Relationship between plant growth and organic acids exudates from ectomycorrhizal and non-ectomycorrhizal *Pinus patula*

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Plant–mycorrhizal interaction is an important association in the ecosystem with significant impacts on the physical, biological and chemical properties of the soil. In the present study, potential relationships that exist between organic acid production by ectomycorrhizal pine seedlings and plant parameters in the absence of any significant environmental stress were investigated. The aim of the study was to investigate the contribution of organic acid production to plant growth. Four different ectomycorrhizal fungi were used in a mycorrhizal synthesis experiment to colonise roots of *Pinus patula*. Ectomycorrhizal and non-ectomycorrhizal plants were used in a pot trial experiment that lasted for 24 weeks. After harvesting, plant materials as well as soil samples underwent different analyses, which included the determination of pH, organic acids, plant biomass, and foliar and root phosphorus and potassium. The results indicated a significant interaction ($P < 0.0001$)

between fungal type and organic acid production. This reflects the influence of fungal type on organic acid production. However, it was observed that organic acids secreted into the soil do not have a direct link to the quantity of nutrients detected in either the root or shoot, but seemed to positively influence plant growth as reflected in the result from root and shoot biomass.

Introduction

Nutrient uptake by plants can be significantly increased by plant associations with mycorrhizal fungi, which are capable of solubilising unavailable mineral resources in the soil (Azcon-Aguilar et al. 1986; Jones et al. 2003; Smith and Read 2008; Adeleke et al. 2012). Traditionally, the rhizosphere provides a platform for such interactions in the ecosystem, thereby supporting a diverse number of microorganisms that stimulate plant growth (Richardson et al. 2009). In ectomycorrhizal (ECM) associations, fungal partners are beneficial and are able to colonise the roots of compatible plants by forming an association that is intercellular, just outside the root cells and the hyphae never penetrate the root cells (Smith and Read 2008). ECM hyphae are able to absorb nutrients to the benefit of their host plants, especially when nutrients are in short supply or otherwise unavailable to plants. Such nutrients are subsequently passed on to the host in exchange for carbon supplied by the plant (Bolan 1991; Smith and Read 2008). ECM fungi operate in the rhizosphere by using a wide range of mechanisms that enhance biogeochemical nutrient-cycling processes, phosphorus solubilisation, degradation of aromatic pollutants as well as playing a key role in healthy ecosystems (Dakora and Phillips 2002; Jones et al. 2003; Smith and Read 2008).

The roles of ECM fungi are enhanced by their ability to produce different metabolites such as organic acids (OAs). Although both ECM and non-ectomycorrhizal (NMC) plants are capable of releasing OAs into the soil environment, there are usually higher concentrations of OAs in soils containing mats of ectomycorrhizal fungi than in non-mat soils (Fox and Comerford 1990; Grierson 1992; Griffiths et al. 1994; Wallander and Wickman 1999; van Schöll et al. 2006a; Adeleke et al. 2012).

Both the ECM root tips and the ECM hyphae in the soil are known to produce low-molecular-weight OAs (Landeweert et al. 2001). Examples include oxalic, citric, acetic, lactic, tartaric, malic and malonic acids (Wallander and Wickman 1999; Adeleke et al. 2010, 2012). The hyphae of ECM fungi can alter the soil chemistry through the production of OAs, a process that is essential for increased nutrient absorption by plants *vis-a-vis* plant health (Rasanayagam and Jeffries 1992; Jones et al. 2003; Adeleke et al. 2012). Nutrient absorption and plant growth are vital aspects of agro-processing that control crop production and ecosystem activities (Dakora and Phillips 2002; Jones et al. 2003). Production of root exudates containing OAs are therefore part of a complex set of processes and interactions that occur in the rhizosphere, which aid the efficient absorption of mineral nutrients (Jones et al. 2003; Smith and Read 2008; Adeleke et al. 2012).

The aim of the present study was to investigate and establish correlation potential between OA production and plant health using EMC and NMC *Pinus patula* plants. The experiment was designed to investigate the relationship between OAs released into the rhizosphere and plant growth under no conditions of significant environmental stressors. Plant parameters such as root and shoot biomass as well as root and shoot K and P were evaluated.

Materials and methods

Origin of ectomycorrhizal fungi

The origin and identity of ectomycorrhizal fungal isolates used are as follows: *Pisolithus tinctorius* # PT 7 (Plant Health Care, Inc., Pittsburgh, PA, USA), *Paxillus involutus* NOF 2340 (Canada), *Laccaria bicolor* UAMH 8232 (Canada) and *Suillus tomentosus* UAMH 6252 (Canada).

Preparation of seeds

Pinus patula was chosen because it is a well-recognised host of several ECM fungi (Smith and Read 2008). Seeds of *Pinus patula* Schltdl. et Cham. were obtained from Komatiland Forests, Sabie, South Africa. The seeds were surface-sterilised in 30% (v/v) H_2O_2 for 15 min, washed continuously under distilled water for 3 min and then soaked overnight in autoclaved distilled water containing a drop of Tween-20. After 24 h, the seeds were resterilised in 10% (v/v) sodium hypochlorite (NaOCl) for 60 s. This was followed by washing 3–5 times under distilled water before inoculation onto 15% (v/v) water agar plates where they were pre-germinated for 4 weeks. Germinants were considered ready for a mycorrhizal synthesis experiment when radicles were 1–2 cm long.

In vitro *ectomycorrhizal synthesis experiment*

The experiment was conducted using autoclavable Magenta boxes (Magenta Corporation, Chicago, IL, USA). Aliquots (50 ml) of Modified Melin Norkrans (MMN) medium (Marx 1969) containing malt extract (3 g L^{-1}), $(NH)_2HPO_4$ (0.25 g L^{-1}), $MgSO_4.7H_2O$ (0.075 g L^{-1}), $CaCl_2.H_2O$ (0.067 g L^{-1}), NaCl (0.025 g L^{-1}), $FeCl_3$ (1%), thiamine (100 µg L^{-1}) and agar (10 g) were used. Glucose was omitted from the medium composition in order to starve the fungi of a carbon source, which could be obtained from the host plant through the

mycorrhizal association. The autoclaved medium was poured into the boxes, and then covered with a layer of autoclaved cellophane paper, in order to prevent the ectomycorrhizal root from penetrating the medium.

Three plugs of ECM mycelia (6 mm each) were placed on the cellophane paper equal distances apart and incubated at 28 °C. After 5 d, germinated seedlings of *Pinus patula* were carefully introduced into the centre of the flasks containing growing mycelia of the fungi. The boxes were incubated under sterile conditions under the following conditions for a period of 12 weeks: 23 °C/16 °C (day/night), 16 h photoperiod and 80% humidity.

The control seedlings (non-mycorrhizal) were transferred to Magenta boxes containing the same MMN medium but lacking ECM fungi. After 12 weeks, root samples were selected at random and carefully examined for signs of mycorrhizal association using a dissecting Leica S4E microscope (Leica Microsystems Imaging Solutions, Cambridge, UK). This was carried out according to Smith and Dickson's (1997) method.

Soil treatments

The growth medium was 100% sand soil obtained from Sable Marco, Inc., Pont-Rouge, Québec, Canada. The soil was soaked in 0.1 M HCl overnight in order to remove fine dust and exchangeable bases. After 24 h, the soil was washed continuously for several hours under distilled water and later dried in an oven for 3 d. The soil was sieved with an electroformed sieve to particle sizes of 0.25–0.59 mm. The sieved soil was sterilised in an autoclave at 121 °C for 30 min and allowed to cool overnight before the sterilisation was repeated at the same temperature and for the same duration. The soil was kept sterile until the beginning of planting.

Planting

Plastic pots (80 mm × 80 mm × 70 mm) were sterilised by soaking in 3% (v/v) NaOCl overnight and washed several times with distilled water. The pots were filled to the brim with the above-mentioned autoclaved/treated soil. Planting holes large enough to hold seedlings were created in the soil. In each hole, healthy seedlings of ectomycorrhizal colonised (EMC) and non-mycorrhizal colonised (NMC) plants were introduced and covered with soil. The seedlings were incubated at 23 °C/16 °C (day/night), 16 h photoperiod and 80% humidity. This experiment lasted for 24 weeks. All treatments were performed with four replicates.

Watering and nutrient supply

Seedlings were watered every alternate day and nutrient solution was applied twice weekly. The nutrient solution used in this experiment was a Hoagland solution (van Schöll et al. 2006b). The final Hoagland solution contained the following components: 7 mL of 1 M $Ca(NO_3)_2$, 2 mL of 1 M $KH_2(PO_4)$, 5 mL of 1 M $NH_4(PO_4)$ and 5 mL of 1 M $MgSO_4$; trace elements: 1 ml (H_3BO_3 2.8 g L^{-1}, $MnCl_{l2}.4H_2O$ 1.8 g L^{-1}, $ZnSO_4.7H_2O$ 0.2 g L^{-1}, $CuSO_4.5H_2O$ 0.1 g L^{-1} and $Na_2MO_4.2H_2O$ 0.025 g L^{-1}) and 1 ml FeEDTA (15 g L^{-1}).

Harvesting

All plants were harvested at the end of 24th week but application of the nutrient solution was stopped in the 23rd week. The first step in harvesting included careful separation of the seedling from the soil by sieving. The roots were thereafter severed from the shoot system, washed free of soil and transported to the laboratory for further processing. In the laboratory, the root samples were thoroughly washed again with distilled water to remove any trace of soil and then prepared for light microscopic examination to determine the percentage of root

colonisation. Soil samples were divided into two for pH determination and organic acid analysis.

Organic acid and pH analyses

Four grams of the harvested soil was placed into 25 ml centrifuge tubes and 20 ml of 10 mM NaH_2PO_4 were added (Mimmo et al. 2008). The mixture was shaken for 4 h at room temperature and then centrifuged at $9\,687 \times g$ for 8 min at 20 °C. Ten millilitres of the supernatant were collected and stored at -20 °C until further analysis. A 1 ml subsample was collected and evaporated to dryness on a SAVANT SpeedVac Plus SC210A evaporator (Fisher Scientific, ON, Canada) and then resuspended in 200 µl demineralised water, vortexed and left at room temperature for 15 min. The resuspended samples were vortexed, transferred to 1.5 ml tubes and centrifuged at $18\,928 \times g$. Fifty microlitres of each sample were analysed by high pressure liquid chomatography (HPLC). The chromatographic conditions were a modification of the separation method described by Schneider et al. (1987). The HPLC analytic system was controlled by Waters Empower software (Waters, Milford, MA, USA) and was composed of a Model 1525 pump, a Model 717 Plus autosampler, and a Model 2487 dual absorbance detector. Organic acids were separated on a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA, USA), eluted isocratically at 25 °C at a flow rate of 0.6 mL min^{-1} with 0.008 N sulphuric acid and detected on a dual absorbance detector set at 210 nm. Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included oxalic acid, citric acid, malonic acid and maleic acid, which were well separated under the described chromatographic conditions.

Measurement of the pH (H_2O) of the sand was carried out using a digital pH meter.

Plant root and shoot analyses

Roots and shoots of the harvested plants were dried at 65 °C for 48 h. Dry weights (root dry mass [RDM] and shoot dry mass [SDM]) were recorded. The root and shoot samples were further analysed for potassium and phosphorus content with a inductively coupled plasma-atomic emission spectrometer (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA) (Adeleke et al. 2012).

Statistical analyses

The statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC, USA). The experiment was a factorial design involving the following treatments: (1) ectomycorrhizal-colonised plants (EMC) and (2) NMC controls. The statistical analyses involved a comparison of the two treatments. For OA analysis (oxalic, citric, maleic and malonic acids) and other dependent variables, a two-way analysis of variance was used and considered the EMC plants colonised by the four fungal types, i.e. *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Laccaria bicolor* (LBR) and *Suillus tomentosus* (ST), as well as NMC plants. Following identification of significant effects, multiple comparisons with the step-down Bonferroni method were conducted to identify the differences between the treatments.

Results

The entire experiment depended on the successful colonisation of the pine roots by ECM fungi during the mycorrhizal synthesis experiment. About 40–100% colonisation was recorded for all four types of ECM fungi used and more than half of the pine seedlings had greater than 60% root colonisation rates. It is important to establish the colonisation of the roots by the ECM fungi but this outcome was acceptable for this particular study.

Organic acids and pH

The statistical analyses revealed that there was a statistically significant interaction ($P < 0.0001$) between the fungal type and OAs produced during the experiment.

In general, citric acid was the highest produced organic acid detected in all the soil samples of all the treatments (Figure 1a). Ectomycorrhizal treatments of PT and ST had the highest amount of citric acid which was significantly higher than the levels in LBR, PI and NMC.

However, for oxalic acid (Figure 1b) and maleic and malonic acids (Figure 1c and d), there was no significant difference in the quantities produced among the EMC and the NMC treatments. A similar trend was recorded among the EMC treatments as there was no significant difference in the amount of soil OAs for all treatments except for malonic acid. The PT treatment had a significantly higher value of malonic acid, compared with that of the other ECM treatments (ST, PI and LBR) (Figure 1d).

The soil pH was significantly affected by fungal type ($P < 0.0001$). A pH range of 4.98–5.29 was recorded in all treatments. All soil samples in EMC treatments were more acidic than in the NMC treatment (Table 1). The ST treatment had a high concentration of soil citric acid as well as low pH values, indicating that there may be a relationship between OA secretion and soil pH (Table 1).

With no statistically significant difference among treatments (EMC and NMC) (Table 1), the mycorrhizal status seemed not to directly affect the pH status of the soil. The highest value of foliar K for the EMC treatment was recorded in the PI treatment, whereas the lowest value was in the LBR treatment (Table 1). These results indicated that there was no significant difference in foliar K in the EMC and NMC treatments except for the LBR treatment with a significantly lower value than the NMC treatment. In contrast, the highest value of foliar P
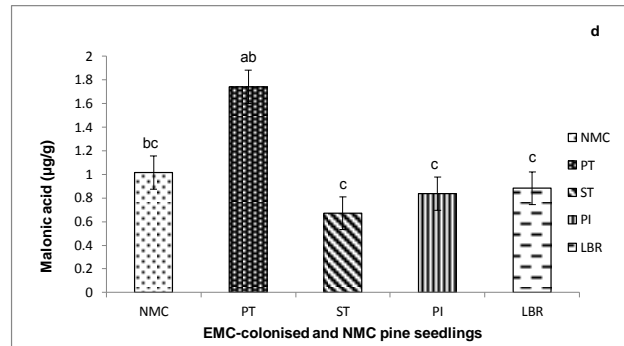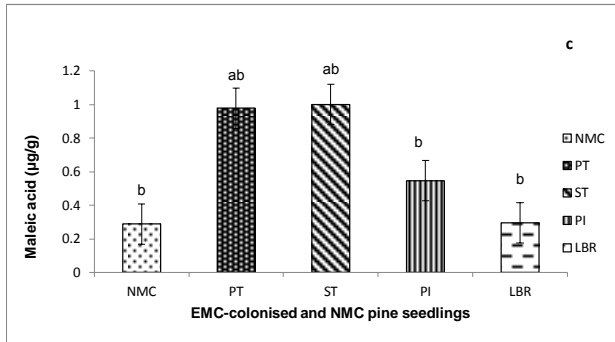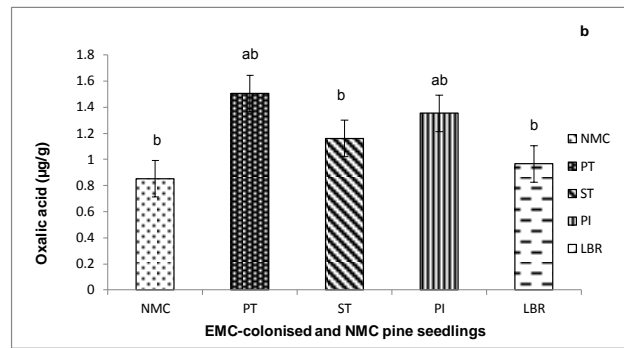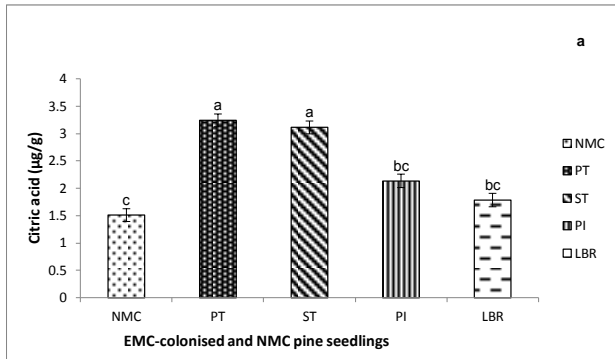
Figure 1: Amount of Citric acid (a), Oxalic acid (b), Malonic acid (c) and Maleic acid (d) released by the four EMC (LBR, PI, PT, ST) and the NMC roots. Values are the mean of four replicates. Error bars represent the SE. For each type of organic acid, bars with the same letters are not significantly different (*P* < 0.05). PT = *Pisolithus tinctorius*, PI = *Paxillus involutus*, LBR = *Laccaria bicolor*, ST = *Suillus tomentosus*

Table 1: Foliar potassium (K) and phosphorus (P), root K and P, soil K and P as well as pH, and root and shoot dry mass (DM) for non-ectomycorrhizal fungi (NMC) and the ectomycorrhizal fungi *Laccaria bicolor* (LBR), *Paxillus involutus* (PI), *Pisolithus tinctorius* (PT) and *Suillus tomentosus* (ST). Means followed by the same letter within a column are not significantly different ($P < 0.05$). P-values for individual effects of each parameter are also indicated

Fungi	Root P	Root K	Foliar P	Foliar K	pH	Soil K	Soil P	Root DM	Shoot DM
NMC	1 140.85c	5 878.48b	1 830.34a	13 738.00ab	5.15bc	21.71a	3.58b	0.12e	0.31e
LBR	1 372.08ab	6 151.50a	1 799.55a	12 551.00c	4.98bc	16.65b	4.59a	0.41d	0.59c
PI	971.05d	4 716.02c	1 693.67ab	14 126.00a	5.32b	15.89b	3.39b	0.35c	0.50d
PT	1 263.26bc	3 776.53d	1 426.80b	13 366.00b	5.08b	11.25d	3.31b	0.82a	0.95a
ST	884.67d	6 698.07a	1 340.11b	13 161.00bc	5.29ab	14.11c	3.38b	0.68b	0.87b

was recorded in the NMC treatment and was significantly higher than that of the PT and ST treatments.

The NMC treatment had significantly higher root K values than the PI and PT treatments but significantly lower values than the ST and LBR treatments. Pine seedlings in the NMC treatment retained a lesser amount of root P than the LBR and PT treatments but contained a significantly higher root P than the PI and ST treatments.

Statistical analyses of the soil nutrient status after the experiment revealed that there was a higher amount of soil K in the NMC treatment than in all of the EMC treatments. However, the soil P value of the NMC treatment was not significantly different from the EMC treatments except for the LBR treatment (Table 1).

Fungal type significantly affected both SDM and RDM ($p < 0.0001$). The highest values of SDM and RDM were recorded in the ST treatment, whereas the lowest values were in the NMC treatment (Table 1).

Discussion

Quality and quantity of plant root exudates, especially OAs, can be improved by plant associations with rhizospheric microorganisms (Dakora and Phillips 2002; Smith and Read 2008). Plant–mycorrhizal associations offer the benefits of releasing high amounts of OAs into the rhizoshere, thereby creating a special zone of interest in the soil for nutrient exchange and microbial activities. This zone is a gradient where the maximum number and diversity of microorganisms occur in the vicinity of the root and decrease with distance from the root (López-Bucio et al. 2000). With such characteristics, plant growth and crop productivity are influenced by high numbers and diversity of soil bacteria and fungi. Hence changes such as solubilisation of nutrients, and changes in root morphology and shoot:root ratio may be experienced (Jones 1998; Badri and Vivanco 2009; Adeleke et al. 2012).

Among the four organic acids analysed from the soil samples in this study, citric acid was produced in the highest quantities, followed by oxalic acid (Figure 1a–d). The study has shown that the quantities of OAs secreted by the ECM fungi varied and were species-specific. For instance, only the PT and ST treatments had a significantly higher amount of citric acid than the NMC treatment. In contrast, there was no significant difference between the amount of other OAs secreted and NMC for all the other treatments. This may indicate that the beneficial effects of EMC due to their secretion of OAs are also species-specific, hence such benefits cannot be generalised. This is similar to the outcome of previous studies on related investigations (van Schöll et al. 2006a, Adeleke et al. 2012). For instance, Adeleke et al. (2012) reported that the weathering benefits of OAs secretion of ECM fungi are species-specific and could not be directly linked to their weathering potential.

With higher secretion of citric and oxalic acids, such outcome is not unexpected as both OAs are important in many biological processes in the rhizosphere (Wallander and Wickman

1999; Ahonen-Jonnarth et al. 2000; van Schöll et al. 2006a; Smith and Read 2008). For instance, citric acid is involved in nitrogenase activity by acting in the uptake and stability of metal ions that form the prosthetic group of nitrogenase (Hoover et al. 1987; Liang et al. 1989). Similar effects of oxalic acid have also been reported, especially in phosphate solubilisation, metal detoxification and altering the surface features of soil particles (Suthipradit et al. 1990; Bolan et al. 1997; Adeleke et al. 2012). The OAs often bind with aluminium (Al) to form stable chelate complexes in Al-toxic soil to assist in the reduction of Al toxicity in such soils. In addition, OAs in the soil are associated with the maintenance of soil pH level. For instance, an initial rise in soil pH during decomposition of organic residue was observed by Haynes and Mokolobate (2001) and this led to a corresponding increase in crop growth and higher yields in acid soils.

This study further investigated the role of citric and oxalic acids in relation to plant nutrient and growth. As shown in Figure 1a–d and Table 1, there was no consistent trend in the nutrient (P and K) concentrations of the root and shoot samples of all ECM and NMC treatments, hence no correlation could be established between these two sets of parameters. However, there was an indication that EMC-treated plants could have utilised absorbed nutrients for growth, as results indicated that the plants had higher biomass of both shoots and roots (Table 1). In addition, this study also revealed the potential of ECM roots to exploit the soil better compared with their non-ECM counterparts as indicated in the lower concentrations (higher nutrient uptake) of nutrients (K) in soil with ECM seedlings.

The results presented in this study are of particular importance to crop production and soil remediation. The more OAs that can be produced by plants, the better plant growth. This relationship was observed in both PT and ST treatments with significantly higher values of biomass. Plants have been reported to grow under low nutrient conditions because of their ability to produce OAs. In such a process, the OAs dissolve insoluble phosphates by lowering

the pH, chelating cations and competing with phosphate for adsorption sites in the soil (Nahas 1996; He et al. 2002). The release of OAs could also protect the root apex by chelating Al in the rhizosphere (Kochian 1995), thus rendering it non-phytotoxic. This is an advantage in soil management for crop production.

The outcome of this study endorses the results of a previous study by Calvaruso et al. (2010) in which impacts of OAs secretion on weathering were investigated. The study showed that colonisation by *Lacarria bicolor* S238N did not cause a significant increase in biotite weathering compared with non-mycorrhizal pine, but significantly contributed to plant health through the absorption of weathered nutrients. Such an outcome supports the observation in the present study that absorption of nutrients played a significant role in overall plant growth.

Conclusion

Regardless of their mycorrhizal status, pine roots are able to produce OAs. Nevertheless, ECM plants can be more effective in nutrient mobilisation compared to NMC plants, depending on the species. However, caution must be exercised in the interpretation of the OA results because the response could vary with soil type, treatment and storage (Mimmo et al. 2008).

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