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ORIGINAL PAPER

Association with arbuscular mycorrhizal fungi influences alkaloid synthesis and accumulation in *Catharanthus roseus* and *Nicotiana tabacum* plants

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Abstract Frequently disregarded, plant associations with arbuscular mycorrhizal fungi (AMF) can influence plant specialized metabolism with important ecological and/or economic implications. In this study, we report on both the influence of mycorrhization on the content of a wide range of alkaloids and differential gene expression of some enzymes involved in alkaloid biosynthetic pathways in the leaves and roots of *Catharanthus roseus* and *Nicotiana tabacum* plants. These plants were divided into several treatments: mycorrhizal, inoculated with AMF; non-AMF inoculated plants; and non-AMF inoculated plants with an extra supply of phosphorus. The contents of vindoline, vinblastine, vincristine, catharanthine, ajmalicine and serpentine in *C. roseus* and of nicotine, anabasine and nornicotine in *N. tabacum* tobacco plants were determined. Mycorrhizal inoculation increased ajmalicine and serpentine contents in *C. roseus* roots suggesting that mycorrhization had a greater influence on the accumulation of alkaloids in roots than it did in shoots. The youngest leaves of mycorrhizal *C. roseus* plants showed lower transcript levels of the genes analysed; however, in older leaves, the expression levels were higher when compared with the leaves of non-mycorrhizal plants. In the case of tobacco, higher leaf to root ratios for nicotine and anabasine were found in plants with a mycorrhizal association. Our results showed that mycorrhization changed the alkaloid content and expression pattern of the genes analysed in both species; however, differences were found between the roots and shoots. In nature, such changes may have a direct influence on the interactions between plants and insects (herbivory) and pathogens. These interactions must be studied further to reveal the ecological influence mycorrhizae may have on chemical defences in a broader sense.

Keywords Mycorrhizae · Specialized metabolites · Catharanthus · Nicotiana

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Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in soils. AMF belong to a group of beneficial microorganisms that can mutualistically associate with the roots of the vast majority of terrestrial plants (Smith and Read 2008). The influence of arbuscular mycorrhizae (AM) on plant specialized metabolite accumulation and biosynthesis is still understudied (Toussaint 2007; Vannette and Hunter 2009), although quantitative and qualitative changes in several classes of plant specialized metabolites have been attributed to mycorrhization (Abu-Zeyad et al. 1999; Kapoor et al. 2007; Zubek et al. 2011). These alterations have frequently been connected with the higher biomass yields of mycorrhizal plants and are usually linked to the nutritional benefits of mycorrhization (Liu et al. 2007; Zubek et al. 2010).

The expression of different genes related to the plant defence system is directly influenced by AM, with consequences for whole-plant fitness and its response to biotic stressors (Pozo and Azcón-Aguilar 2007). In this way, it is

increasingly recognised that AM have a significant impact on the interaction between plants and other organisms both above- and belowground. Positive and negative effects on plant resistance to attackers, depending on their life styles and the plant organ affected, have previously been observed (Hartley and Gange 2009; Wooley and Paine 2011). AM are also known to alter general hormone balance in plants by activating defence systems, which in turn may induce the synthesis of specialized metabolites (Yuan et al. 2007). Therefore, there is strong evidence of the influence of AM on the accumulation of different specialized compounds in plant organs, both above- and belowground (Abu-Zeyad et al. 1999; Asensio et al. 2012; Khaosaad et al. 2008; Zubek and Blaszkowski 2009).

Catharanthus roseus, commonly named Madagascar periwinkle, is a tropical perennial plant belonging to the family Apocynaceae and is a source of several monoterpene indole alkaloids (MIAs) of pharmacological importance, such as vinblastine, vincristine, ajmalicine, vindoline, catharanthine and serpentine (El-Sayed and Verpoorte 2007). Currently, *C. roseus* is cultivated in many tropical and subtropical countries and even in temperate regions (Lata 2007). One of the main obstacles for the commercial exploitation of *C. roseus* alkaloids is their low concentration in plant tissue. Leaves have an alkaloid content of approximately 1 % (based on dry weight), and roots have an alkaloid content between 2 and 9 %; therefore, depending on the compound, a large amount of plant material is necessary to obtain commercial quantities (Tyler 1988), not considering that for some alkaloids, it is laborious work to collect roots. For biological and technical reasons, cell and tissue cultures and chemical synthesis are not economically viable as alternatives to meet the commercial demand for MIAs (El-Sayed and Verpoorte 2007). For this reason, field cultivation continues to be the only viable commercial source of *C. roseus* alkaloids (DiCosmo and Misawa 1995).

MIA biosynthesis is widely studied (Fig. S1) and involves highly compartmentalised and regulated enzymatic machinery (El-Sayed and Verpoorte 2007; Roepke et al. 2010). Except for a few steps, most of the genes involved in MIA biosynthesis have been described (Rischer et al. 2006; Verma et al. 2012). Although this accumulation of knowledge would be favourable for the study of MIA biosynthesis, the influence of common symbiotic associations with AMF on MIA biosynthesis and accumulation in *C. roseus* has never been deeply evaluated. A few reports indicate that depending on the AMF species, the total alkaloid content or the abundance of specific MIAs, such as vincristine and/or vinblastine in leaves or ajmalicine in roots and may increase due to the AM association (De la Rosa-Mera et al. 2011; Ratti et al. 2010).

The biosynthetic route of pyridine alkaloids (PAs) from plants in the genus *Nicotinana* and their role in chemical defence against aboveground herbivores have also been extensively studied, although the biosynthesis of these compounds (Fig. S2) occurs mainly in the roots (Baldwin and Schmelz 1996). Nicotine, for example, is produced in the root cortex and is transported via xylem to the aerial parts of the plant, where it is stored in the vacuoles of leaf cells (Dawson 1942) and secreted at the leaf surface by trichomes (Thurston et al. 1966). One of the key enzymes in the nicotine biosynthetic pathway is putrescine methyl transferase (PMT), which is located in the cortical cells of roots (Biastoff et al. 2009), the same site as AMF root colonisation (Smith and Read 2008). A622 is another protein composing the enzymatic complex of "nicotine synthase" and is known to be involved in nicotine, anabasine and anabatine synthesis in tobacco (DeBoer et al. 2009). The *A622* gene encodes an NADPH-dependent oxidoreductase, which is also expressed in the cortical cells of roots (DeBoer et al. 2009; Leete 1983). Nicotine can be converted into nornicotine in tobacco roots and leaves by an *N*-demethylation reaction that requires enzymes from the cytochrome P450 family (Chakrabarti et al. 2008; Lewis et al. 2010).

As with MIAs in *C. roseus*, the relationship between AMF and PA biosynthesis and accumulation in tobacco is largely unknown. Because PA biosynthesis is strongly influenced by jasmonates (Baldwin and Schmelz 1996) and AMF may induce significant changes in the level of this hormone in the roots (Hause et al. 2002; López-Ráez et al. 2010), it might be speculated that there may be a mycorrhizal effect on PA synthesis.

The objective of the present study was to evaluate the influence of AM association on the biosynthesis of alkaloids and their accumulation in the leaves and roots of *C. roseus* and *N. tabacum* plants. These species were chosen based on the vast knowledge that has been accumulated on their alkaloid metabolism, which allows us to evaluate the expression of genes positioned in key steps of each biosynthesis route. UPLC-MS/MS was used to determine the concentrations of vindoline, catharanthine, vincristine, vinblastine, ajmalicine and serpentine in the leaves and roots of *C. roseus* and of nicotine, nornicotine and anabasine in the leaves and roots of *N. tabacum* plants. To distinguish between the direct effects of mycorrhization and indirect effects due to the altered nutritional status of AM plants, a treatment with an extra P supply was included to maintain well-nourished, non-mycorrhizal control plants.

Materials and methods

Experimental design

Two independent greenhouse experiments were conducted in a completely randomised design, with seven replicates.

The study plant for experiment 1 was *Catharanthus roseus* (L.) G. Don, while the study plant for experiment 2 was *Nicotiana tabacum* L. Treatments included the following: M, with AMF inoculation; NM, without AMF inoculation; and NM + P, without AMF inoculation and with an extra phosphorus supply.

Fungal material and conditions for plant growth

Mycorrhizal inoculation was carried out when the seeds were sown. On each vase, a volume of 10 mL of soil inocula containing a mixture of colonised root fragments, hyphae and spores of the AMF *Glomus etunicatum* (Becker and Gerdemann) in experiment 1 and *Glomus intraradices* (Smith and Schenck) in experiment 2 was spread. The inocula were kindly supplied by Dr. Adriana Parada Dias da Silveira, from the Center of Soil and Environmental Resources of the Agronomic Institute (IAC). Non-mycorrhizal treatments received washings of the soil–inoculum mixture filtered through Whatman 42 paper.

For both experiments, plastic pots were filled with 2 L of a sand and vermiculite mixture (3:1, v/v) and sterilised for 1 h at 120 °C in an autoclave. *C. roseus* and *N. tabacum* seeds had previously been surface-sterilised in 2.5 % NaClO for 10 min. Six seeds were sown in each pot and AMF inocula was added to the growing substrate at sowing. After emergence, seedlings were thinned to two plants per pot and began receiving the following nutrient solution: $N–NO_3$, 154.6 mg L^{-1}; $N–NH_4$, 19.5 mg L^{-1}; $S–SO_4$, 18.7 mg L^{-1}; Ca, 151.2 mg L^{-1}; K, 70.9 mg L^{-1}; Mg, 18.8 mg L^{-1}; P, 5 mg L^{-1}; B, 0.53 mg L^{-1}; Fe, 1.99 mg L^{-1}; Mn, 0.97 mg L^{-1}; Cu, 0.076 mg L^{-1}; Zn, 0.3 mg L^{-1}; and Mo, 0.15 mg L^{-1} (Furlani and Furlani 1988). Distilled water was supplied on alternate days. Plants in the extra P treatment received a nutrient solution containing 10 mg L^{-1} of P supplied as KH_2PO_4. *C. roseus* plants were allowed to grow for 16 weeks, and *N. tabacum* plants for 90 days before being harvested.

Measurements and analytical determinations

At the harvest of both experiments, shoots and roots were separated and washed under tap water. The root subsamples were separated into three groups: (a) stored in 50 % ethanol for determination of mycorrhizal colonisation; (b) frozen in liquid N_2 and stored at 80 °C for use in molecular assays; and (c) lyophilised and stored in a vacuum desiccator for alkaloid analysis. Just before root harvest in experiment 1, the first (L1) and third (L3) youngest leaves were collected from the main and secondary branches of the plants from three pots per treatment; some of the collected leaves were stored at 80 °C for the use in molecular assays, while others were lyophilised and maintained in a vacuum desiccator for alkaloid analysis. At the harvest of experiment 2, the youngest three leaves from three replicates per treatment were collected and conditioned as in experiment 1. In both experiments, tissue samples for alkaloids and molecular determinations were simultaneously collected at the same period of the day, between 8:00 and 10:00 a.m. The shoots of the other four plants in each treatment were dried at 60 °C, weighed and ground to a powder in a mortar with a pestle. P, K, Ca, Mg, S, B, Cu, Fe, Mn and Zn concentrations in the shoots were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; JobinYvon, JY50P Longjumeau, France) after HNO_3–$HClO_4$ digestion.

The percentage of the root length that was colonised by AMF was estimated using a slide method (Giovannetti and Mosse 1980), where the roots were first cleared with 2.5 % KOH and then stained with 0.05 % trypan blue (Phillips and Hayman 1970). For each sample, a total of 30 root segments, each one of approximately 1 cm in length, were observed under an Olympus BX 40 light microscope (Olympus Optical Co., Tokyo, Japan).

In experiment 1, the concentration of MIAs were determined from leaf and root extracts of the freeze-dried *C. roseus* leaves and roots from three experimental replicates per treatment, following an adaptation of Tikhomiroff and Jolicoeur (2002) and Hughes et al. (2004). A methanolic extraction was performed as follows: freeze-dried plant tissues were powdered with liquid N_2 in a mortar and homogenised with 100 % methanol in a 1:50 (m/v) ratio. Homogenates were sonicated for 30 min and centrifuged at 10,000 rpm for 10 min, and the supernatants were collected in HPLC vials. Chromatographic separation and identification were performed using a UPLC-MS system with a C-18 reverse phase column (Acquity, Waters, Manchester, UK) using an isocratic method with at a flow rate of 1 mL min^{-1}. The mobile phase consisted of 98.9 % deionised water, 1.0 % acetonitrile and 0.1 % formic acid. Authentic standard solutions (Sigma-Aldrich) and sample solutions were injected (2 µL) into the system. Peak areas were extracted using the Mass Lynx software, and concentrations were calculated between 1 and 600 µg/mL. Mass spectra were acquired between 100 and 500 m/z in the positive ion mode in a TQD (triple quadrupole) mass spectrometer (Acquity, Micromass Waters, Manchester, UK) under the following conditions: capillary 3,500 (V); cone 30 (V); source temperature, 150 °C; and desolvation temperature, 300 °C.

In experiment 2, PAs were extracted from the freeze-dried *N. tabacum* leaves and roots from three experimental replicates per treatment. Approximately 0.05 g per sample were powdered with liquid N_2 in a mortar and homogenised with acid water (deionised water + 0.01 % formic acid) in a 1:1 (m/v) ratio. Homogenates were sonicated for

30 min and centrifuged at 10,000 rpm for 10 min, and the supernatants were collected in HPLC vials. Chromatographic separation and identification were undertaken using a UPLC-MS system with a C-18 reverse phase column (Acquity, Waters, Manchester, UK) using an isocratic method with at a flow rate of 1 mL min^{-1}. The mobile phase consisted of 98.9 % deionised water, 1.0 % acetonitrile and 0.1 % formic acid. Authentic standard solutions (Sigma-Aldrich) and sample solutions were injected (2 µL) into the system. Peak areas were analyzed using the Mass Lynx software, and concentrations were calculated between 1 and 600 µg mL^{-1}. Mass spectra were acquired between 100 and 500 m/z in the positive ion mode in a TQD mass spectrometer (Acquity, Micromass Waters, Manchester, UK) under the following conditions: capillary 3,500 (V); cone 30 (V); source temperature, 150 °C; and desolvation temperature, 300 °C.

RNA isolation and quantitative real-time PCR (RT-qPCR) analysis

Total RNA was isolated from the leaf and root samples that were stored at −80 °C using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified using Quant-It™ Assays (Invitrogen, Carlsbad, CA, USA). Then, 3.0 µg of total RNA was treated with Turbo DNase-free (Ambion, Austin, TX, USA), and the first-strand cDNA synthesis was carried out using the reverse transcription polymerase Superscript III RT (Invitrogen). The first-strand cDNA was diluted 50 times with nuclease-free water and used in RT-qPCR reactions. Each reaction contained a mixture of 4.5 µL of diluted cDNA aliquots, 0.6 µL of mixed primers (1.25 pmol mL^{-1}), 7.5 µL of QuantiFast™ SYBR® Green PCR Kit (Quiagen, Hilden, Germany) and 2.4 µL of nuclease-free water. The list of genes and primer pairs used for the RT-qPCR is shown in Table 1. The RT-qPCR amplification was carried out in 96-well plates on an iCycler iQ™ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The cycling parameters consisted of a 95 °C hold for 3 min, followed by 40 cycles of a 95 °C denaturing step for 10 s and a 60 °C annealing/extension step for 30 s. PCR efficiency was estimated using LinReg Software and was between 90 and 97 % for all primers used (Ramakers et al. 2003). Confirmation of amplicon specificity was based on the dissociation curve at the end of each run, using a cDNA pool. Each primer analysed was performed in triplicate, and PCR reactions without a template were also performed as negative controls for each primer pair. The relative gene expression was quantified using the comparative CT (threshold cycle) method (Schmittgen and Livak 2008). Ribosomal 40S protein S9 (*RpS9*) and elongation factor-1 (*EF-1α*) were used as internal reference genes for *C. roseus* (Liscombe et al. 2010; Wei 2008), and ribosomal protein (*NtL25*) and elongation factor-1 (*NtEF-1α*) genes were used as internal reference genes for *N. tabacum* (Schmidt and Delaney 2010).

Statistical analysis

The data obtained for each parameter studied were analysed using a one-way analysis of variance (ANOVA), with a Tukey's test at 5 % probability for means comparisons. Data that were expressed as a percentage were arcsine-square-root transformed prior to statistical analyses.

Results

Catharanthus roseus

The highest shoot-mass production was observed in plants that were not inoculated with AMF, but that were supplied with extra P (NM + P), followed by the inoculated plants (M) and then plants without AMF inoculation and without the extra P supply (NM) (Fig. 1a). In plants that received the AMF inoculation, approximately 31 % of the root length exhibited the characteristic structures of AM symbiosis, such as intra- and extraradical hyphae and arbuscules (not shown). As expected, the plants that were not inoculated with AMF did not show any mycorrhizal structure. In general, the K, Ca, Mg, Cu and Fe concentrations in *C. roseus* leaves were similar in plants across the three treatments (Table 2). However, NM + P plants had higher P and S concentrations than M or NM plants. Foliar Mn concentrations were significantly lower and B and Zn concentrations were higher in M plants than those in the NM or NM + P treatments (Table 2).

Vindoline, vinblastine and vincristine were detected in the roots (Fig. 2). In general, the concentrations of vindoline (Fig. 2a), vinblastine (Fig. 2b), catharanthine (Fig. 2d), ajmalicine (Fig. 2e) and serpentine (Fig. 2f) were highest in the youngest leaves (L1), diminishing with age (Fig. 2). The difference was not as clear for vincristine (Fig. 2c). Ajmalicine and serpentine concentrations were higher in the roots than in the leaves (Fig. 2e, f, respectively). The catharanthine concentration was higher in the leaf tissue than in the roots (Fig. 2d). When concerning the effects of the different inoculation treatments on alkaloid accumulation, the largest differences were observed in the youngest leaves (L1), where the NM plants had the highest concentrations of five of the six alkaloids analysed (all except serpentine) (Fig. 2f). For most of the MIAs, the M and NM + P plants exhibited similar concentrations in their leaves. In general, the contents of catharanthine (Fig. 2d), ajmalicine (Fig. 2e) and serpentine (Fig. 2f)

Table 1 Genes, the proteins they encode, accession numbers, oligonucleotides primers and amplicon sizes used for RT-qPCR differential expression analysis

Gene	Enzyme/protein (accession no.)	Primer pairs	Amplicon (bp)
TDC	Tryptophan decarboxylase (J04521)	5′-TCCGAAAACAAGCCCATCGT -3′ 5′-AAGGAGCGGTTTCGGGGATA-3′	126
STR	Strictosidine synthase (X61932.1)	5′-CCTACGCATCTCCCTTCTG-3′ 5′-CCCACCTTCTTTTCCAACC-3′	167
SGD	Strictosidine *β-glucosidase* (AF112888)	5′-CTATGGGGGATGGCAGCA-3′ 5′-GTTCTATTTTCCTCAACCACACCAC-3′	123
DAT	Deacetylvindoline 4-O-acetyltransferase (AF053307)	5′-GACCTAGTCCTTCCCAAACG-3′ 5′-CCTCCATCAGCAACTTTGTG -3′	128
PRX1	*Catharanthus roseus* Peroxidase 1 (AM236087)	5′-ACGGGGAATCAAGGTGAA-3′ 5′-CGCATAATAAACTCATCAAGAACTAA-3′	192
RpS9	40S Ribosomal protein S9 (AJ749993)	5′-TTGAGCCGTATCAGAAATGC-3′ 5′-CCCTCATCAAGCAGACCATA-3′	122
EF-1α	Elongation factor-1 alpha (EU007436)	5′-TCAGGAGGCTCTTCCTGGTGA-3′ 5-AGCTCCCTTGGCAGGGTCAT-3′	115
PMT	Putrescine *N*-methyltransferase (AF126810.1)	5′-GCAGCATTCATTTTACCATCTTT-3′ 5′-CGTCGCATTTCACTTATTTATTCA-3′	132
A622	PIP family oxidoreductase (D28505)	5′-TCTGCGGTGACTCTATCAAACT-3′ 5′-CAAAAGCACTGAAGCGAAAA-3′	80
CYP82E4	Nicotina *N*-demethylase (HM802352.1)	5′-CAAAACCCTTACCACCGAAA-3′ 5′-AGAAAAGCTGGACGATTGGA-3′	241
L25	Ribosomal protein (L18908)	5′-CCCCTCACCACAGAGTCTGC-3′ 5′-AAGGGTGTTGTTGTCCTCAATCT-3′	51
EF-1α	Elongation factor 1-α (AF120093)	5′-TGAGATGCACCACGAAGCTC-3′ 5′-CCAACATTGTCACCAGGAAGTG-3′	51

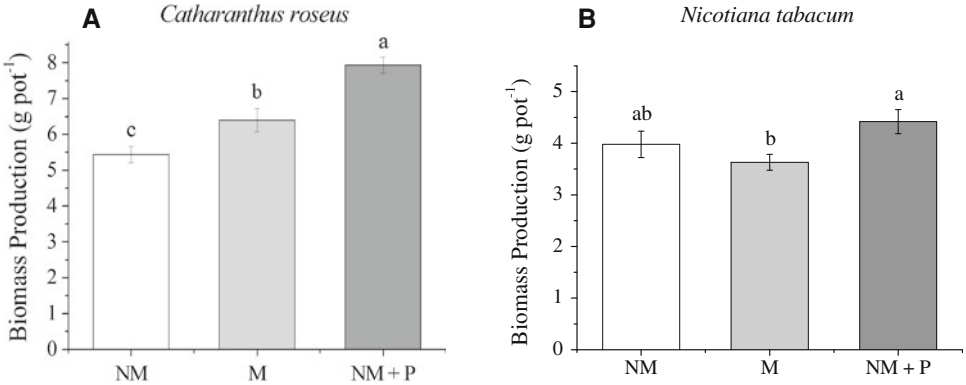


Fig. 1 Shoot mass (g pot^{-1}) of non-mycorrhizal (*white*), mycorrhizal (*light grey*) and non-mycorrhizal plus extra phosphorus supply (*dark grey*) *Catharanthus roseus* (**a**) and *Nicotiana tabacum* (**b**) plants. *Different letters* denote statistically significant differences among the treatments, as indicated by a Tukey's test ($P < 0.05$) ± standard error, $n = 7$

were higher in the M than in the NM and NM + P plants, although there was some variance among them.

The expression levels of the genes that encode the key enzymes of the biosynthetic pathways of MIAs were compared in the leaves and roots of the plants using RT-qPCR analysis (Fig. 3). In general, higher expression of all analysed genes was observed in the older leaves. In these leaves (L3), there was a clear trend toward a higher gene expression in the M plants, except for the expression of *SGD*, although the trends were not statistically significant for all genes and treatments. In the youngest leaves (L1), the highest expression of genes was observed in the NM plants, while they were similar in the M and NM + P plants, except for the expression of *DAT*. In the roots, the M plants exhibited lower TDC and STR transcript levels than plants without AMF inoculation. Similar *SGD* transcript numbers were found in the roots of the plants from all of the treatments. In contrast, *PRX1* expression was

Table 2 Nutrient concentrations in the leaves of non-mycorrhizal (NM), mycorrhizal (M) and non-mycorrhizal plus extra phosphorus supply (NM + P) *Catharanthus roseus* and *Nicotiana tabacum* plants

Treatment	K (g kg^{-1})	P (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	S (g kg^{-1})	B (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})
Catharanthus roseus										
NM	9.6 a	0.62 b	9.9 a	3.15 a	1.17 b	30.2 b	157 a	3.40 a	87 a	7.3 b
M	9.9 a	0.65 b	10.0 a	3.35 a	1.17 b	36.8 a	105 b	3.62 a	99 a	9.2 a
NM + P	10.9 a	0.80 a	9.4 a	2.92 a	1.30 a	37.6 a	169 a	3.65 a	169 a	9.3 a
Nicotiana tabacum										
NM	18.2 a	0.82 a	16.6 a	6.6 a	1.9 b	26.5 a	89 a	9.1 a	482 a	33 a
M	18.9 a	0.85 a	17.6 a	6.2 a	2.6 a	27.0 a	46 b	10.8 a	411 a	31 a
NM + P	19.1 a	0.92 a	17.6 a	7.0 a	2.0 b	29.3 a	106 a	9.0 a	461 a	34 a

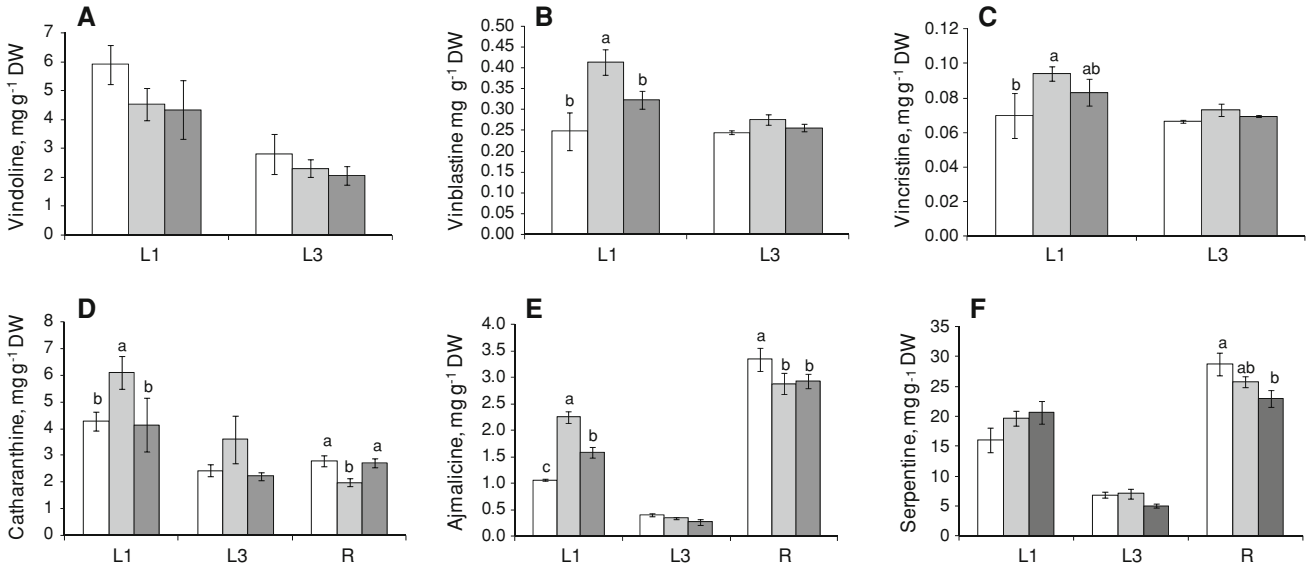


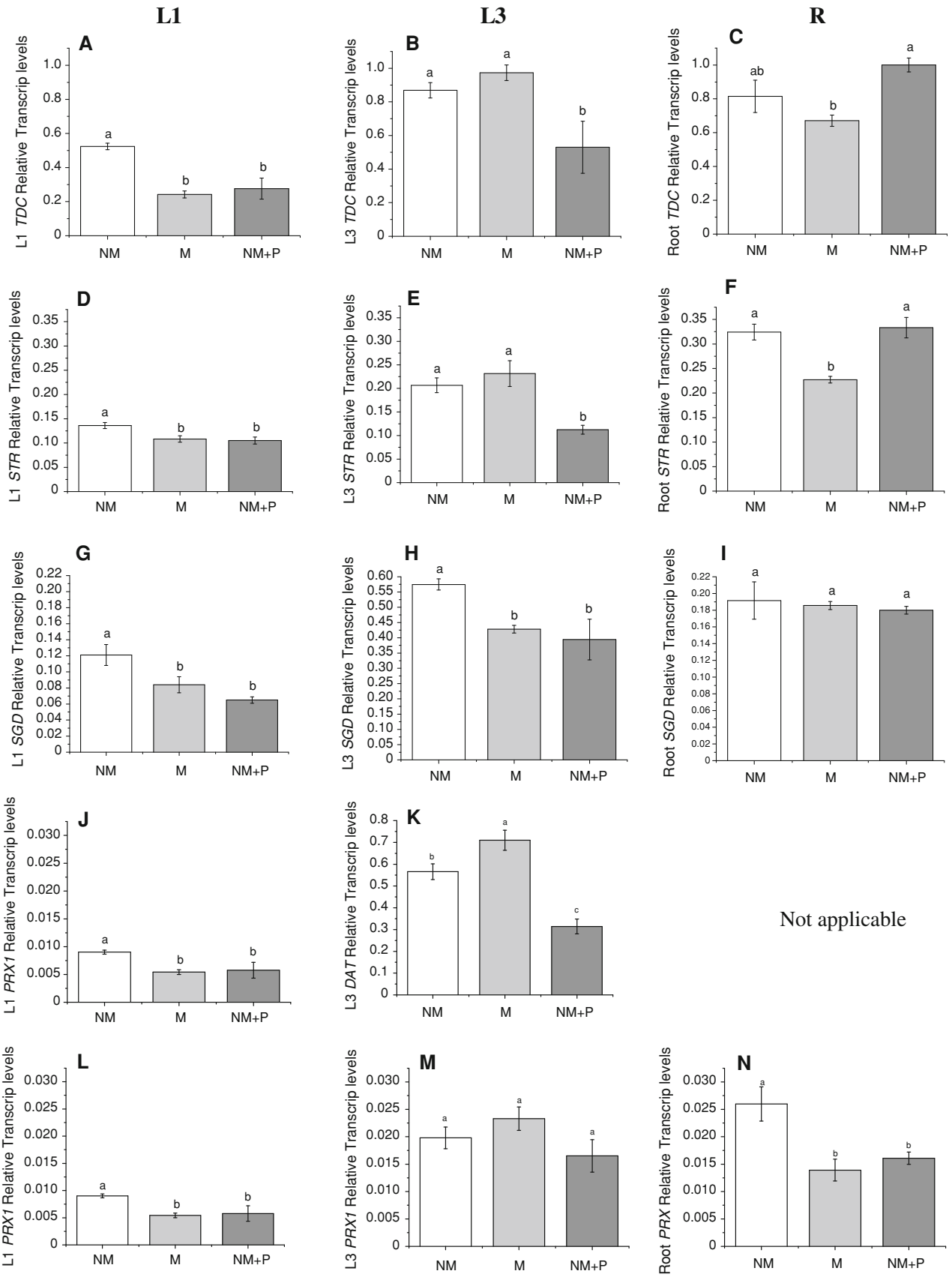
Fig. 2 Accumulation of vindoline (**a**), vinblastine (**b**), vincristine (**c**), catharanthine (**d**), ajmalicine (**e**) and serpentine (**f**) in the first (*L1*) and third (*L3*) youngest leaves and/or in the roots (*R*) of non-mycorrhizal (*white*), mycorrhizal (*light grey*) and non-mycorrhizal plus extra phosphorus supply (*dark grey*) *Catharanthus roseus* plants. Significant differences among the treatments at $P < 0.05$ were determined using a one-way analysis of variance (ANOVA) followed by a Tukey's test and are indicated by *different letters*; ± standard error, $n = 3$

higher in the roots of the NM plants than in the roots of the M and NM + P plants.

Nicotiana tabacum

G. intraradices was very effective in colonising tobacco roots, with an average of 90 % of the colonised root length showing the characteristic structures of AMF symbiosis, such as intra- and extraradical hyphae, vesicles and arbuscules, and ranging from 81 to 94 % (not shown). Roots from non-AMF inoculated treatments had no mycorrhizal structures, indicating that there was no contamination. Despite the high percentage of the root length that was colonised by *G. intraradices*, mycorrhization did not cause an increase in growth. NM + P tobacco plants had significantly higher shoot dry mass than NM plants, followed by the AMF-inoculated plants (Fig. 1b). The contents of K, Ca, Mg, B, Cu, Fe and Zn in the *N. tabacum* leaves did not vary significantly among plants in the three

Fig. 3 Relative transcript levels of terpene indol alkaloid related ▶ enzymes (**a–n**) in the first (*L1*) and third (*L3*) youngest leaves and/or in the roots (*R*) of non-mycorrhizal (*white*), mycorrhizal (*light grey*) and non-mycorrhizal plus extra phosphorus supply (*dark grey*) *Catharanthus roseus* plants. Significant differences among the treatments at $P < 0.05$ were determined using a one-way analysis of variance (ANOVA) followed by a Tukey's test and are indicated by *different letters*; ± standard error, $n = 3$. Quantifications of the transcript levels of *TDC*, *STR*, *SGD*, *DAT* and *PRX1* genes were calculated in relation to *RpS9* reference gene transcript levels


L1
L3
R
A
B
C
L1 TDC Relative Transcrip levels
L3 TDC Relative Transcrip levels
Root TDC Relative Transcrip levels
D
E
F
L1 STR Relative Transcrip levels
L3 STR Relative Transcrip levels
Root STR Relative Transcrip levels
G
H
I
L1 SGD Relative Transcrip levels
L3 SGD Relative Transcrip levels
Root SGD Relative Transcrip levels
J
K
L1 PRX1 Relative Transcrip levels
L3 DAT Relative Transcrip levels
Not applicable
L
M
N
L1 PRX1 Relative Transcrip levels
L3 PRX1 Relative Transcrip levels
Root PRX Relative Transcrip levels
NM
M
NM+P

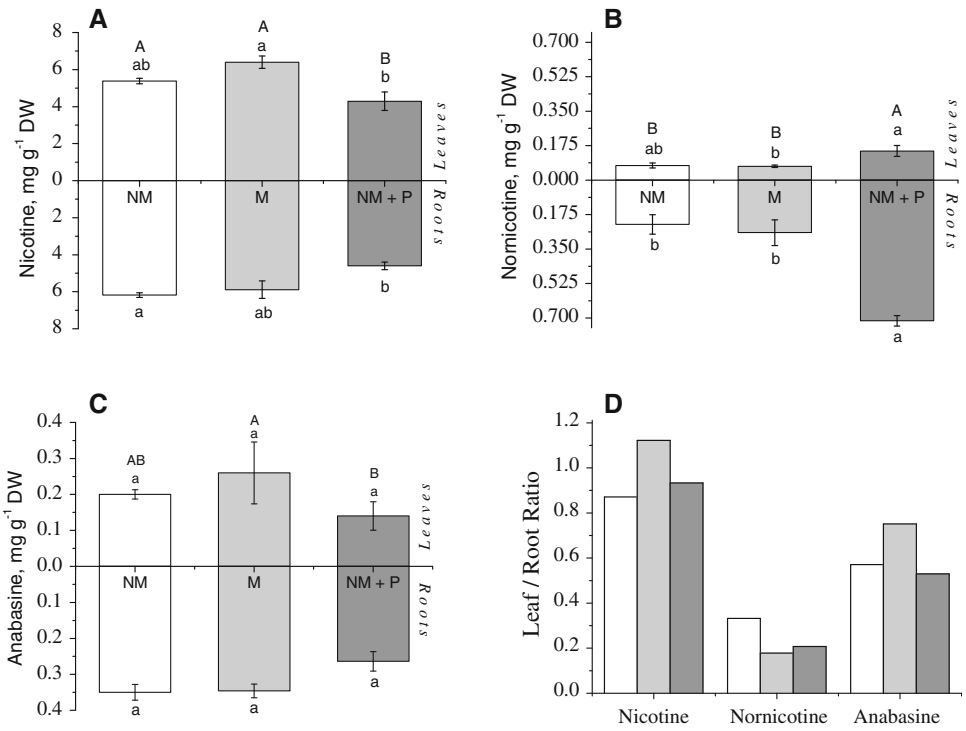


Fig. 4 Nicotine (**a**), nornicotine (**b**) and anabasine (**c**) contents in leaves and roots and their leaf to root ratio (**d**) in non-mycorrhizal (*white*), mycorrhizal (*light grey*) and non-mycorrhizal plus extra phosphorus supply (*dark grey*) *Nicotiana tabacum* plants. *Different letters* denote statistically significant differences among the treatments, as indicated by a Tukey's test ($P < 0.05$); ± standard error, $n = 3$

treatments. Higher S and lower Mn concentrations were observed in the leaves of the M plants when compared with plants from the non-mycorrhizal treatments (Table 2).

Nicotine, anabasine and nornicotine were analysed in the leaves and roots of the *N. tabacum* plants. Nicotine was the main alkaloid found, followed by anabasine and nornicotine, respectively (Fig. 4). The nicotine concentration in the leaves was circa 50 % higher in the M plants than in the NM + P plants (Fig. 4a). Foliar nicotine in the NM plants was lower than in the leaves of the M plants, even though this difference was not statistically significant. In the roots, nicotine concentration followed the same trend observed for the leaves (i.e., it was lower in the NM + P plants than in the M and NM plants, which showed similar contents) (Fig. 4a). The nornicotine concentration was lower in the leaves of the M plants, and its concentration in the roots was much higher in plants that received the extra P supply than in those without a P supply (Fig. 4b). Although no differences in anabasine concentrations were observed in the roots, the M plants had a higher total anabasine concentration in the leaves than the NM + P plants (Fig. 4c). The M plants exhibited higher leaf to root ratios of nicotine and anabasine content than the NM plants and, interestingly, had lower or similar leaf to root ratios of nornicotine than the NM or NM + P plants, respectively (Fig. 4d).

The relative transcript levels of *PMT* and *A622* were determined in the roots, while the leaves were used to determine the relative transcript levels of *CYP82E*. *PMT* was the gene with the highest expression level, which was observed in the roots of the M plants (Fig. 5a). The NM + P plants exhibited the lowest *PMT* and *A622* expression levels when compared with the other treatments studied (Fig. 5b), while the transcript level of *CYP82E4* in the leaves was similar in plants under the different treatments (Fig. 5c).

Discussion

This study demonstrates that AMF inoculation in *C. roseus* and *N. tabacum* plants influences both alkaloid synthesis and accumulation to a different extent, depending on the species or plant organ examined. Roots showed a higher influence of mycorrhizae on alkaloid accumulation, but alteration of specific alkaloids was also observed in the leaves. These results clearly show that AM association can lead to alterations of plant specialized metabolism by altering gene expression.

Mycorrhization of *C. roseus* and MIA alkaloids

Although the growth of the *C. roseus* plants inoculated with *G. etunicatum* was lower than the plants receiving an extra P supply, it was higher than the control plants that were not inoculated and did not receive extra P. Therefore,

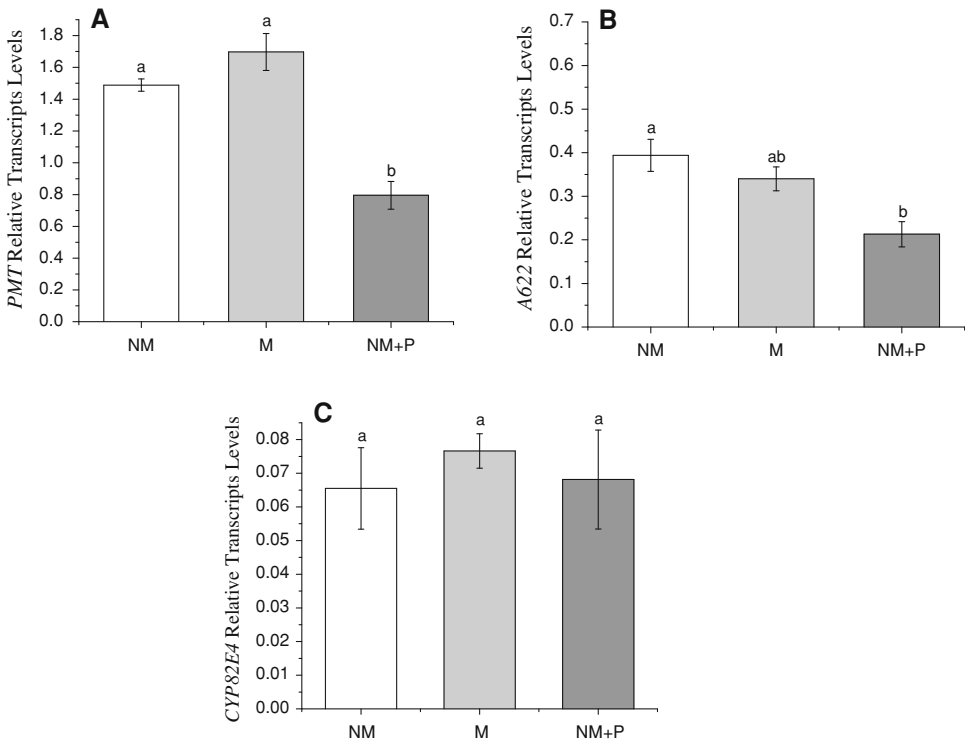


Fig. 5 Relative transcript levels of the tobacco alkaloids related-enzymes PMT (**a**), A622 (**b**) and CYP82E4 (**c**), in relation to elongation factor EF-1α transcript levels, in non-mycorrhizal (*white*), mycorrhizal (*light grey*) and non-mycorrhizal plus extra phosphorus supply (*dark grey*) *Nicotiana tabacum* plants. Significant differences among the treatments at $P < 0.05$ were determined using a one-way analysis of variance (ANOVA) followed by a Tukey's test and are indicated by *different letters*; ± standard error, $n = 3$

these results are in accord with other reports, which have also shown higher mass yields due to mycorrhization in this plant species (Ratti et al. 2010; Cartmill et al. 2008). In contrast, a non-significant effect of AMF inoculation on *C. roseus* growth has also been reported (De la Rosa-Mera et al. 2011; Cartmill et al. 2008). The percentage of the root length colonised by *G. etunicatum* was within the range already reported by others for *C. roseus* plants, which was 31 % on average (Karthikeyan et al. 2008; Ratti et al. 2010). Although there was a relatively low degree of root colonisation, *G. etunicatum* was an efficient symbiont, promoting plant growth when compared to the NM plants and corroborating the frequently observed lack of correlation between the extent of intraradical colonisation and mycorrhizal response (Gange and Ayres 1999). Despite such growth, mycorrhization did not enhance the foliar P concentration when compared with non-inoculated plants (NM and NM + P), suggesting that a P dilution effect could have occurred due to increased shoot growth (Marschner and Dell 1994).

In the present study, it was evident that the AM association of *C. roseus* plants influenced the MIA contents to a greater extent in the roots than in the leaves. Mycorrhization increased the concentrations of ajmalicine and serpentine in the roots. A possible explanation for these enhanced concentrations could be the influence of AM association on plant hormones, which interferes with different metabolic signalling cascades and leads to increased activity of the biosynthetic pathways of defence-related compounds. Changes in phytohormone balance induced by AMF have been suggested to increase terpenoid in plants (Copetta et al. 2006; Toussaint et al. 2007). Nevertheless, at the moment, there is no evidence that root alkaloid concentrations may regulate AMF root colonisation. However, it has been suggested that several specialized compounds produced by plants, such as essential oils, may be synthesised as a chemical defence against the presence of mycorrhizal fungi in the roots (Copetta et al. 2006). A similar role may be suggested for the ajmalicine and serpentine concentrations in mycorrhizal roots, as the increased concentrations of these alkaloids may play a role in controlling AMF colonisation in *C. roseus* roots.

Independent of the mycorrhizal treatment, we found that the youngest leaves (L1) of *C. roseus* had higher MIA concentrations than older leaves (L3). In the case of vincristine and vinblastine, the accumulation of 3,4-anhydrovinblastine, a precursor of these dimeric MIAs, was found to be much higher in the young, but completely developed leaves than in the youngest leaves that still did not have well-developed foliar tissues (Roepke et al. 2010). However, the results were presented as the amount of 3,4 anhydrovinblastine in the whole leaf (g/leaf); the opposite result may have emerged if the data were presented as a concentration (amount of alkaloid per leaf dry mass), as the youngest leaf was very small in size. Thus, the concentration in the older leaves would have been smaller than in the young leaves, corroborating our results. Therefore, it is expected that higher MIA concentrations should be found

in younger tissues and developing leaves (St-Pierre et al. 1999), where they may have a defensive and protective role against herbivorous predators or phytopathogens (Luijendijk et al. 1996).

An important goal of this study was to find the possible alterations that the mycorrhizal status of *C. roseus* plants might have on the expression levels of genes related to enzymes of the MIA biosynthetic pathway (see Fig. S1). The RT-qPCR analyses showed that the expression level of the determined genes was only positively correlated with the concentrations of the analysed alkaloids in the case of L1 leaves, with serpentine being an exception. In these leaves, *TDC*, *STR*, *SGD* and *PRX1* transcript levels paralleled the amounts of catharanthine, vindoline, vinblastine, vincristine and ajmalicine, where increased levels were found in the NM plants and lower expression levels were found in the M or NM + P plants. No special correlation between expression levels and the MIA content was observed in the L3 leaves or in the roots. For example, transcript levels of *STR*, a key enzyme involved in the formation of strictosidine, were lower in mycorrhizal roots and did not correspond to the higher contents of catharanthine, ajmalicine and serpentine observed.

PRX1, an alkaline class III peroxidase, was found to be involved in the dimerisation of vindoline and catharanthine to form 3,4-anhydrovinblastine in *C. roseus* leaves (Sottomayor et al. 2004). Vacuolar class III peroxidases were also suggested to mediate the conversion of ajmalicine to serpentine in cell suspensions of *C. roseus* (Blom et al. 1991; Costa et al. 2008). More recently, Costa et al. (2008) isolated and characterised a class III peroxidase gene from *C. roseus* leaves whose protein was located in the vacuole. This peroxidase was expressed predominantly in leaves and absent or expressed at very low levels in roots, as we observed here. However, the participation of this enzyme in the conversion of ajmaciline to serpentine has yet to be proven.

Independent of treatment, the catharantine content in the L1 leaves had a positive and direct correlation with the expression of *TDC*, *STR* and *SGD*, enzymes that are positioned upstream in the biosynthesis pathway. However, the same pattern was not observed in the L3 leaves or the roots. While vindoline and the dimeric MIAs are found exclusively in the leaves and stems of *C. roseus*, catharanthine can also be found in the roots (Balsevich and Bishop 1989). The lack of a correlation between catharanthine accumulation and the expression levels of *STR* has also been found in *C. roseus* hairy root cultures (Wang et al. 2010). Increased catharanthine contents in hairy roots were related to the over-expression of the key enzymes of the MEP pathway, which are involved in the route of secologanin biosynthesis (Wang et al. 2010), confirming that the iridoid pathway is probably the main limiting factor of MIA synthesis in *C. roseus* plants (Moreno et al. 1993).

SGD transcript levels have been shown to limit metabolite flux through the MIA biosynthesis pathway, causing the feeding of upstream metabolites to be ineffective in *ORCA3*-overexpressed *C. roseus* hairy root cultures (Peebles et al. 2009). Our results demonstrated that *SGD* expression levels and catharanthine or vindoline contents in the leaves or catharantine, ajmalicine or serpentine concentrations in the roots were also not correlated.

DAT and the earlier step gene *D4H* are mainly expressed in specific cells, such as the idioblasts and laticifers of *C. roseus* leaves, but not in roots (St-Pierre et al. 1999). In our experiments, *DAT* transcripts were found only in the leaves; additionally, expression levels increased with leaf age, likely because vindoline synthesis mainly occurs in well-developed leaves that have formed idioblasts and laticifers (Roepke et al. 2010) that will probably be defective in the L1 leaves. In the third youngest leaves (L3) of the M plants, *DAT* expression levels increased in relation to the non-mycorrhizal plants, which may be related to a probable higher number of idoblast or laticifers induced by mycorrhization. Mycorrhized *Ocimum basilicum* and *Oreganum vulgare* plants exhibited an increased number of peltate glandular hairs and trichomes, known to be sites of essential oil synthesis, and therefore accumulated a higher oil content than non-mycorrhizal plants (Copetta et al. 2006; Morone-Fortunato and Avato 2008). It would be interesting to investigate the influence of mycorrhization on idioblast and laticifer density in *C. roseus* plants to verify the possible higher expression of enzymes specifically located in these cells (St-Pierre et al. 1999).

The plant growth hormone, abscisic acid (ABA) enhanced ajmalicine contents in *C. roseus* cell suspension and promoted the pathway towards vindoline biosynthesis (Pan et al. 2010). Jasmonates, salicylic acid, auxins, ABA and cytokinins are all hormones that are known to be altered upon the establishment of AM (Hause et al. 2007; Pozo and Azcón-Aguilar 2007). Furthermore, some of these hormones have been shown to regulate the transcription levels of some MIA biosynthetic enzymes (Pasquali et al. 1992).

It is worth noting that in some cases, the specialized metabolite concentration is positively correlated with plant biomass production, eliminating any possible explanation based on concentration/dilution effects caused by different plant sizes (Koricheva 1999). However, the results must be carefully interpreted because variations in metabolite concentrations may represent differences in size among or within plants rather than in metabolite biosynthesis rates (Koricheva 1999). We may consider that this occurred in the young leaves of the NM plants, which grew less than the plants in the other treatments, accounting for their higher MIA content. The same might be true for the roots, particularly for serpentine and ajmalicine, because the roots of the NM + P plants exhibited increased growth.

The mismatches between the alkaloid levels and gene expression in different plant parts may be because very precise phenological and spatial regulation processes occur during alkaloid biosynthesis (Mahroug et al. 2007). Multicellular transportation of intermediates also occurs during the process of MIA assembly, further complicating the map of MIA biosynthesis and distribution (Burlat et al. 2004; St-Pierre et al. 1999). In addition, abiotic and biotic factors and the balance of phytohormones, are known to influence MIA biosynthesis and accumulation (El-Sayed and Verpoorte 2007). Post-transcriptional events or transcriptional regulators or repressors may also explain why a positive relationship between transcript abundance and its related protein activity was not found. Although the MIA pathway is very well studied, it constitutes one of the most complex cases of compartmentalised plant specialized metabolism (Ziegler and Facchini 2008). The results presented here corroborate the extraordinary complexity of alkaloid synthesis and accumulation; these processes become still more complex when considering plant interactions with mutualistic symbionts such as AMF, which certainly alter alkaloid contents in *C. roseus* plants.

Mycorrhization of *N. tabacum* and pyridine alkaloids

Mycorrhizal and non-mycorrhizal plants had similar levels of performance regarding nutritional status and mass accumulation; this allowed for an interpretation of the data on alkaloid biosynthesis and content that was not necessarily correlated with an increase in nutrient resources. Comparable shoot mineral concentrations in tobacco plants, whether mycorrhizal or not, were possibly due to the high availability of nutrients supplied along with the nutrient solution in this experiment. As in previous studies using *N. tabacum* and the same fungal species used here (Fester et al. 1999; Janouskova et al. 2007), the roots were highly colonised by *G. intraradices* and vesicles, arbuscules and abundant extra- and intraradical hyphae were observed. Thus, despite the high rate of colonisation, positive effects on growth and nutrient uptake were not observed. This "unresponsiveness" to AM inoculation may be related to a phosphorus/carbon imbalance between the symbionts, as the experimental condition of irrigation with a nutrient solution may have minimised the positive mycorrhizal response on plant growth (Smith et al. 2011).

Tobacco is a classic example of a plant that synthesises specialized metabolites in its roots, which are transported to the shoots where they will play a role in plant defence (Erb et al. 2008). In tobacco, the synthesis of the main PAs in roots and their concentration in the leaves may increase in response to different mechanical, chemical or biotic stressors (Baldwin et al. 1994; Shi et al. 2006; Steppuhn et al. 2004). We observed that mycorrhization increased nicotine and anabasine in the roots and shoots as compared to non-mycorrhizal plants, especially in the NM + P plants. Increases in nicotine synthesis and transport to the aerial parts of the plant have been related to the protective role of these alkaloids against herbivore attacks in the shoots (Baldwin and Schmelz 1996). However, attacks on the roots have also been observed to enhance nicotine production in *Nicotiana* species. For example, the infestation of tobacco roots with the nematode *Meloidogyne incognita* greatly increased foliar nicotine contents (Hanounik and Osborne 1977). However, to our knowledge, only a congress communication has related the increase of nicotine in tobacco to AMF root colonisation (Subhashini and Krishnamurty 1995), cited by (Abu-Zeyad et al. 1999). As cortical cells in the roots are the common site for tobacco alkaloid biosynthesis (Shoji et al. 2000) and intraradical AMF establishment (Smith and Read 2008), and considering that AM symbiosis is as ancient as the colonisation of land ecosystems by plants (Brundrett 2002), the interaction between plant alkaloid production and AM may represent an important but nevertheless overlooked factor in the evolution of plant defences (Kempel et al. 2010).

Although our main objective was to study the effect of AM association on tobacco alkaloid synthesis and accumulation, the extra P supply treatment unexpectedly had a significant effect on PA synthesis. Although plants exhibited similar foliar P concentrations, absolute P contents (P concentration × plant mass) were higher in the NM + P plants (data not shown). Reports on the effects of P availability on the alkaloid content are scarce in the literature, but a P deficiency has been shown to lead to a 20 % reduction of caffeine concentration in the leaves of coffee seedlings (Mazzafera 1999). Hol (2011) discussed the effects of nutrients on pyrrolizidine alkaloids in several *Senecio* species, and (Koricheva 1999) gave excellent insights into phenotypic variations and their relationship with phytochemical concentrations. Particularly in the specific case of P in *N. tabacum*, it is possible to infer that the extra P supply chiefly affected the accumulation of nornicotine.

The *A622* gene encodes an NADPH-dependent oxidoreductase, involved in both of the last steps of nicotine and anabasine formation (DeBoer et al. 2009). The lower expression levels of this enzyme in the NM + P plants may be correlated with the lower total content of anabasine found in these plants. In addition to the role of the A622 protein in anabasine and anabatine synthesis (Fig. S2), this enzyme promotes the condensation of 1-methyl-Δ^1-pyrrolinium with nicotinic acid to form nicotine (DeBoer et al. 2009) and is possibly part of the enzymatic complex named nicotine synthase (Friesen and Leete 1990).

In roots, *PMT* and *A622* transcript levels mirrored the trend observed for nicotine and anabasine, with the highest expression levels found in the M plant roots. However, the

marked increase of nornicotine in the roots of the NM + P plants was not followed by an increase in the transcript levels of *CYP82E4*, a gene expressed that mediates the conversion of nicotine to nornicotine (Fig. S2). In our experiment, *CYP82E4* expression was similar among the plants of different treatments. In Nicotiana species that accumulate nicotine, such as *N. tabacum*, *CYP82E4* transcript accumulation is negligible and occurs mainly in the leaves and during leaf senescence and air-curing (Chakrabarti et al. 2008; Lewis et al. 2010; Siminszky et al. 2005). Therefore, when compared with the expression levels of *PMT* and *A622*, *CYP82E4* was much less expressed (Fig. 3), and at such low expression levels, significant differences may be difficult to detect. However, the primers used here to assay the *CYP82E4* transcript levels were common to several other known members of the CPY82E gene's family [*CYP82E2*, *CYP82E4v2*, *CYP82E4v1*, *CYP82E2*, and *CYP82E3* (Chakrabarti et al. 2008; Gavilano et al. 2006; Gavilano and Siminszky 2007; Siminszky et al. 2005; Xu et al. 2007)], except for a cytosine/adenine substitution in *CYP82E10* (Lewis et al. 2010) for the forward primer. Therefore, able to recognise other members of *CYP82E* family and excluding the possibility that other gene was responsible for the conversion of nicotine to nornicotine. Alternatively, a second possible explanation for these results that was not investigated here would be a decreased conversion of anabasine to other alkaloids downstream in the metabolic route of PAs, such as myosmine and nicotyrine. Our results indicate that such a response may be the direct effect of P nutrition.

Conclusions

Mycorrhizal symbiosis has direct effects on the physiology of the whole plant, including changes in specialized metabolism in both above- and belowground organs. Our results support previous reports and, for the first time, show the influence of mycorrhization on a wide range of alkaloids in the leaves and roots of *C. roseus* and *N. tabacum* plants, as well as the gene expression of key biosynthetic enzymes of these alkaloids. Mycorrhization of these plants altered alkaloid synthesis and accumulation in the shoots, but especially in the roots. Besides genetic variation, environmental factors, pathogen and/or pest attacks, and associations with beneficial microorganisms seem to play an important role in defining the content and profile of specialized metabolites in plants. Further studies may show whether such interactions are affected by AMF association.

Author contributions S.A.L. Andrade performed the experiments and together with P. Mazzafera conceived, designed the experiments and wrote the paper, S. Malik helped in experiments and laboratorial analysis, A.C.H.F. Sawaya was responsible for UPLC-MS analysis of plant alkaloids and A. Bottcher designed primers for quantitative PCR. All authors read the final manuscript.

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