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Effect of 1-naphthaleneacetic acid on organic acid exudation by the roots of white lupin plants grown under phosphorus-deficient conditions

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SUMMARY

The effect of NAA (1-naphthaleneacetic acid) on organic acid exudation in white lupin plants grown under phosphorus deficiency was investigated. Plants were sampled periodically for collecting of organic acids (citrate, malate, succinate), and also were used to study the effect on proton extrusion and release of Na^+, K^+, Ca^{2+} and Mg^{2+}. The tissues were later processed to quantify the organic acids in tissues, the phosphorus content and the effects on plant biomass. The exogenous addition of NAA led to an increase in organic acid exudation, but this response was not proportional to the concentration of the dose applied, noticing the largest increments with NAA 10^{-8} M. In contrast the increase in root weight was proportional to the dose applied, which shows that with higher doses the roots produced are not of proteoid type. Proton extrusion and the release of cations were related to the NAA dose, the first was proportional to the dose applied and the second inversely proportional. Regarding the analysis of tissues, the results of citrate and phosphorus content in shoots show that the overall status of these parts are the main responsible of the organic acids exuded. NAA served as an enhancer of the organic acid exudation that occurs under phosphorus deficient conditions, with a response that depends on the dose applied, not only in its magnitude, but also in the mechanism of action of the plant hormone.

Introduction

The exudation of organic acids such as citrate, malate, oxalate, succinate and fumarate by plant roots constitute a response mechanism to different stress situations, which may be caused mainly by a nutritional deficiency (Carvalhais et al., 2011; Hoffland et al., 2006; Lipton et al., 1987), the presence of a toxic element (Chiang et al., 2006; Zeng et al., 2008) or anoxic conditions (Xia and Saglio, 1992). Generally this process is limited to specific zones of the roots, for example the apices in rape (*Brassica napus* L.) (Hoffland et al., 1989), and the cluster of rootlets in roots of white lupin (*Lupinus albus* L.) (Gardner et al., 1982); in both cases is largely known the enhancement of exudation under phosphorus-deficient conditions. This exudation is accompanied by a rhizosphere acidification (Dinkelaker et al., 1989).

The study of the effect of auxins in white lupin roots started with the observation that plants grown in P-sufficient conditions do not develop roots with clusters (named proteoid roots), and the exudation of organic acids is very low (Peñaloza et al., 2002). With the application of natural and synthetic auxins like the indole-3-acetic acid (IAA) (Neumann et al., 2000), indole-3-butyric acid (IBA) (Hocking and Jeffery, 2004) and 1-naphthaleneacetic acid (NAA) (Gilbert et al., 2000; Skene and James, 2000) has been possible to simulate the formation of proteoid roots in plants grown in P-sufficient conditions, with very similar morphologies to those produced naturally in P-deficient conditions, but these roots do not exude organic acids (Hocking and Jeffery, 2004). Under P deficiency the most relevant acids exuded are citrate and malate, and in terms of amount exuded citrate is the most important anion, this has been demonstrated both in experiments in soil (Dessureault-Rompré et al., 2006) and in hydroponics (Neumann and Römheld, 1999; Zhu et al., 2005).

Moreover Neumann et al. (2000) studied the effect of TIBA (2,3,5-triiodobenzoic acid) and NPA (N-(1-naphtyl)phtalamic acid), two inhibitors of the polar shoot-to-root transfer of auxins in higher plants, on the production of clusters in the roots: both compounds suppressed the formation of these structures, which shows that the development of clusters is under hormonal control.

IAA, the most common naturally-occurring auxin, has an important role in the generation of resistance to different stresses. It has been shown that can reduce the toxic effect of Cu (II) in sunflower

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roots (*Helianthus annuus* L.) (Ouzounidou and Ilias, 2005). The opening of the stomata by direct interaction of IAA with the extracellular part of the anionic channels of occlusive cells has also been demonstrated (Marten et al., 1991). Furthermore, the effect of IAA on root organic acid exudation has been shown in wheat exposed to Al, being the anion malate the most important exudate as response mechanism against the toxic element. The increase in the exudation with the auxin was only seen in presence of Al, the addition of IAA alone had no effect on the efflux of malic acid (Yang et al., 2011).

As described above, it is known the role that auxins have in root development of white lupin plants. Its function could go further, being also involved in the exudation of organic acids. In this study we selected a synthetic auxin, NAA, to measure its effect on root development and exudation of three organic acids (citrate, malate and succinate), as well as in the power of acidification of the roots. These responses, which are the most relevant for the improvement of growth medium, are studied in the light of the effects with importance at physiological level (organic acid and phosphorus content in tissues, and the release of K^+, Na^+, Mg^{2+} and Ca^{2+} by the roots). For comparison purposes, the same NAA treatments were applied to plants grown with or without P supply.

Materials and methods

Plant material

White lupin seeds (*Lupinus albus* L. cv. Marta) were surfaced sterilized and germinated on moist filter paper at 28 °C in the dark for 3 days. Seedlings were transferred to a hydroponic culture system with 3500 mL ¼ strength basal nutrient solution for a week. At the end of this time plants had developed two complete leaves, they were selected by their visual homogeneity and transferred to containers filled with 10 L of entire nutrient solution (composition: 2 mM $Ca(NO_3)_2$, 4 mM KNO_3, 1 mM $MgSO_4$, 36 μM Fe-EDDHA, 33 μM $MnSO_4{\cdot}H_2O$, 1.6 μM $ZnSO_4{\cdot}7H_2O$, 1.6 μM $CuSO_4{\cdot}5H_2O$, 46 μM H_3BO_3 and 0.1 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$; phosphorus was supplied as 0.25 mM KH_2PO_4, and was replaced by 0.25 mM KCl in P-deficient treatments). The solutions were changed every four days, and plants grew until day 37 after seed germination. Plant culture was performed in a growth chamber with a light period of 13 h, 520 μmol m^{-2} s^{-1}, 25 °C/20 °C day/night temperature and a relative humidity of 40%/60%.

Hormonal treatment

After one week in entire nutrient solution, the first NAA (α-naphthaleneacetic acid, Sigma, St. Louis, MO, USA) application was performed, adding an aliquot of 50 mL of a stock auxin solution to obtain a concentration of NAA 10^{-7} M, NAA 10^{-8} M and NAA 10^{-9} M in each container of both deficient (−P) and with phosphorus supply (+P) treatments. This volume is large enough for an adequate mix with the nutrient solution. The addition of NAA was performed every four days (5 applications along the experiment). Control treatments (without auxin) received an aliquot of 50 mL of distilled water at the time of hormone addition.

Exudate sampling

The first sampling of root exudates was performed on day 21 after seed germination, every 4 days until day 37. The exudates were collected from the entire roots following the procedure of Peñaloza et al. (2002). Plants were removed of the nutrient solution containers, the roots were washed twice in 0.25 mM $CaSO_4$ and transferred to 100 mL containers filled with 40 mL of distilled water previously aerated, where the roots remained submerged for two hours (two plants per replicate). During this time the containers were periodically agitated. A sample of 10 mL was taken for the analysis of organic acids by anion exclusion chromatography, and the remaining was frozen at −20 °C for later analysis of cations. Weight measurements were done for the whole plant and roots and shoots separately, and the tissues were frozen in liquid nitrogen for the analysis of organic acids and phosphorus content.

Organic acid extraction from tissues

Organic acids were extracted from the entire roots and shoots according to the procedure of Peñaloza et al. (2002). The tissues were treated with 2% (v/v) acetic acid, and the extract cleaned later with chloroform in proportion 2:1. The aqueous phase was diluted and stored at −20 °C until analysis by anion exclusion chromatography.

Analysis of organic acids

Twenty μL of the exudates, and of the final aqueous extract for the determination in tissues were injected into a HPLC (Metrohm) fitted with an anion exclusion column (250 mm × 7.8 mm, 6.1005.200 Metrosep Organic Acids, Metrohm). The mobile phase was a mixture of 0.2 mM H_2SO_4 with 5% acetone, and the flow was set at 0.6 mL/min. Organic anions were identified by comparison with the elution times of standards of citric, malic and succinic acids (Fluka) using a conductivity detector, the amounts of each one in the samples were determined from the peak areas in the samples with those for the standards using MagIC Net software (Metrohm).

Phosphorus content

Total phosphate was determined on roots and shoots dried at 70 °C, the material was ground and digested with a mixture containing HNO_3:H_2O_2:H_2O (3:2:8) for 30 min in closed containers under pressure (Lozano-Rodriguez et al., 1995). After digestion P content was measured colorimetrically (Kitson and Mellon, 1944).

Cations in exudates

For the determination of K^+, Na^+, Mg^{2+} and Ca^{2+} in root exudates, samples were directly measured by means of atomic absorption spectroscopy (Analyst 800, Perkin Elmer). The cation release by proteoid roots was calculated for each ionic species from the concentration difference between the exudation solution and the corresponding blank solution.

Statistical analysis

Data were analyzed with one-way analysis of variance (ANOVA) followed by Duncan's test for all the measurements (SPSS 16.0 for Windows). To ensure homogeneity of variances, data were transformed where necessary. Data were processed separately for each compound or element quantified.

Results and discussion

Organic acids exudates and plant biomass

After five applications of the auxin to the nutrient solution in three different concentrations (NAA 10^{-7} M, NAA 10^{-8} M and NAA 10^{-9} M), the effect of the hormone on root exudation of citrate, malate and succinate is shown in Fig. 1. Using the exudates sampling system selected for the experiments, we quantified citrate for the first time the day 25 after seed germination (second auxin