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Effect of auxin on cluster roots induction in *Embothrium coccineum* J.R. Forst. & G. Forst. in phosphorus deficiency condition

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Auxins play an important role in cluster root (CR) development in some species under low P conditions. However, the mechanism for CR induction in *Embothrium coccineum* J.R. Forst. & G. Forst remains unknown. Therefore, the aim of this work was to determine the effect of auxin on CR induction in *E. coccineum*, under low P conditions. For this, plants of *E. coccineum* were grown under P sufficiency and deficiency, and were treated with exogenous auxin and an auxin transport inhibitor. Phosphorous concentration in leaves, proportion of the total root allocated to CR mass (CRA), CR number (CRN), CR mass (CRm), and individual CR mass (ICRm), were measured. The number of CR showed a negative correlation with P concentrations in leaves. Furthermore, application of auxin in plants grown under P deficiency increased the CRA in a 50% and CRN in a 60%. The values of all parameters measured in plants grown under P deficiency conditions were reduced to less than half that observed in control plants, after foliar application of auxin transport inhibitor. However, no difference was observed when auxin was applied in plants grown with P supply. These results suggest that P and auxin are important factors for CR induction in *E. coccineum*.

Key words: Auxin transport inhibitor, naphthalene acetic acid, n-1-naphthylphthalamic acid, plant hormones.

INTRODUCTION

Cluster roots (CR) are those portions of lateral roots with dense clusters of rootlets (at least 10 per centimeter) (Purnell, 1960). Cluster roots are present in several families including Betulaceae, Casuarinaceae, Cucurbitaceae, Eleagnaceae, Fabaceae, Moraceae, Myricaceae, and Proteaecae (Shane and Lambers, 2005). This type of roots increases the absorption surface area and modifies the chemistry of the rhyzosphere through the release of large amounts of organic acids, phenolic compounds, enzymes, mucilage, and water (Peñaloza et al., 2002; Lambers and Poot, 2003; Shane et al., 2003).

Some reports in *Lupinus albus* L. (Fabaceae) have shown that CR induction is dependent on P, N, Fe, Mg or Zn deficiency (Shane et al., 2003; Shen et al., 2003). In this species, the mass of CR is more than 50% of the total root mass obtained in plants under low P conditions. The mass allocated to CR decreases ten times in plants with sufficient P supply (Johnson et al., 1994). In *Hakea prostrata* R. Br. (Proteaceae) studies based in a split root experiment showed that CR growth was favored by a low shoot P status (Shane et al., 2003). Similar studies in *Grevillea crithmifolia* R. Br. (Proteaceae) found that the regulation of CR induction was dependent of shoot P concentration. The authors suggested a systemic suppression of CR formation under higher P supply conditions (Shane and Lambers, 2006). Plant hormones play an important role in CR development in low P conditions (Gilbert et al., 2000; Neumann et al., 2000; Skene and James, 2000). For example, application of exogenous auxin in *Grevillea robusta* A. Cunn. ex R. Br. (Proteaceae) and *L. albus* grown in P supply conditions mimics CR induction in a P deficiency condition (Skene and James, 2000; Gilbert et al., 2000). A practical tool to investigate the hormonal CR induction has been the use of hormone transport inhibitors. For instance, application of naphthylphthalamic acid (NPA), an auxin transport inhibitor, can cause decrease of CR formation in *L. albus* grown in P deficiency conditions (Gilbert et al., 2000).

Embothrium coccineum J.R. Forst. & G. Forst. is a tree species of the Proteaceae family that grows between 35 to 56° S lat (Rodríguez et al., 1983) and 1300 m a.s.l. (Alberdi and Donoso, 2004). *Embothrium coccineum* grows successfully in nutrient impoverished soil in South Chile (Donoso-Ñanculao et al., 2010) like other members of *Proteaceae* (Skene, 1998). These environmental conditions could favor the development of CR in *E. coccineum* (Donoso, 2006). In addition, a recent report

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indicates that low P availability induces CR formation in *E. coccineum* (Zúñiga-Feest et al., 2010). However, little is known about hormonal effects in CR induction in this species. Therefore, in the present study we evaluate the hypothesis that the induction of CR in *E. coccineum* in low P conditions is auxin dependent. The objective of this work was to determine the effect of auxin in the CR induction of *E. coccineum*, under low P conditions.

MATERIALS AND METHODS

Plant material and growth conditions

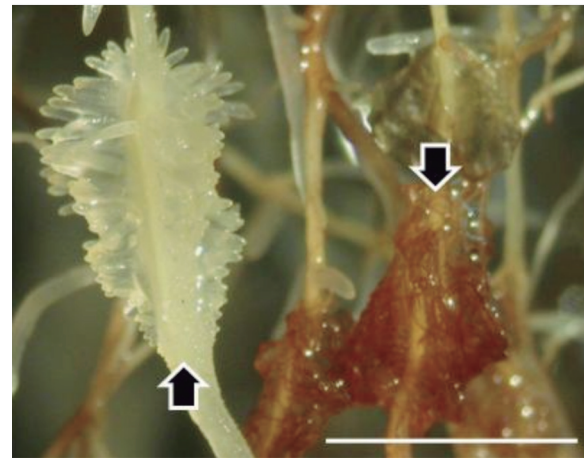
Seeds of *E. coccineum* were germinated in a growth chamber on sandy loam soil for optimal germination conditions, at 22 ° C by 1 wk. Seedlings were grown in a growth chamber under fluorescent light (80 μmol m^{-2} s^{-1}, 14:10 h photoperiod) at 22 ºC light/28 ºC dark by 1-mo on sandy loam soil and then were transplanted to perlite in plastic pots of 300 cm^3. Seedlings were supplemented twice a week with Hoagland solution that contain: 2.5 mM $Ca(NO_3)_2$, 3 mM KNO_3, 1 mM $MgSO_4$, 1 mM KH_2PO_4 (only for P supply solution), 1 mM KCl (only for P deficiency solution), 22 μM H_3BO_3, 4 μM $MnCl_2 \times 4H_2O$, 0.4 μM $ZnSO_4$, 1.6 μM $CuSO_4$, 0.05 μM $Na_2MoO_4 \times 2H_2O$, and 12 μM Fe(III)EDTA. Seedlings of 3-mo were irrigated once a week for 2-mo with exogenous auxin (1×10^{-8} M of naphthalene acetic acid, NAA). Moreover, auxin transport inhibitor (10 nM of N-1-naphthylphthalamic acid, NPA) was applied once a week on leaves during 2-mo. The treatment combinations were: P deficiency (-P); P deficiency plus NAA (-P NAA); P deficiency plus NPA (-P NPA); P deficiency plus NAA and NPA (-P NAA NPA); P supply (+P); P supply plus NAA (+P NAA); P supply plus NPA (+P NPA); P supply plus NAA and NPA (+P NAA NPA). At the end of the experiment, plants were harvested and leaves were frozen in liquid nitrogen for P determination. The root system was carefully removed from pots, adhering perlite was removed and root was weighed. The proportion of the total root allocated to CR mass (CRA), CR number (CRN), CR mass (CRm), and individual CR mass (ICRm), was measured in every seedling. Only healthy CRs were considered (Figure 1).

Phosphate inorganic determination in leaf

Inorganic phosphate (Pi) was determined in leaves of seedlings of *E. coccineum* grown under low and high P conditions. Leaves were frozen and ground in liquid nitrogen. Inorganic phosphate was extracted from leaves with 2% (v/v) acetic acid and quantified as described by Murphy and Riley (1962).

Statistical analysis

The experiment consisted of a complete randomized design with nine to eleven replicates. Data were analyzed with three-way ANOVA (where the factors were: P, NAA, and NPA). Differences between individual treatments were tested using LSD Fisher ($P \leq 0.05$). All analyses were performed using INFOSTAT (Di Rienzo et al., 2011).

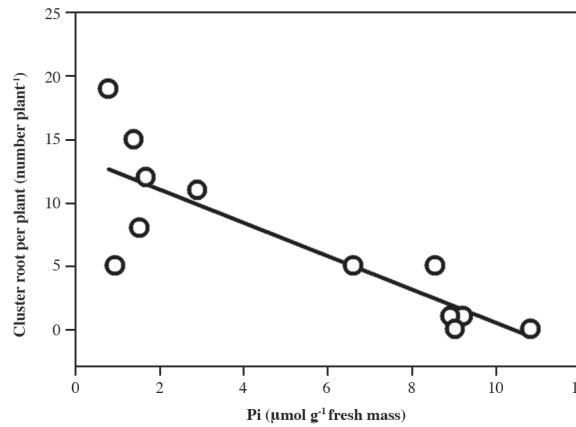


Left arrow, healthy cluster roots; right arrow, senescent cluster roots. White bar represents 5 mm.

Figure 1. Cluster roots in perlite grown *Embothrium coccineum*.

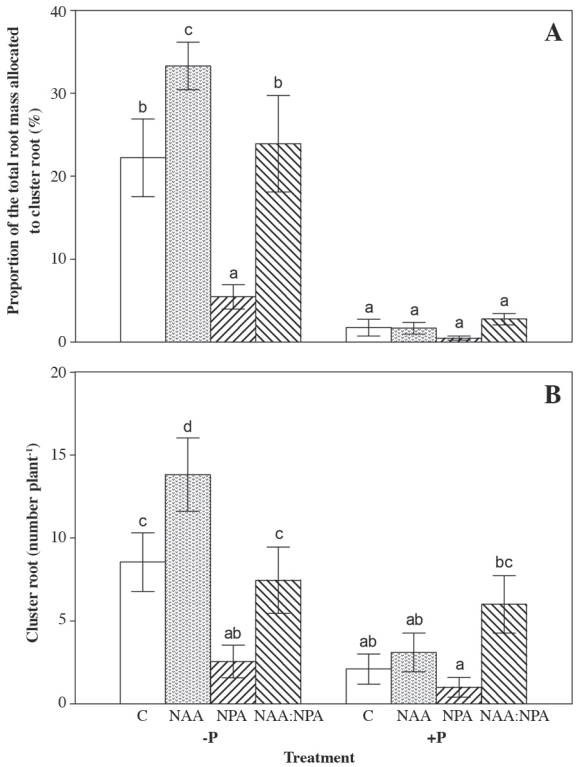
RESULTS AND DISCUSSION

The CRN had a negative correlation with the P concentrations in leaves ($r^2 = 0.69$; $P \leq 0.05$) (Figure 2). The highest number of CR observed was 19 in seedlings with leaf P concentration of 0.8 μmol g^{-1} fresh mass. The results are consistent with other reports in *L. albus*, *G. crithmifolia, H. prostrata* that show that internal P concentration regulates CR formation (Gilbert et al., 2000; Shane et al., 2003; Shane and Lambers, 2006). This result suggests that leaf P concentration over 9 μmol g^{-1} fresh mass could be critical leaf concentration for CR inhibition in *E. coccineum*.



Circle represent a CR numbers on different leaf P concentration. Each point represents one plant ($y = 13.711 - 1.323x$; $r^2 = 0.694$; $P \leq 0.05$).

Figure 2. Relationship between leaf P concentration and cluster root (CR) number of *Embothrium coccineum*.



-P: Without P; +P: with P; NAA: naphthalene acetic acid; NPA: N-1-naphthylphthalamic acid.
Treatments resulted from a factorial combination of: NAA and NPA; C: control without NAA and NPA; NAA, exogenous auxin, NPA, auxin transport inhibitor; NAA:NPA, exogenous auxin plus auxin transport inhibitor.
The experiment was conducted in the presence (+) or absence (-) of P. Error bar represent a standard error (n = 9-11). Letter indicates difference between treatments. For all three way ANOVA and LSD Fisher test was used ($P \leq 0.05$).

Figure 3. Effects of exogenous auxin and auxin transport inhibitor on proportion of the total root mass allocated to cluster root (CRA) (A), and number of cluster root (CRN) (B) in *Embothrium coccineum* in presence or absence of P.

Three way ANOVA showed an effect of P on CRA, CRN, CRm and ICRm, NAA on CRA and CRN, and NPA on CRN, CRm and ICRm (Table 1). The interactions were between P and NAA affecting CRA, and between P and NPA affecting CRA, CRN and CRm. No other interaction was observed. The CRA in plants without addition of P was near to 25%, which was similar to observed in plants grown under NAA and NPA without addition of P (Figure 3A). This proportion was lower than observed in plants grown under -P conditions and simultaneously treated with NAA (35%). In plants treated with NPA and/or P, the CRA were below 5%. These results suggest that P and NPA are effective inhibitors of CRA in *E. coccineum*.

Plants grown under -P had higher CRN than plants grown under +P conditions (Figure 3B). These results showed that low P condition increased the CRN of *E. coccineum* as it has been reported previously in other species (Shane et al., 2003; Shen et al., 2003; Shane and Lambers, 2006). In absence of P, hormonal treatment of plants with NAA caused an increase of 61% in CRN and NPA application caused a decrease in 70% in CRN. No differences between plant treated with NAA plus NPA and plants grown without P, was shown. In presence of P, plants treated with NAA plus NPA shown higher CRN than plant grown in presence of NPA. Interestingly, NAA application in presence of P did not caused an increase in CRN.

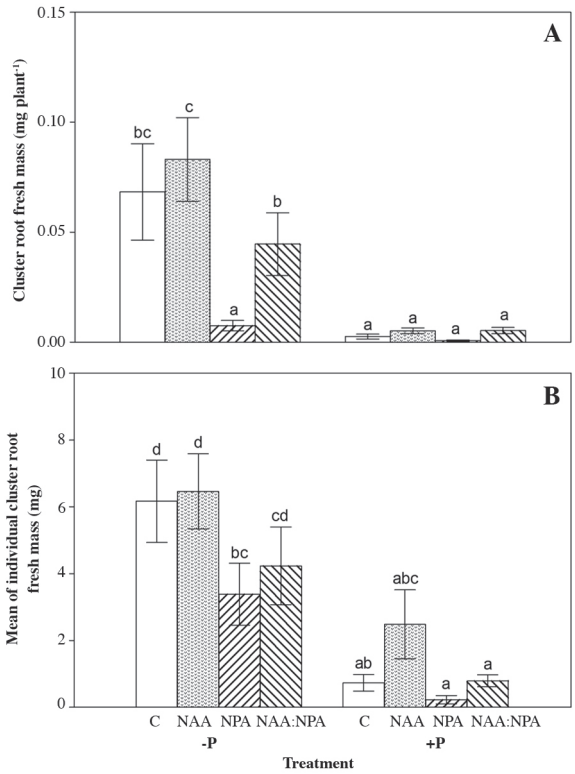
Application of NPA caused a decreased in 89% of CRm in plants grown without P (Figure 4A). No difference in CRm was observed between treatment with NAA plus NPA and control plants grown without P. All treatment grown in presence of P presented the lowest CRm. The ICRm was negatively affected by P and NPA application (Figure 4B). NPA application caused and decreased in 45% the ICRm. In all of cases, plants grown in presence of P presented a low ICRm (< 2.5 mg). The biggest ICRm value was observed in plants grown in absence of P (6.2 mg) and in NAA-treated plants without P (6.4 mg).

An increase in the CRN in P deficiency conditions was observed previously in other experiment using plants of *E. coccineum* (Zúñiga-Feest et al., 2010). The decreases in all parameters measured in CR of seedling of *E. coccineum* grown under NPA, suggest that CR induction in *E. coccineum* could be mediated by basipetal transport of auxin. These results are similar to those reported in *L. albus* and *Arabidopsis* which showed that application of NPA in seedlings grown without P caused inhibition of CR and lateral root, respectively (Reed et al., 1998; Gilbert et al., 2000). These results were confirmed after application of NAA in plants grown in presence of NPA without P, where plants developed similar CR phenotype observed in control plants. This shows that auxins have a fundamental role in the induction of CR in *E. coccineum*, as well as was observed in lateral root of *Arabidopsis* and CR in *L. albus* (Gilbert et al., 2000; Casimiro et al.,

Table 1. Level of significance of ANOVA of cluster root evaluation by P, naphthalene acetic acid (NAA) and naphthylphthalamic acid (NPA) treatments.

Dependent variable	P	NAA	NPA	P × NAA	P × NPA	NAA × NPA	P × NAA × NPA
CRA	***	**	ns	*	*	ns	ns
CRN	***	**	*	ns	*	ns	ns
CRm	***	ns	*	ns	*	ns	ns
ICRm	***	ns	*	ns	ns	ns	ns

Level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.001$; $^{***}P \leq 0.0001$; ns: non-significant.
NAA: naphthalene acetic acid; NPA: N-1-naphthylphthalamic acid; CRA: proportion of the total root mass allocated to cluster root; CRN: cluster root number per plant; CRm: cluster root fresh mass per plant; ICRm: mean of individual cluster root fresh mass.



-P: Without P; +P: with P; NAA: naphthalene acetic acid; NPA: N-1-naphthylphthalamic acid.
Treatments resulted from a factorial combination of: NAA and NPA; C: control without NAA and NPA; NAA, exogenous auxin, NPA, auxin transport inhibitor; NAA:NPA, exogenous auxin plus auxin transport inhibitor.
The experiment was conducted in the presence (+) or absence (-) of P. Error bar represent a standard error (n = 9-11). Letter indicates the difference between treatments. For all three way ANOVA and LSD Fisher test was used ($P \leq 0.05$).

Figure 4. Effects of exogenous auxin and auxin transport inhibitor on total cluster root mass (CRm) (A) and individual cluster root mass (ICRm) (B) in *Embothrium coccineum* in presence or absence of P.

2001; Linkohr et al., 2002). However, the application of NAA in plants of *E. coccineum* grown in presence of P did not cause any effect. Which was different from that reported in *L. albus*, where NAA application reverses the inhibitory effect of P on CR induction (Gilbert et al., 2000). Probably, other hormones, such as cytokinins could be related with P stress in *E. coccineum*.

Cytokinins could be related with a negative modulation of systemic control of Pi starvation responses (Martín et al., 2000). However, the effect of cytokinin was not determined in *E. coccineum*. Cytokinins have an important role in the inhibition of CR development in *L. albus* (Neumann et al., 2000) and in the regulation of lateral root induction in -P or +P conditions in *Arabidopsis* (Franco-Zorrilla et al., 2005). Recent reports show that cytokinins mediated repression of initiation of lateral roots is caused by arrest of cell cycling of the pericycle founder cells. This effect could not be rescued with exogenously applied auxin (Li et al., 2006). Similar effect was observed when exogenous auxin was applied to *E. coccineum* grown in +P treatment. This result suggests that auxin is not the only factor involved in CR induction and that probably exists an inhibitor, like cytokinins, probably implicated in CR development in +P treatment. Additional studies are necessary to unravel the relationships between auxin and cytokinins levels to produce in CR induction in *E. coccineum*.

CONCLUSIONS

In *E. coccineum*, the CR induction is regulated by P concentration on leaf. This regulation could be associated with the transport of endogenous auxin. However, according to the results obtained, we suggest that auxin is not the unique factor related with CR induction. This work is an important step for elucidating the role of exogenous and endogenous factors in cluster root induction in *E. coccineum*.

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