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Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition

J. -P. Toussaint · F. A. Smith · S. E. Smith

Abstract The potential of three arbuscular mycorrhizal fungi (AMF) to enhance the production of antioxidants (rosmarinic and caffeic acids, RA and CA) was investigated in sweet basil (*Ocimum basilicum*). After adjusting phosphorus (P) nutrition so that P concentrations and yield were matched in AM and non-mycorrhizal (NM) plants we demonstrated that *Glomus caledonium* increased RA and CA production in the shoots. *Glomus mosseae* also increased shoot CA concentration in basil under similar conditions. Although higher P amendments to NM plants increased RA and CA concentrations, there was higher production of RA and CA in the shoots of AM plants, which was not solely due to better P nutrition. Therefore, AMF potentially represent an alternative way of promoting growth of this important medicinal herb, as natural ways of growing such crops are currently highly sought after in the herbal industry.

Keywords *Ocimum basilicum* · Phytochemicals · Rosmarinic acid · Caffeic acid · Plant phosphorus nutrition

Introduction

Medicinal herbs are known as sources of phytochemicals, or active compounds, that are widely sought after worldwide for their natural properties. Members of the Lamiaceae have been used since ancient times as sources of spices and flavourings (Hirasa and Takemasa 1998) and for their

J. -P. Toussaint (✉) · F. A. Smith · S. E. Smith
School of Earth and Environmental Sciences,
Waite Campus DP 636, The University of Adelaide,
Adelaide, South Australia 5005, Australia
e-mail: jeanpatrick.toussaint@adelaide.edu.au

pharmaceutical properties (Bais et al. 2002). It is generally accepted that the medicinal properties of this family are due to secondary metabolites such as phenolic compounds (including flavonoids and phenylpropanoids) as well as anthocyanins (Phippen and Simon 1998, 2000; Kahkonen et al. 1999). Important phenolic compounds within the Lamiaceae include rosmarinic acid (RA), an ester of caffeic acid (CA), which is commonly recognised to have a number of biological activities, predominantly as an antioxidant but also antibacterial, antiviral and anti-inflammatory (Petersen and Simmonds 2003). Antioxidants have been used in the food processing industry for more than 50 years (Cuvelier et al. 1994), and natural antioxidants such as RA have recently gained recognition because of major concerns about toxic side effects of synthetic antioxidants like BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) in food (Pizzale et al. 2002; Sacchetti et al. 2004).

Sweet basil (*Ocimum basilicum*) belonging to the Lamiaceae has been traditionally used for the treatment of many ailments, such as headaches, coughs and diarrhoea (Phippen and Simon 1998; Javanmardi et al. 2002). Sweet basil is generally recognised as safe (GRAS) and is a rich source of phenolic antioxidant compounds and flavonoids (Juliani and Simon 2002; Jayasinghe et al. 2003). Rosmarinic acid is probably the major compound that confers antioxidant activity in sweet basil (Javanmardi et al. 2002; Juliani and Simon 2002; Jayasinghe et al. 2003). This compound is reported to be mainly produced in the leaves, but it has been pointed out that considerable amounts are produced in the roots, and that it may be released specifically under microbial challenge (Bais et al. 2002).

Many species belonging to the Lamiaceae, including sweet basil, form arbuscular mycorrhizas (AM; Wang and Qiu 2006). In addition to increasing uptake of poorly

available nutrients such as phosphorus (P) and nitrogen (N; Smith and Read 1997; Smith et al. 2003; Toussaint et al. 2004) or conferring protection against pathogens (St-Arnaud et al. 1995; Cordier et al. 1996; Fillion et al. 1999), arbuscular mycorrhizal fungi (AMF) can also induce changes in the accumulation of secondary metabolites, including phenolics, in host plant roots (Vierheilig et al. 2000; Devi and Reddy 2002; Rojas-Andrade et al. 2003; Yao et al. 2003). Relatively little is known about the effects of AM colonisation on the accumulation of active phytochemicals in shoots of medicinal plants, which are often the harvest products. However, it was recently reported that *Glomus mosseae* directly increases the essential oil content in shoots of *Origanum* sp. (Khaosaad et al. 2006) as well as sweet basil (Copetta et al. 2006). The overall aim of the present study was to see if different AMF species can provide an efficient and natural way of improving the growth of this medicinal herb and, at the same time, increase the production of RA and its precursor CA. More specifically, we have investigated whether increases in RA and CA are the direct result of AM establishment or are an indirect effect of altered P nutrition via the fungi.

Materials and methods

Seeds of sweet basil (*O. basilicum* L., Genova strain, Yates Seed Ltd) were surface-sterilised by soaking in a 30% sodium hypochlorite solution (12.5% commercial bleach) for 10 min, rinsed with reverse osmosis (RO) water and sown directly into pots. The pots contained 1,400 g of sterilised sand (three parts coarse sand and one part fine sand) and soil (3:1 w/w) with or without AMF inoculum. Nutrients were mixed with the soil at the following rates (mg/kg dry soil): K_2SO_4, 75; $CaCl_2 \cdot 2H_2O$, 75; $CuSO_4 \cdot 5H_2O$, 2.1; $ZnSO_4 \cdot 7H_2O$, 5.4; $MnSO_4 \cdot H_2O$, 10.5; $CoSO_4 \cdot 7H_2O$, 0.39; $MgSO_4 \cdot 7H_2O$, 45.0; $Na_2MoO_4 \cdot 2H_2O$, 0.18; NH_4NO_3, 85.7. Phosphorus (P) was added separately as $CaHPO_4$ in the soil/ sand mix at three different rates for the non-mycorrhizal (NM) treatments: 0.1, 0.2 or 0.3 g/kg of $CaHPO_4$ (P1, P2 and P3, respectively) and at 0.2 g/kg for AM treatments. These rates corresponded to approximately 0.02, 0.05 and 0.07 g/kg of actual P added. Preliminary experiments had revealed that when grown with 0.2 g/kg of $CaHPO_4$, basil plants colonised by *Glomus intraradices* or NM had similar tissue P concentrations. Moreover, this P level allowed extensive AM fungal colonisation of basil roots. Accordingly, different P amendments were used for NM plants, with the aim of obtaining matched tissue P concentrations between the NM and AM plants, in the experiment described here.

The mycorrhizal treatments consisted of fresh inoculum (i.e. 350 g/kg dry soil) of one of the following AMF: *G.*

intraradices, Schenk and Smith (BEG 159), *Glomus caledonium* (BEG 162) and *G. mosseae* (NBR 1–2). Inoculum was obtained from pot cultures from the collection of Soil and Land Systems, School of Earth and Environmental Sciences, University of Adelaide, South Australia. Each consisted of a mixture of sand/soil and root pieces of leek (*Allium porrum* L. cv Vertina) that had been grown for at least 8 weeks with the different fungal species. The experiment consisted of three P levels and four inoculation treatments (three AM and one NM), with six replicates (one plant/pot) per treatment, in a complete randomised design.

Sweet basil plants were grown in a glasshouse at the Waite Campus, University of Adelaide. The mean diurnal temperature range was 28°C day/18°C night. Light levels averaged 400 μmol m^{-2} s^{-1}, and relative humidity averaged 40% during the day and 55% at night. The plants were watered as required in the first few days until the seedlings were well established, then to field capacity three times per week for the remainder of the experiment. After 7 weeks growth, the plants were harvested, and the following parameters were recorded: shoot and root fresh and dry weights, number of leaves, plant P concentration and content, RA and CA concentrations and content and percent AM root colonisation.

Harvesting and sampling

At harvest, roots and shoots were separated, washed and gently dried with paper towel before being weighed. Total fresh weights of roots and shoots were determined, and a weighed root sample (100–200 mg) was taken for determination of mycorrhizal colonisation. Root and shoot samples were dried at ~45°C for 3–4 days before dry weight was determined. This temperature was chosen to prevent any breakdown of RA and CA. The total root dry weight was determined from the total fresh weight and the fresh/dry weight ratio of the sample.

Mycorrhizal colonisation

Root samples were prepared for determination of percent colonisation using a modified staining method of Vierheilig et al. (1998). Roots were cut into ~1-cm segments and cleared in 10% KOH for 2 days at room temperature, rinsed several times in RO water and stained in a 5% ink–vinegar solution (black ink, Shaeffer) for 30 min at 80°C. Roots were subsequently destained overnight in acidified water (using a few drops of vinegar). Colonisation was determined according to the magnified intersections method of McGonigle et al. (1990) as modified by Cavagnaro et al. (2001). Root pieces mounted on slides were examined at ×100 magnification, using an Olympus BH-2 light microscope contain-

ing an ocular crosshair eyepiece. One hundred intersections between roots and crosshairs were observed for presence or absence of AM structures (internal and external hyphae, arbuscules and/or vesicles). At each intersection, the incidences of the structures were recorded, and the percent incidence of each structure over total intersections was calculated, and the total percent colonisation was determined from the presence of any colonised cells.

Determination of P concentration in plants

Phosphate concentration in plant tissue was determined using the method of Hanson (1950). Approximately 50 mg of dried material (root or shoot) was ground and digested overnight in a nitric–perchloric acid mixture (6:1) by heating on a programmed Tecator R digestion block at 70 to 180°C. Digests were then diluted to 25 ml in RO water, and an 8 ml aliquot was made up to 25 ml with 2 ml of colour reagent (nitric acid, 0.25% ammonium vanadate and 5% ammonium molybdate, 1:1:1 $v/v/v$) and RO water. After 30 min, absorbances were read at 390 nm on a Shimadzu UV-1601 spectrophotometer. A standard curve was prepared using a range of P concentrations from 0 to 10 μg/ml.

High performance liquid chromatography (HPLC) analysis

Sample preparation

Ethanol used for sample preparation was of analytical grade, the methanol was of HPLC grade, and the water was purified with a MilliQ apparatus. RA and CA standards were purchased from Adelab Scientific, Australia. The extraction and analytical methods were adapted from those described by Wang et al. (2004). Briefly, ~50 mg of dried material (root or shoot) were ground and extracted with 25 ml ethanol/water (30:70, v/v) with 10 min of sonication. The mixture was then centrifuged for 5 min at 4,500 rpm in a Sorvall Legend RT centrifuge (Kendro Laboratory Products, Germany) and the supernatant transferred to a 50 ml tube. The residue was further extracted with 20 ml ethanol/water followed by 5 min of sonication and centrifuged again. The supernatants were combined and further centrifuged at 4,500 rpm for an additional 20 min. The resulting supernatant was filtered through a 0.45-μm filter and transferred to 2-ml amber vials before injection to HPLC.

Instrumentation

The HPLC apparatus used was an Agilent 1100 Series liquid chromatograph system comprising a quaternary pump, thermostated column compartment, vacuum degasser, autosampler and diode array detector. An Apollo C18 column from Alltech Associates, USA (Model 36511) 5 μm, 250× 4.6 mm was used for the analyses and maintained at 30°C. The solvents used for separation were 0.1% orthophosphoric acid in water (v/v; eluent A) and 0.1% orthophosphoric acid in methanol (v/v; eluent B). After trying various gradient steps to isolate RA and CA, it was found that the best way to separate the compounds was to use an isocratic step. Hence, eluents A and B were maintained at 50% for 12 min with a flow rate of 1.0 ml/min and an injection volume of 50 μl. The detection wavelength was 330 nm, and the compounds' chromatographic peaks were confirmed and quantified by comparing their retention times with those of the standards after obtaining a calibration curve.

Statistical analyses

Treatment effects were determined by one-way analyses of variance (ANOVA), and differences between treatments were determined using Tukey's pairwise comparison test at a significance level of 95% (SYSTAT 11®) except where otherwise indicated in the text. When needed, the data were transformed to meet the assumptions of ANOVA (i.e. normality of data and evenness of variance).

Results

After 7 weeks growth, percent colonisation (%±SE) was relatively low for plants colonised by *G. caledonium* and *G. mosseae* (15±4 and 39±5%, respectively), whereas plants colonised by *G. intraradices* had a significantly higher percent colonisation (76±6%). Overall, the dry weight in NM plants increased with increasing P supply. At P1, NM plants had a significantly lower dry weight than other treatments (Fig. 1), and at P2, NM and AM plants had a similar dry weight.

NM plants had similar shoot P concentrations regardless of treatment. Concentrations at P1 and P2 were similar to plants colonised by *G. caledonium* (Fig. 2a). Plants colonised by *G. intraradices* and *G. mosseae* had significantly higher shoot P concentrations, which were similar to NM plants at P3. Thus, *G. caledonium*-colonised plants matched NM plants at P1 and P2, and *G. intraradices*- and *G. mosseae*-colonised plants matched NM plants at P3. Phosphorus concentrations in the roots of NM and AM plants were similar between treatments, except for plants colonised by *G. mosseae*, which had higher root P concentration than all NM plants (Fig. 2b). Total plant P content of NM plants increased as P supply increased (Fig. 3), and all AM plants had similar P content to NM plants grown at both P2 and P3.

Shoots of NM plants had significantly lower RA concentrations at P1 than other NM treatments (Fig. 4a).
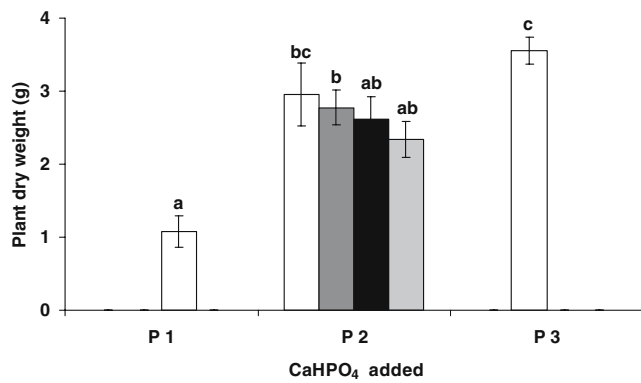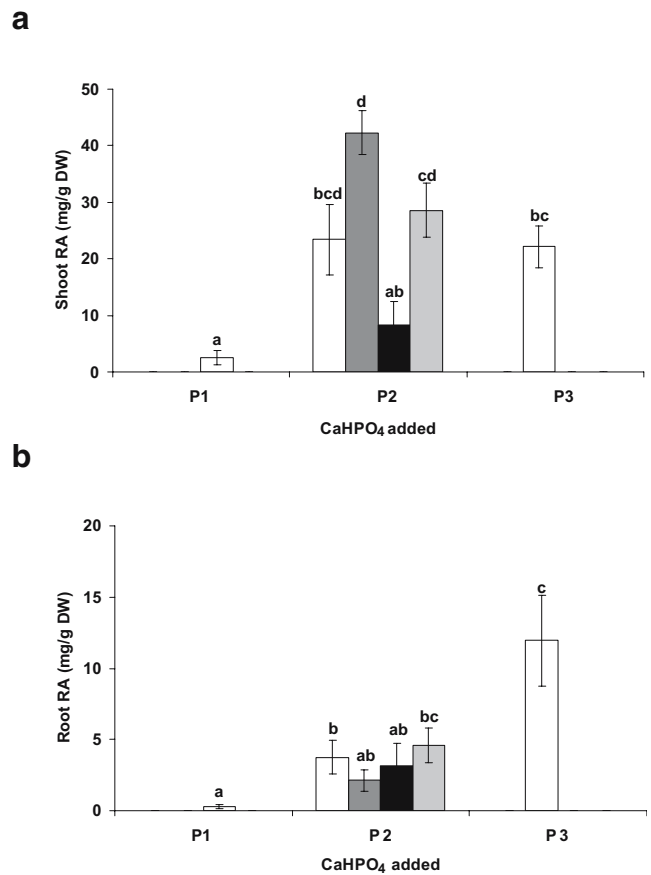
Fig. 1 Total plant dry weight (DW, g) of *O. basilicum* grown under three different P amendments. The plants were non-mycorrhizal (NM, *white bars*) or colonised by one of the following AMF: *G. caledonium* (*dark grey bars*), *G. intraradices* (*black bars*) or *G. mosseae* (*light grey bars*). Means (*n*=6, except for *G. caledonium* where *n*=4) *and* SE bars are shown. *Different letters* indicate significant differences according to Tukey's test (*p*=0.05)
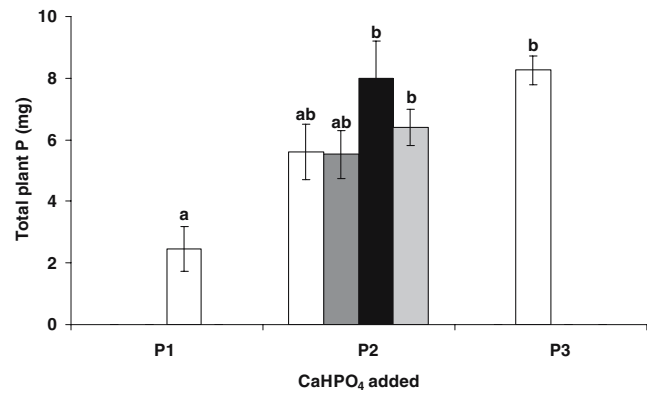
Fig. 3 Total plant phosphorus (P) content (mg) in *O. basilicum* grown under three different P amendments. The plants were non-mycorrhizal (NM, *white bars*) or colonised by one of the following AMF: *G. caledonium* (*dark grey bars*), *G. intraradices* (*black bars*) or *G. mosseae* (*light grey bars*). Means (*n*=6, except for *G. caledonium* where *n*=4) *and* SE bars are shown. *Different letters* indicate significant differences according to Tukey's test (*p*=0.05)
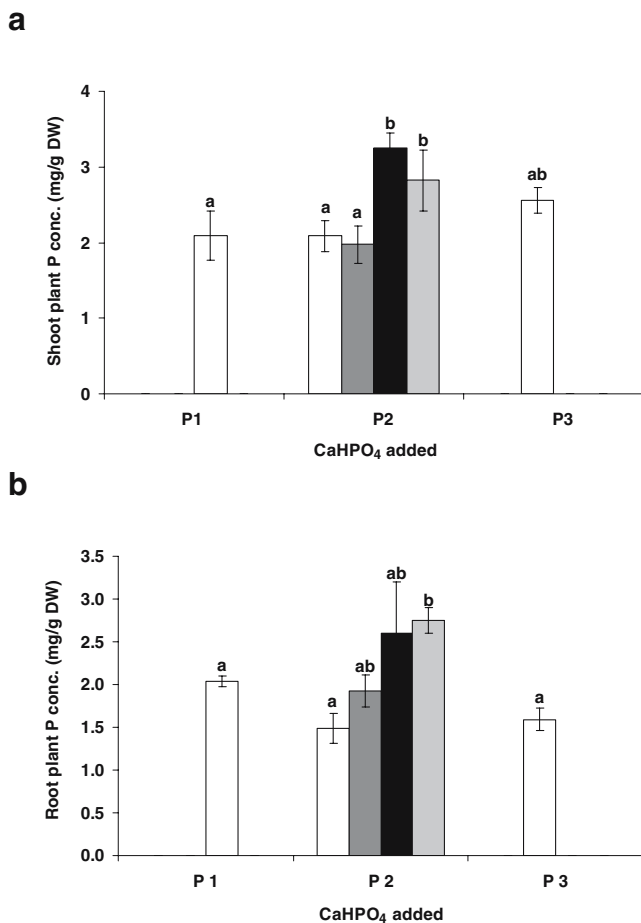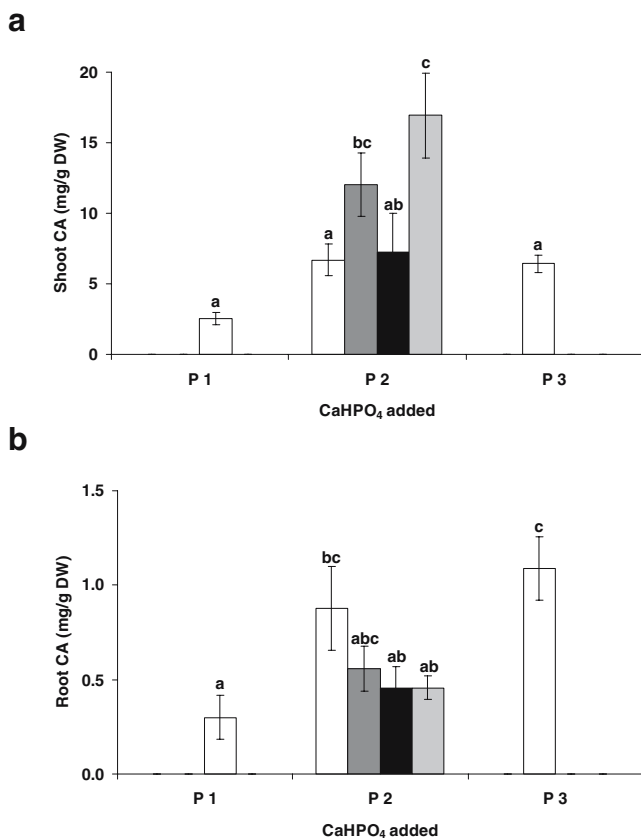

Fig. 2 **a** Shoot and **b** root P concentrations (mg/g DW) in *O. basilicum* grown under three different P amendments. The plants were non-mycorrhizal (NM, *white bars*) or colonised by one of the following AMF: *G. caledonium* (*dark grey bars*), *G. intraradices* (*black bars*) or *G. mosseae* (*light grey bars*). Means (*n*=6, except for *G. caledonium* where *n*=4) *and* SE bars are shown. *Different letters* indicate significant differences according to Tukey's test (*p*=0.05)

Fig. 4 **a** Shoot and **b** root rosmarinic acid (*RA*) concentrations (mg/g DW) in *O. basilicum* plants grown under three different P amendments. The plants were non-mycorrhizal (NM, *white bars*) or colonised by one of the following AMF: *G. caledonium* (*dark grey bars*), *G. intraradices* (*black bars*) or *G. mosseae* (*light grey bars*). Means (*n*=6, except for *G. caledonium* where *n*=4) *and* SE bars are shown. *Different letters* indicate significant differences according to Tukey's test (*p*=0.05)

Plants colonised by *G. caledonium* and *G. mosseae* had shoot RA concentrations similar to or greater than those of NM plants at P2 and P3, with higher values for *G. caledonium* (marginally significant at $p=0.08$) than all other treatments. RA concentrations in plants colonised by *G. intraradices* were low and similar to NM P1 plants. Root RA concentrations were generally much lower than shoot concentrations (Fig. 4a and b). Those of NM plants increased significantly as P supply increased. All AM plants had similar root RA concentrations, which were not significantly different from NM plants at P2 (Fig. 4b). Overall, shoot CA concentrations showed a similar pattern to shoot RA concentrations. Those of NM plants were similar regardless of P treatment and were significantly lower than concentrations in plants colonised with *G. caledonium* or *G. mosseae* at P2 (Fig. 5a). Shoot CA concentrations in plants colonised with *G. intraradices* were not significantly different from NM plants or *G. caledonium*-colonised plants, but were significantly lower than in plants colonised by *G. mosseae*. Root CA concentrations were again much lower than shoot concen-
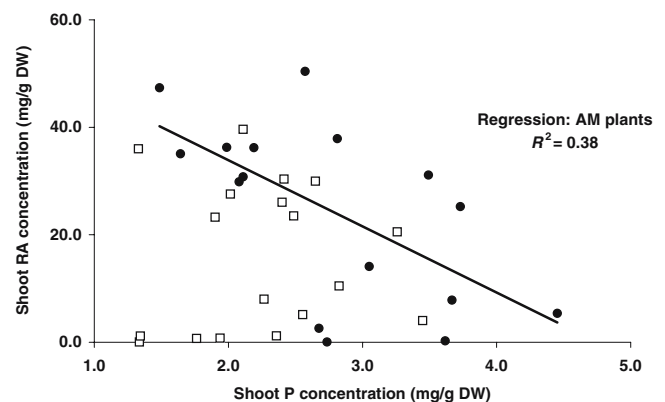
trations (Fig. 5a and b). Concentrations of CA in roots of NM plants increased as P supply increased; concentrations in AM plants did not differ between fungal treatments (Fig. 5b) and were not significantly different from NM plants at P2.

Regression analyses showed that there was no significant correlation between dry weight and total P content in AM plants, in either shoots or roots. There was a significant correlation between dry weight and RA concentrations in both roots ($R^2=0.49$; $y=13.6x-3.50$; $p=0.001$) and shoots ($R^2=0.73$; $y=12.44x-7.35$; $p=0.000$) of NM plants. Total P content and RA concentrations were also significantly correlated in both roots ($R^2=0.58$; $y=9.32x-4.21$; $p=0.000$) and shoots ($R^2=0.43$; $y=3.51x+0.51$; $p=0.03$) of NM plants. There was a negative relationship ($R^2=0.34$; $y=-12.31x+58.49$; $p=0.01$) between P concentrations and RA concentrations but only in shoots of AM plants (Fig. 6).

Discussion

We have demonstrated that in AM and NM sweet basil plants with matched tissue P concentrations: (1) AM plants grow as well as NM plants and (2) plants colonised by *G. caledonium* yield higher concentrations of RA and CA in their shoots compared to NM plants of the same P status. Colonisation by *G. mosseae* also increased CA concentrations in the shoots compared to NM plants. These findings show that RA and CA concentrations in the shoots of these plants are influenced both by improved P nutrition possibly mediated by the fungus and by a direct effect of the AM symbiosis. The negative relationship between shoot P concentration and shoot RA concentrations in AM plants as well as the clear effect of P addition on the production of RA and CA in NM plants further support these conclusions. Altogether, our results indicate that it is possible to grow

Fig. 5 **a** Shoot and **b** root caffeic acid (*CA*) concentrations (mg/g DW) in *O. basilicum* plants grown under three different P amendments. The plants were non-mycorrhizal (NM, *white bars*) or colonised by one of the following AMF: *G. caledonium* (*dark grey bars*), *G. intraradices* (*black bars*) or *G. mosseae* (*light grey bars*). Means (*n*=6, except for *G. caledonium* where *n*=4) *and SE bars* are shown. *Different letters* indicate significant differences according to Tukey's test (*p*=0.05)

Fig. 6 Correlation between shoot RA concentrations and shoot P concentrations for all AM (*black circles*) and NM plants (*open squares*). Each symbol represents one plant. A regression line is shown only for the AM plants, together with the R^2 value

sweet basil plants equally well using AMF or added P supply to produce more biomass with higher concentrations of active compounds.

Until recently, few investigations have focussed on AM effects on the production of phytochemicals in shoots of plants that are commonly used for human consumption. Exceptions include the increased production of essential oils in coriander and dill colonised by *Glomus fasciculatum* or *Glomus macrocarpum* (Kapoor et al. 2002a,b) and mint colonised by *G. fasciculatum* or a suite of AMF (Gupta et al. 2002; Freitas et al. 2004). However, AM and NM plants were not matched for tissue P concentrations in these studies, and effects might have been due to an improved P nutrition via the AM fungus. Nevertheless, recent studies showed increases in essential oils in shoots of sweet basil plants colonised by *Gigaspora rosea* (Copetta et al. 2006) and in oregano plants colonised by *G. mosseae* (Khaosaad et al. 2006), which were not due to an improved P status of the AM plants. Although the effect of AMF on phenolic accumulation has been reported for roots (Morandi et al. 1984; Grandmaison et al. 1993; Larose et al. 2002), we provide evidence that AMF can alter the production of phenolic compounds, such as RA and CA, also in the shoots of sweet basil.

A possible mechanism by which *G. caledonium* and *G. mosseae* increased phytochemical concentrations in sweet basil could be through improved N nutrition. Tyrosine and phenylalanine are important precursors to the production of CA and RA (Petersen and Simmonds 2003). Therefore, possible higher N assimilation in AM plants (Smith and Read 1997; Toussaint et al. 2004) might have contributed to the production of these amino acids and, subsequently, to higher production of the phenylalanine ammonia-lyase, one of the main enzymes involved in the production of CA and RA. Another possible mechanism could reside in the potential of AMF to induce changes in phytohormone levels in the host plant, such as cytokinins or gibberellin (Allen et al. 1980, 1982). These possibilities remain to be tested.

The variability of RA and CA concentrations we observed in plants colonised by different AMF highlights the functional diversity that exists between fungal isolates belonging to the same genus. Other reports also indicate variations in effectiveness between different AMF species in the production of active compounds (Kapoor et al. 2002b; Copetta et al. 2006). Moreover, the relatively low colonisation by *G. caledonium* and *G. mosseae* indicates that AMF can have considerable effects on the host plant physiology, even when their biomass in roots is low. These variations may have significant ecological implications via effects on the aboveground interactions between herbivores and host plants through the alteration of defense compounds (Gehring and Whitham 2002).

Our study has extended earlier findings (e.g. Kapoor et al. 2002a,b) by providing evidence that the increased accumulation of phytochemicals in sweet basil is mediated by direct effects of the AMF and is not solely the indirect result of improved P nutrition. In NM plants, growth with higher P amendments was related to higher biomass and concentrations of active compounds. From our results, we suggest two alternatives to produce sweet basil plants with higher biomass and higher phytochemical concentrations: (1) either use a conventional approach to grow basil plants, using higher P amendments or (2) inoculate the plants with AMF at lower P amendment. The latter approach would represent a more "natural alternative", as is currently highly sought after in the herbal industry. More work is needed to elucidate the mechanisms by which, in our study, *G. caledonium* specifically affected the production of RA and CA in sweet basil, as our experimental design did not allow us to answer this question. We also need to further investigate the accumulation of RA and CA in sweet basil challenged with a wider range of AMF. We are currently considering using a metabolomic approach to screen the various metabolites involved in the biochemical pathway of RA synthesis, which might be altered by AMF.

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