibitor of shoot growth

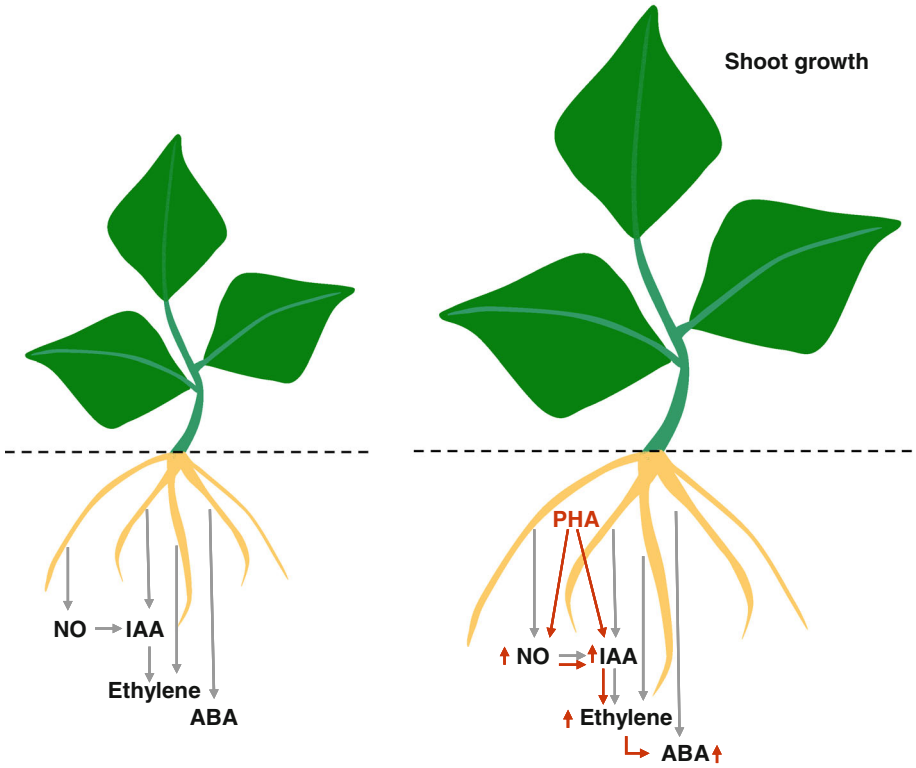

Fig. 5 Proposed mechanism of PHA action on shoot growth through increase of NO and IAA in root

(Davies 1995; Munns and Cramer 1996). However, studies using ABA-deficient mutants and inhibitors of ABA synthesis are changing the view of the role of ABA from the traditional idea that the hormone is generally involved in growth inhibition (Sharp 2002). Additionally, an important role of endogenous ABA is to limit ethylene production, and this interaction is involved in the effects of ABA status on shoot and root growth (Spollen and others 2000). In this line, Hussain and others (2000) indicated that, for unknown reasons, stressed relative to non-stressed plants require an increased concentration of ABA to prevent excess ethylene production. Data from the present work show that the presence of an inhibitor of ethylene synthesis (Co^{2+}) is associated with a decrease in root ABA levels indicating a relation between the ethylene synthesis and ABA accumulation in +PHA plants (Fig. 4b). It is possible that PHA-treated cucumber plants sense the decrease of ABA and this effect triggers a negative feedback mechanism controlling ethylene production (Hussain and others 2000; Sharp 2002) and subsequently maintaining shoot growth at levels of the control −PHA (Fig. 1b).

We also suggest that the accumulation of ABA in roots might also be involved in the main mechanism of PHA action on shoot growth, probably by affecting water-nutrient root uptake as a result of higher hydraulic conductivity mediated by aquaporin-activation (Mahdieh and Mostajeran 2009).

Further research is needed to elucidate the role of ABA in the main mechanism involved in the improvement of shoot growth caused by PHA as well as the functional relationships between this mechanism and that related to both nitrate and CK root-to-shoot translocation (Mora and others 2010; Jannin and others 2012).

Conclusions

In summary, our results present further evidence that the promoting action of PHA on cucumber shoot growth involves a complex mechanism probably mediated by increases in root of NO, IAA, ethylene and ABA concentrations. The results suggested that the effect of PHA on root IAA concentration involves a NO-dependent pathway but also another pathway that is NO-independent. The increase in root ethylene production is also regulated by an IAA-dependent pathway, as in the case of −PHA plants. Finally, the PHA-mediated increase on root ABA concentration is expressed through IAA- and ethylene- dependent pathways (Fig. 5).

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