Mycorrhiza
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ORIGINAL ARTICLE

Rhizophagus irregularis as an elicitor of rosmarinic acid and antioxidant production by transformed roots of *Ocimum basilicum* in an in vitro co-culture system

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Abstract Arbuscular mycorrhiza is a symbiotic association formed between plant roots and soil borne fungi that alter and at times improve the production of secondary metabolites. Detailed information is available on mycorrhizal development and its influence on plants grown under various edapho-climatic conditions, however, very little is known about their influence on transformed roots that are rich reserves of secondary metabolites. This raises the question of how mycorrhizal colonization progresses in transformed roots grown in vitro and whether the mycorrhizal fungus presence influences the production of secondary metabolites. To fully understand mycorrhizal ontogenesis and its effect on root morphology, root biomass, total phenolics, rosmarinic acid, caffeic acid and antioxidant production under in vitro conditions, a co-culture was developed between three *Agrobacterium rhizogenes*-derived, elite-transformed root lines of *Ocimum basilicum* and *Rhizophagus irregularis*. We found that mycorrhizal ontogenesis in transformed roots was similar to mycorrhizal roots obtained from an in planta system. Mycorrhizal establishment was also found to be transformed root line-specific. Colonization of transformed roots increased the concentration of rosmarinic acid, caffeic acid and antioxidant production while no effect was observed on root morphological traits and biomass. Enhancement of total phenolics and rosmarinic acid in the three mycorrhizal transformed root lines was found to be transformed root line-specific and age dependent. We reveal the potential of *R. irregularis* as a biotic elicitor in vitro and propose its incorporation into commercial in vitro secondary metabolite production via transformed roots.

Keywords Antioxidants · Elicitor · Transformed roots · In vitro · *Ocimum basilicum* · *Rhizophagus irregularis* · Rosmarinic acid

Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with roots of plants and influence their primary and secondary metabolic pathways (Schliemann et al. 2008; Ceccarelli et al. 2010). Roots of species such as basil, tomato and clover grown in soil have been extensively studied for the influence of mycorrhizal colonization on their polyphenolic profile and content (Toussaint et al. 2007; Lopez-Raez et al. 2010; Zhang et al. 2013). Additionally, *Agrobacterium rhizogenes*-derived transformed (hairy) roots have also been examined for their polyphenolic content (Weremczuk-Jeżyna et al. 2013; Thiruvengadam et al. 2014; Srivastava et al. 2016a), however, no information is available on the effect of mycorrhizal colonization on the polyphenolic content of transformed roots.

Transformed roots have served as model systems in mycorrhizal research to examine the similarities and differences between natural and in vitro mycorrhizal ontogenesis, production of mycorrhizal propagules in axenic conditions, evaluations of host dependence, nutrient assimilation and

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genotyping studies (Declerck et al. 1996; Tiwari and Adholeya 2002; Toussaint et al. 2004; Kumar et al. 2013), however, only one study (Mrosk et al. 2009) has briefly examined the effect of mycorrhizal colonization on secondary metabolite production by transformed roots.

Roots of *Ocimum basilicum* (Sweet basil) are rich, alternative reserves of rosmarinic acid that have antioxidant, anti-inflammatory, anti-diabetic and neuroprotective-like biological properties (Srivastava et al. 2014, 2016b). The aerial parts of *O. basilicum* have been examined for the impact of mycorrhizal colonization on their essential oils, total phenolics, chicoric acid, and rosmarinic acid concentrations under greenhouse conditions (Copetta et al. 2006; Toussaint et al. 2007; Lee and Scagel 2009; Scagel and Lee 2012) however, their soil grown and transformed roots have been largely unexplored.

In our previous work (Srivastava et al. 2014, 2016a), we developed an in vitro growth system for the selection of elite (high rosmarinic acid producing) cultivars of *O. basilicum* followed by their *A. rhizogenes*-mediated transformation for the selection of three high rosmarinic acid producing transformed root lines. Here, we report development of a co-culture system in vitro between three transformed (selected) roots of *O. basilicum* and *Rhizophagus irregularis* for the characterization of mycorrhizal ontogenesis in transformed roots. The developed co-cultures (mycorrhizal transformed roots) and control (non-inoculated) transformed roots were used to investigate the effect of age on their morphological and biochemical traits. Comparisons between the control and mycorrhizal roots at specific ages were undertaken to investigate the potential of *R. irregularis* as an elicitor in in vitro. Furthermore, we determined the optimum age and co-culture system for enhanced production of rosmarinic acid.

Materials and methods

Establishment of the co-culture system in vitro

For the development of the co-culture system, three transformed root lines of *O. basilicum* selected from our earlier study (Hairy root (HR) line 2, HR 4 and HR 5; 20 days old, Srivastava et al. 2016a) were used as the plant host. A stock culture (CMCCROC3) of *R. irregularis* formerly known as *Glomus intraradices* was obtained from the Centre of Mycorrhizal Culture Collection (CMCC), TERI, India. For raising the co-culture system, spores were harvested from stock culture (75 days old; axenically developed co-culture of *R. irregularis* with transformed roots of *Daucus carota* var. 'Pusa Kesar' maintained on 100 ml of minimal [M] media in Jam jars at 26 °C) in 100 ml of sodium citrate buffer (Doner and Becard 1991) using a shaker (Kuhner Shaker, Basel, Switzerland) at 25 °C for 60 min at 100 rpm. The deionized buffer (without roots) was then sieved through a 325 British Standard (300 μm) Sieve (BSS) (Fritsch, Idar-Oberstein, Germany) and the spores retained on the sieve mesh were washed with sterile distilled water (twice). After washing, the spores were finally collected into 5 ml of sterile distilled water (all steps were performed under aseptic conditions). One hundred harvested spores per plate were inoculated onto the centre of Petri plates (90 mm in diameter) that contained 35 ml of M medium supplemented with 1 % phytagel (Puri and Adholeya 2013). Four root tips (approximately 2 cm in length) of the HR 2 (20 days old) were gently placed on the medium surface one cm away from the spores, the plates were then labeled, sealed and placed in the dark at 26 °C within an incubator (ET-650-8, Lovibond, Dortmund, Germany). Similar steps were repeated to raise a co-culture between *R. irregularis* and the other transformed root lines (HR 4 and 5). The inoculated plates were observed weekly to monitor the growth of the symbionts using a stereomicroscope (×10–40, SZ16, Olympus, Japan).

Assessment of mycorrhizal ontogenesis

Light microscopy, confocal laser scanning microscopy and scanning electron microscopy were utilized for detailed analysis and characterization of the ontogenetic stages of the in vitro mycorrhizal symbiosis. For each technique, three replicate co-cultures were established for each transformed root line for the assessment of ontogenesis (3 transformed root lines × 3 replicates per technique) as described below.

Light microscopy

Weekly observations of mycorrhizal developmental stages were made for up to 120 days after co-culture initiation under a stereomicroscope (×10–40, SZ16, Olympus, Japan) and digital images were captured.

Confocal laser scanning microscopy

For the laser-induced autofluorescence study of mycorrhizal structures, ten colonized root tips of each co-culture at 30, 60 and 90 days were mounted on a microscopic cover glass (22 × 40 mm, Blue Star, New Delhi, India) in lactoglycerol and used for imaging. Confocal laser scanning microscopy (CLSM) was performed using an Axio observer Z1 microscope (LSM-710, Carl Zeiss Microimaging GmbH, Jena, Germany). Inbuilt software Zen version 2010 was used to control the microscope, laser type, its intensity (2–3 %) and image acquisition. Ar 488 nm was used as the excitation wavelength (Dreyer et al. 2006).

Scanning electron microscopy (SEM)

Colonized root tips of HR 2, 4 and 5 at 30, 60 and 90 days were screened (for mycorrhizal colonization), selected and transversely sectioned into 0.5-cm long segments under a stereomicroscope. The segments were fixed in 2.5 % glutaraldehyde (Sigma, Bangalore, India) for 3 h and then washed three times in phosphate buffer (pH 7). After washing, the root sections were dehydrated through an ethanol series of 30, 40, 50, 60, 70, 80, and 90 % ethanol in water and finally 100 % ethanol for 20 min each. Dehydrated root sections were then subjected to critical point drying (CPD) using liquid CO_2 (EMITECH 6850, TABB, Birkshire, UK) at 500 to 1000 psi. After CPD, the root sections were mounted onto clean aluminium stubs using adhesive, conducting carbon tape (TABB, Birkshire, UK). The samples were coated with gold palladium for 90 s at 15 mA (SC7620, Sputter Coater, Quorum, Birkshire, UK) before examination with a SEM (EVO MA10, Carl Zeiss Microimaging GmbH, Jena, Germany). A voltage of 5 kV was maintained while imaging samples.

Assessment of root colonization and determination of number of spores

As described above, three replicate of co-cultures were established for each transformed root line for assessment of the percentage of the total root length that was colonized by *R. irregularis* and for spore counts. Colonized roots of HR 2, 4 and 5 (90 days) were harvested into 100 ml of 10 mM sodium citrate buffer (Doner and Becard 1991) to remove the phytagel. The harvested roots in buffer were sieved through a 300-μm filter (spores thereby collected were kept for spore number determination) and roots were collected in a Petri dish. Collected roots were washed in distilled water three times. Washed roots were cleared by placing them in a test tube containing 3 % potassium hydroxide (*w*/*v*) in water (Qualigens, Mumbai, India) at 65 °C for 10 min in a water bath (Heto HMT 200, Heto Lab Equipment, Allerod, Denmark). The cleared roots were then added to 15 ml of freshly prepared 1 % (*v*/*v*) hydrochloric acid (HCl) in water (Fischer scientific, Mumbai, India) for 5 min. After incubation, hydrochloric acid was decanted and roots were stained with 3 % (*v*/*v*) blue ink (PG Stationary Private Limited, New Delhi, India) in acetic acid solution (5 %, (*v*/*v*) acetic acid in water) overnight (Vierheilig et al. 1998). The following day excess stain was removed using 5 % (*v*/*v*) acetic acid in water (Qualigens, Mumbai, India) and specimens were then stored in lactoglycerol until observed. For the determination of percentage root length colonized by *R. irregularis*, ten root segments (1 cm) from each mycorrhizal co-culture were mounted per slide and observed with a compound microscope (CH, Olympus, Japan). Ten slides were prepared for each co-culture. A root segment was considered colonized if hyphae, arbuscules, and/or vesicles were observed. Root length colonization percentage was calculated from a frequency distribution chart as per the method developed by Biermann and Linderman (1981).

Spores of the 90-day-old co-cultures that were collected on sieves were washed with distilled water and finally collected in 100 ml of water in a beaker for spore number determination. To facilitate counting, the spore suspension was thoroughly mixed with a glass rod, and 1 ml of the water taken by a pipette was placed as ten drops on one half of a Petri dish. The number of spores in each drop was counted under a stereomicroscope using a tally counter (Right way, New Delhi, India). The abovementioned process was repeated five times for each replicate and the total number of spores present in 100 ml of water was calculated.

Establishment of mycorrhizal symbiosis in planta

A conventional in planta mycorrhization experiment was set up to compare the in vitro developed mycorrhizal symbiosis with natural mycorrhizal development. For this, the seeds of the B3 cultivar of *O. basilicum* were surface sterilized and set for seed germination as described by Srivastava et al. (2014). Germinated seedlings were transferred to black root trainers (1 L, Rajdeep Agri Products Private Limited, New Delhi, India) containing sterilized soil (750 g, pH = 7.8, *EC* = 312.4 μS, *N* = 0.75 % and Olsen's *P* = 8.46 mg/ml) and 100 spores of *R. irregularis* (obtained as described in establishment of the co-culture system above) were inoculated in close proximity to roots. The control (non-inoculated) and mycorrhizal plants with three replicates for each treatment (30, 60, 90 days) were grown in a greenhouse under natural light at 25–28 °C, 28.426 N (latitude) and 77.150 E (longitude). All plants from each treatment were harvested and roots were washed in water to remove the adhering soil particles. To compare the stages of mycorrhizal ontogenesis in planta with in vitro transformed mycorrhizal roots, the cleaned roots were used for ink vinegar staining (as described in the "Assessment of root colonization and determination of number of spores" section), confocal and scanning electron microscopy observations.

Examination of *R. irregularis* as a biotic elicitor in vitro

The effect of root age and colonization by *R. irregularis* on root morphology, root biomass, total phenolics, polyphenolics and antioxidant production of three transformed root lines (HR 2, 4 and 5) was examined to assess the potential of *R. irregularis* to act as a biotic elicitor. A square (3 cm^2) portion from the developed co-cultures of HR 2 (90 days old) that had mycorrhizal structures and six root tips (approximately 3 cm in length) were placed onto separate Petri plates (150 mm, Corning, USA; containing 135 ml of M media)

and incubated in the dark for three ages (30, 60 and 90 days) in three replicates for each at 26 °C. Similar steps were followed for raising the mycorrhizal roots of HR 4 and 5 also. The control (non-inoculated transformed roots) roots for each transformed root line also were raised simultaneously for three ages. Thus, in total, there were two treatments (control and mycorrhizal) for each transformed root line with harvesting done at three ages and three replicates were raised for each analysis described below (3 transformed roots × 2 treatments × 3 ages × 3 replicates).

Root morphological parameters and biomass

After 30, 60 and 90 days, the control and mycorrhizal roots were harvested into sodium citrate buffer to dissolve the adhering phytagel (Doner and Becard 1991). The freshly harvested control and mycorrhizal roots were then characterized for their morphological traits (length, diameter and number of root tips) using image analysis software (WinRHIZO® version Pro2007; Regent Instruments Inc, Quebec, Canada) and a flat bed scanner (EPSON Perfection V 700, Delhi, India). After characterization, the roots were subjected to lyophilization at −94.3 °C and 141 kPa for 48 h (Labconco lyophilizer, Kansas City, USA) and then their dry biomass was recorded.

Extraction and determination of the concentration of total phenolics and individual polyphenolics (rosmarinic acid and caffeic acid)

A method similar to that described by Srivastava et al. (2014, 2016a) was used for the extraction of total phenolics and polyphenolics from the control and mycorrhizal roots of HR 2, 4 and 5 after three time periods of co-culture. Briefly, 25 mg of lyophilized root from each sample was homogenized separately in 500 μl of 60 % (*v*/*v*) methanol (AR grade, Merck, Mumbai, India) in water. After homogenization 14.5 ml of 60 % methanol, was added and the mixture was sonicated for 10 min at 25 °C in a water bath sonicator (Branson Ultrasonics, Danbury, CT, USA). After sonication the extract was centrifuged for 5 min at 10,000 rpm and the supernatant was collected in a 25-ml volumetric flask. The residue was re-extracted in 10 ml of 60 % methanol for 5 min and then centrifuged as previously. The supernatants were then pooled and the final volume was made up to 25 ml with 60 % methanol. This extract was filtered (Millipore Millex HN, 0.45 μm, Merck, Darmstadt, Germany) into HPLC vials and was used for determining the concentration of total phenolics and individual polyphenolics.

A modified Folin's test was used for the estimation of total phenolics (Singleton and Rossi 1965; Srivastava et al. 2016a) and the concentration was expressed as gallic acid equivalents (mg/g GAE DW).

Chromatographic analysis of the root extracts was carried out using an HPLC system (Shimadzu, Kyoto, Japan) equipped with a controller (CBM—20A), quaternary pump (LC—20AT), solvent degasser system (DGU—20 A5), autosampler (SIL—20A) and diode array detector (SPDM—20A). Inbuilt software (Shimadzu, LC solution) was used to control the HPLC pump and acquire the data from the photodiode array. Separations were performed with a C18 Phenomenex column (Gemini-NX 250 mm × 4.6 mm × 5 μm). For the separation of rosmarinic acid and caffeic acid, HPLC grade water + 0.1 % (v/v) *ortho* phosphoric acid (OPA) in water (Mobile phase A) and Methanol (HPLC grade, Merck, Mumbai, India) + 0.1 % OPA (*v*/*v*) in methanol (Mobile phase B) were used in a gradient program similar to that of Srivastava et al. (2014, 2016a, b). The flow rate of the mobile phase was 1.0 ml/min and the wavelength used for detection was 280 nm with an injection volume of 20 μl. Unknown samples were identified by comparison with standard retention times and a standard curve over a concentration range from 20–100 mg/l was used for determining the concentration of rosmarinic and caffeic acids (Sigma, Bangalore, India) in the extracts.

Acidic potassium permanganate-based chemiluminescence assay for the assessment of total and individual compound antioxidant potential

Twenty-five milligrams of roots taken from 60-day-old control and mycorrhizal roots of HR 2, 4 and 5 were used for antioxidant determination in extracts. The methodology used for the extraction and estimation of total and individual compound antioxidant potential in control and mycorrhizal roots followed that described in Srivastava et al. (2014, 2016b). Briefly, for the determination of total antioxidant potential, an HPLC system (Agilent 1200) without column, Minipuls 3 peristaltic pump, bridged PVC tubing, and custom built luminometer (which functions similarly to a conventional flow injection analysis system) was used. Root extracts (50 μl) of the control and mycorrhizal transformed root lines were diluted 100 times using deionized water before injection and merged with 1×10^{-3} M of acidic potassium permanganate. The peak area was recorded after the interaction between the analyte and acidic potassium permanganate to calculate the total antioxidant potential.

For the determination of antioxidant potential of the individual polyphenolics (rosmarinic acid and caffeic acid) present in the root extracts, a post-column acidic potassium permanganate chemiluminescence assay was used as described in detail in Srivastava et al. (2014). The chemiluminescence response generated was detected by the in-house built manifold (Mc Dermott et al. 2011). For comparison, the standards of rosmarinic acid and caffeic acid (1×10^{-3} to 1×10^{-12} M) were prepared in 100 % analytical grade methanol and appropriately diluted with the same solvent.

Statistical analysis

All statistical tests, unless specified, were performed with SPSS Statistics V 22.0 (IBM, New York, USA). Prior to any statistical analyses, all data sets were tested for normality using the Shapiro–Wilk's test and homogeneity of variance by Levene's test. Transformations were performed to meet the homogeneity of variance requirement (arcsine square root for percentage colonization; log for root morphological traits, root biomass, total phenolics, polyphenolics and antioxidants). All data are presented in the form of mean ± standard deviation (SD).

One-way analysis of variance was used to determine the effect of mycorrhizal transformed root line on the colonization percentage and spore count after 90 days using Tukey's HSD (Honestly Significant Difference) at $p \leq 0.05$.

All data obtained on the parameters examined for the control and mycorrhizal roots of each transformed root line for three time periods that had homoscedastic variances were compared using one-way ANOVA with Tukey's HSD while data showing heteroscedasticity were analyzed by Games Howell's test. Pairwise comparisons were made to identify the differences between control and mycorrhizal roots at each age separately using multiple *t* tests based on Holm's Sidak method (Prism 6.02, GraphPad, LaJolla, USA).

A two-way ANOVA was performed to study the effect of mycorrhizal transformed root lines (three levels: HR 2, 4 and 5) and ages (three levels: 30, 60 90 days) and their interaction on total phenolics, rosmarinic acid and caffeic acid. Prior to performing the two-way ANOVA, homogeneity of variances was checked using Levene's test. Pairwise comparisons between the three mycorrhizal transformed root lines and three ages were performed after application of Bonferroni correction ($p = 0.017$). A Ryan-Einot-Gabriel Welsch Range (R-E-G-W-Q) test (for similar sample sizes and good control of Type I error) was used as the post hoc test.

Results

Co-culture development and analysis of mycorrhizal ontogenesis

Mycorrhizal symbiosis development in all transformed roots progressed through presymbiotic (spore germination, hyphal elongation and root epidermal cell invasion), symbiotic (arbuscule and vesicle development) and post-symbiotic (extra-radical sporulation) stages (Fig. 1). Spore germination, hyphal spread and epidermal cell colonization were found to occur after 10–20 days of co-culture initiation in all the transformed root lines (Fig. 1a, b, c). Following root colonization, the highest density of hyphal spread was observed in HR 5 in comparison to HR 2 and 4 (Fig. 1d). After 40 days, the extra-radical sporulation began for all transformed root lines and was found to be highest in HR 2 at 90 days (HR 2; 2567 ± 280a > HR 4; 2145 ± 295ab > HR 5; 1686 ± 190b). Intra-radical spores also were observed in all colonized transformed root lines (data not shown). Terminal/intercalary branching patterns with globose/sub-globose/elliptical, hyaline (juvenile) to brownish (mature) colored terminal spores in loose and aggregated form were observed with the fully colonized roots of HR 2, 4 and 5 (Fig. 1m).

To elucidate the mycorrhizal structures formed during ontogenesis in greater detail, the developed co-cultures were subjected to CLSM and SEM. Interestingly, all mycorrhizal structures such as hyphae, active and collapsed arbuscules, vesicles, intra- and extra-radical hyphae and extra-radical spores showed equivalent autofluorescence on excitation with the Ar laser (excitation wavelength 488 nm) in all the mycorrhizal transformed roots (Fig. 1b, e, f, g, j, k, n, q). Autofluorescence of a *tube-like* structure at the interface of hyphae and a transformed root indicated entry point (Fig. 1b). Both inter- and intra-cellular hyphal progression was observed in transformed roots through SEM (Fig. 1c). After root colonization, intricately branched tree-like structures *arbuscules* having cylindrical/flared trunk and characteristic dichotomous branching were observed inside the cortical cells (Fig. 1h). Fully developed arbuscules (size, 40–50 μm) were found to occupy most of the cortical cell lumen. Reductions in the hyphal width from the trunk to very fine branches of arbuscules also were observed. *Arum* types of arbuscules were observed in all mycorrhizal transformed root lines. In HR 4, collapsed arbuscules characterized by septation in the adjoining hyphae and small (21.31 μm) size in comparison to active arbuscules also showed autofluorescence (Fig. 1f). Concurrent to the arbuscule development, intercalary and terminal hyphal swellings, *vesicles*, on the intra-radical hypha within the cortical cells also were observed (Fig. 1i, j, k, l). Vesicles were observed to be double layered, elliptical in shape and filled with globules (lipid) which also showed autofluorescence (Fig. 1j). Interestingly, anastomoses were observed in mycorrhizal cultures of HR 5 (Fig. 1p, q; both extra-radically and intra-radically). Further, distinct autofluorescence was observed in the spore wall and lipid globules within the extra-radical spores of *R. irregularis* (Fig. 1n). Highest root length colonization percentage was found in HR 2 (31.89 ± 2.11a %) followed by HR 5 and 4 (17.33 ± 1.29b, 18.75 ± 0.98b % respectively).

Mycorrhizal ontogenesis in the in planta system

Similar to the observations made with the co-culture system developed in vitro, mycorrhizal structures such as intra-radical hyphae, arbuscules, vesicles and extra-radical spores also were observed in in planta (Fig. S1a) derived mycorrhizal roots (results of ink vinegar staining not shown). Arbuscules formed in the mycorrhizal roots obtained from the in planta

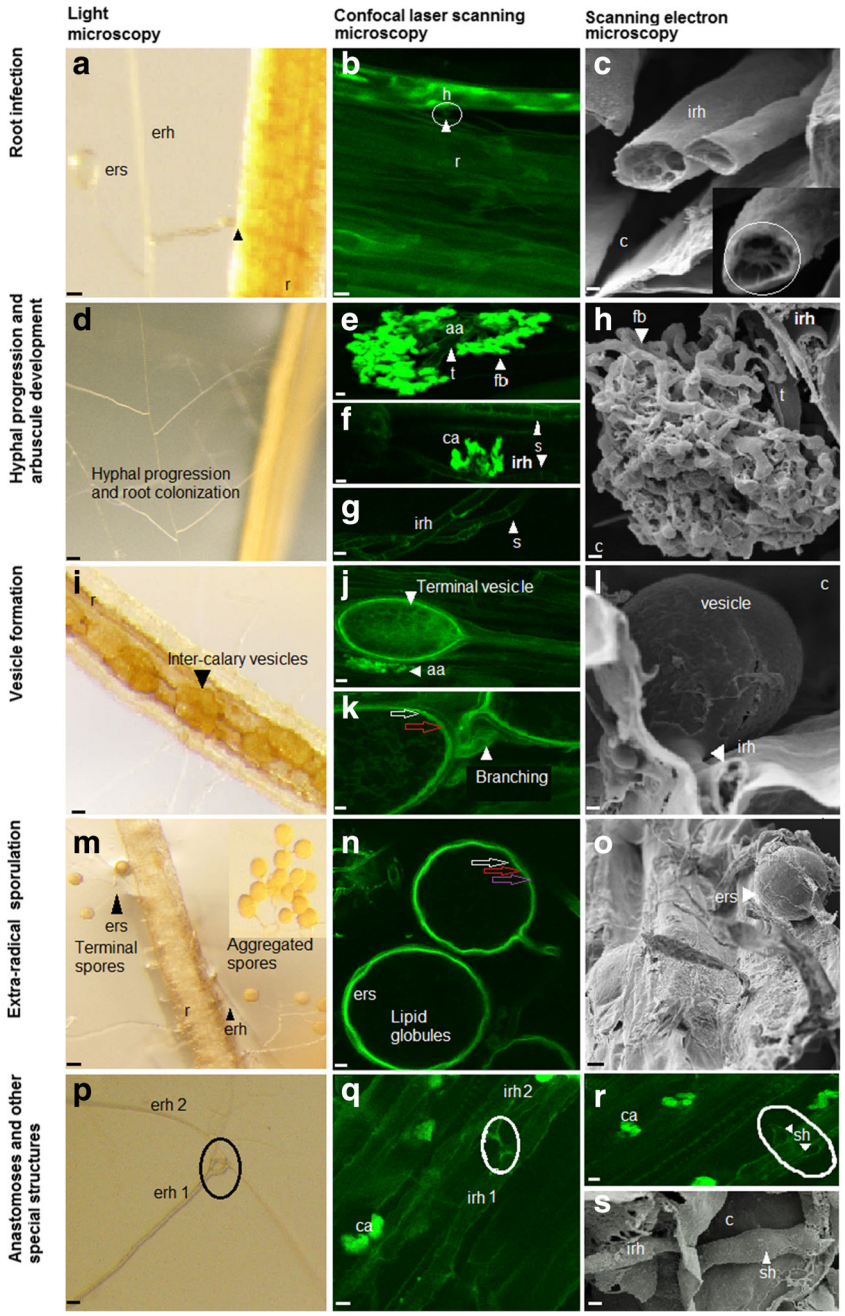

Fig. 1 Imaging of mycorrhizal (*R. irregularis*) ontogeneis in the in vitro co-culture of transformed root lines (HR 2, 4 and 5) by basic and advanced microscopic techniques. **a**–**b** Entry point characterized by the contact between the root (*r*) and extra-radical hyphae (*erh*); *black* (**a**) and *white* (**b**) *arrows* indicate entry point (**a**) and "tube-like" structure (**b**). **c** Hyphae infecting cortical cell (**c**); cylindrical hyphae with spoke-in-the-wheel-like structure (highlighted by *white circle*). **d** Hyphal spread after root colonization. **e**–**g** Arbuscule development; autofluorescing active arbuscules (*aa*; size: 43.60 μm) with flared trunk (*t*) and characteristic dichotomous branching (shown by *arrow*) and fine branches (*fb*); collapsed arbuscule (*ca*; size: 21.31 μm) with adjoining septate (*s*) hyphae (*irh*); mature intra-radical hyphae (*irh*) showing septa (*s*) formation. **h** A mature arbuscule (size: 45.60 μm) in the cortical (*c*) cell with a cylindrical trunk which divides into numerous fine branches (*fb*). **i**–**l** Types of vesicles; intercalary (**i**) and terminal vesicles (**j**; **l**); space within vesicle and double layers (*white* and *red arrows*) of vesicle also showed autofluorescence (**j**; **k**). **m**–**o** Globose type of extra-radical spores (*ers*); lipid globules and three wall layers (*white*: inner layer, *red*: middle layer, *purple*: outer layer) of ers showed autofluorescence (size of ers: 103.30 and 91.15 μm) **p**–**q** Anastomoses in the extra-radical (erh 1 and 2) and intra-radical hyphae (irh 1 and 2); circular (**p**); bridge (**q**) type. **r**–**s** intra-radical hyphae traversing between cells (*irh*) showing characteristic swelling (*sh*). Scale bar of **a**, **d**, **i**, **m**, **p** = 100 μm; **b**, **e**, **f**, **g**, **q**, **r**, **s** = 10 μm; **j**, **k**, **n**, **o** = 20 μm; **c**, **h**, **l** = 2 μm

system also exhibited autofluorescence and showed typical tree-like structures (Fig. S1b). Fully developed and highly branched arbuscules were also observed (Fig. S1c).

R. irregularis as a biotic elicitor in in vitro

Root morphological traits and biomass

A consistent increase in the root length, diameter and number of tips and root biomass was observed with increase in age for all control and mycorrhizal roots (Table 1, Table S1). Colonization with *R. irregularis* had no effect on root morphological traits and biomass of HR 4 as no significant difference was found between its control and mycorrhizal roots. Similar to HR 4, HR 2 and 5 also showed no difference between the control and mycorrhizal roots for all traits except root length (HR 2) and number of tips (HR 5) at 60 days.

Total phenolics

Total phenolics concentration increased with age (from 30 to 90 days) in both the control and mycorrhizal roots of all transformed root lines (Table 2a, Table S1). The highest total phenolic content was found in the 90-day old control and mycorrhizal roots. Colonization with *R. irregularis* increased the total phenolic content of HR 2 and 5 after 60 days ($p \leq 0.05$) while no difference was found in HR 4 at any age.

Polyphenolics (rosmarinic acid and caffeic acid)

Rosmarinic acid was detected as the main polyphenolic in our study (Fig. S2). The concentration of rosmarinic acid increased with age in both the control and mycorrhizal roots of all transformed root lines (Table 2b, Table S1). Mycorrhizal colonization increased rosmarinic acid concentration in all transformed root lines at all ages ($p \leq 0.05$) except for 30-day-old samples of HR 2. The increase in the concentration of rosmarinic acid after colonization with *R. irregularis* was found to be differently associated with age in the three transformed root lines. In HR 2, the concentration of rosmarinic acid was 1.9- and 1.5-fold higher in mycorrhizal roots after 60 and 90 days of co-culture respectively. In contrast to HR 2, the highest increase in rosmarinic acid concentration of 2.1-fold was found in HR 4 after 30 days of co-culture which then

Table 1 Effect of *R. irregularis* on the root morphological traits and root biomass of three transformed (HR) root lines of *O. basilicum* at three ages of co-culture

Transformed root lines						
	HR 2		HR 4		HR 5	
Days after subculturing (d)	Control roots	Mycorrhizal roots	Control roots	Mycorrhizal roots	Control roots	Mycorrhizal roots
(a) Root length (cm)						
30	1771.78 ± 175.78b	2334.34 ± 436.24a	1180.55 ± 233.61c	1323.23 ± 86.05b	598.12 ± 19.78c	610.63 ± 11.35c
60	*2266.12 ± 96.17ab*	*3204.44 ± 49.70a**	4012.54 ± 261.14b	4173.69 ± 264.01ab	2055.19 ± 62.91b	2261.17 ± 195.26b
90	2952.44 ± 225.20a	3525.74 ± 448.17a	4694.98 ± 120.15a	5231.31 ± 488.13a	3029.32 ± 111.47a	3183.10 ± 146.71a
(b) Number of root tips						
30	2786.67 ± 334.89c	3177.67 ± 443.00c	2044.67 ± 1118.80b	2246.00 ± 115.33b	934.00 ± 60.92b	962.33 ± 82.50c
60	4341.33 ± 229.24b	5119.67 ± 629.79b	8423.67 ± 682.95a	9523.00 ± 711.01a	*2923.00 ± 39.23a*	*3704.67 ± 151.11b**
90	8639.00 ± 2077.15a	10762.00 ± 2344.18a	10410.00 ± 1513.64a	9678.33 ± 968.18a	4232.67 ± 502.01a	4354.67 ± 117.89a
(c) Root diameter (mm)						
30	0.38 ± 0.01a	0.46 ± 0.04a	0.28 ± 0.02b	0.31 ± 0.01b	0.31 ± 0.01a	0.33 ± 0.02a
60	0.42 ± 0.03a	0.45 ± 0.03a	0.34 ± 0.01a	0.37 ± 0.01a	0.31 ± 0.01a	0.32 ± 0.00a
90	0.45 ± 0.04a	0.42 ± 0.02a	0.35 ± 0.00a	0.34 ± 0.01b	0.32 ± 0.01a	0.31 ± 0.00a
(d) Root biomass (mg)						
30	245.33 ± 52.87b	267.67 ± 58.09c	246.90 ± 52.87b	285.33 ± 58.08c	95.10 ± 24.90c	112.00 ± 19.02c
60	347.57 ± 31.99ab	427.03 ± 62.13b	441.03 ± 31.99b	487.77 ± 62.12b	182.00 ± 20.24b	188.00 ± 11.72b
90	554.67 ± 32.11a	575.63 ± 63.56a	594.70 ± 32.10a	621.60 ± 63.56a	261.13 ± 45.01a	282.47 ± 31.37a

Values represent the mean ± SD of three replicates. Values followed by the same letter indicate no significant difference between the three ages separately for control and for mycorrhizal root samples of HR 2, 4 and 5 according to Tukey's HSD ($p \leq 0.05$) for root diameter and root biomass and Games Howell's test; ($p \leq 0.05$) for root length and number of root tips. Values in italics and "* " shows significant differences between control and mycorrrhizal roots at $p \leq 0.05$ by *t* tests based on Holm Sidak's method

Table 2 Effect of *R. irregularis* on the concentration of total phenolics, rosmarinic acid and caffeic acid of three transformed (HR) root lines of *O. basilicum* at three ages of co-culture

Transformed root lines						
	HR 2		HR 4		HR 5	
Days after subculturing (days)	Control roots	Mycorrhizal roots	Control roots	Mycorrhizal roots	Control roots	Mycorrhizal roots
(a) Total phenolics (mg/g GAE DW)						
30	250.33 ± 42.09c	340.00 ± 39.69a	260.17 ± 9.12b	267.83 ± 2.31ab	101.10 ± 12.00c	107.14 ± 10.86c
60	*336.33 ± 8.81b*	*385.50 ± 17.84a**	308.87 ± 11.84ab	327.57 ± 13.90ab	*150.63 ± 7.04b*	*194.00 ± 5.41b**
90	403.17 ± 51.03a	425.00 ± 37.00a	354.83 ± 19.94a	417.33 ± 54.42a	283.17 ± 20.79a	343.58 ± 43.20a
(b) Rosmarinic acid (mg/g DW)						
30	31.45 ± 2.47b	32.42 ± 2.62c	*18.19 ± 5.91b*	*37.54 ± 8.84b**	*14.47 ± 2.59c*	*22.88 ± 2.29c**
60	*37.54 ± 1.82ab*	*57.20 ± 10.45b**	*81.82 ± 18.21ab*	*127.87 ± 2.05a**	*35.82 ± 5.29b*	*55.60 ± 7.87b**
90	*50.98 ± 5.58a*	*96.52 ± 18.54a**	*98.66 ± 4.77a*	*140.53 ± 5.26a**	*65.52 ± 6.89a*	*94.97 ± 13.05a**
(c) Caffeic acid (mg/g DW)						
30	*0.35 ± 0.10c*	*2.36 ± 0.13a**	*0.58 ± 0.04ab*	*2.21 ± 0.70a**	*0.32 ± 0.04b*	*0.43 ± 0.03b**
60	*0.75 ± 0.07b*	*1.78 ± 0.50a**	*1.57 ± 0.05a*	*2.46 ± 0.42a**	*0.48 ± 0.03ab*	*0.63 ± 0.07ab**
90	1.68 ± 0.17a	2.04 ± 0.17a	*0.62 ± 0.07ab*	*1.33 ± 0.41a**	*0.56 ± 0.02a*	*0.67 ± 0.06a**

Values represent the mean ± SD of three replicates. Values followed by the same letter indicate no significant difference between the three ages separately for control and for mycorrhizal root samples of HR 2, 4 and 5 according to Games Howell's test ($p \leq 0.05$) except for control and mycorrhizal roots of HR 4 and 5 for caffeic acid (analyzed by Tukey's HSD). Values in italics and "* " shows significant difference between control and mycorrrhizal roots at $p \leq 0.05$ by *t* test based on Holm Sidak's method. *GAE* Gallic acid equivalents, *DW* Dry weight

decreased with age to 1.4 fold at 90 days old. Similar to HR 4, HR 5 also showed the highest (1.6-fold) increase in rosmarinic acid concentration at the earliest age (30 days).

The concentration of caffeic acid increased in the control roots (HR 2 and 5) and mycorrhizal roots (HR 5) with age (Table 2c, Table S1). Mycorrhizal colonization enhanced the concentration of caffeic acid in all except the 90-day co-culture of HR 2. The highest increase in the concentration of caffeic acid was found in the 30-day-old roots of HR 2 (6.7-fold) followed by HR 4 (3.3-fold).

Total and individual polyphenolic antioxidant potential

An increase in the total antioxidant potential was found in the transformed root lines after colonization with *R. irregularis* (Fig. 2a). Four-, two- and five-fold higher levels of total antioxidant potential were observed in the mycorrhizal roots of HR 2, 4 and 5, respectively versus their controls. No difference in the chemiluminescence profile of control and mycorrhizal roots indicated that colonization did not alter the antioxidant profile (Fig. 2b). Rosmarinic acid (14.5 min) was detected as the major antioxidant peak in the extracts of both control and mycorrhizal roots (Fig. 2b). For caffeic acid, a very small chemiluminescence signal was observed (10.08 min). The slight difference in retention time of the same sample to that reported in the quantification of polyphenolic section (RA; 15.28 and CA; 12.08 min respectively) was due to use of different columns and HPLC systems. Similar to the total antioxidant potential, higher levels of antioxidant potential of rosmarinic and caffeic acids were detected in mycorrhizal roots. Five-, four- and 12-fold higher antioxidant potentials of rosmarinic acid were observed in HR 2, 4 and 5 respectively (Fig. 2c). The highest concentrations of rosmarinic acid (329.17 and 261.84 mM/100 g DW) were found in HR 5 and 2, respectively. Similarly, for caffeic acid an approximate two-fold increase above controls was obtained for mycorrhizal roots of HR 2, 4 and 5 (Fig. 2d).

Selection of age and mycorrhizal transformed root line for enhanced production of rosmarinic acid

Ninety days and mycorrhizal roots of HR 4 were found as the optimal age and mycorrhizal root combination for significantly ($p \leq 0.01$) enhancing rosmarinic acid production in vitro. Mycorrhizal colonization and age together were found to affect rosmarinic acid, caffeic acid and total phenolics production in transformed root lines (Table 3). No effect of age was found on caffeic acid concentration in any transformed root line ($p = 0.144$). Thus, HR 4—*R. irregularis* association is the optimum co-culture for enhanced production of rosmarinic acid.

Discussion

We have found that colonization of the three transformed root lines of *O. basilicum* with *R. irregularis* in vitro (i) forms a

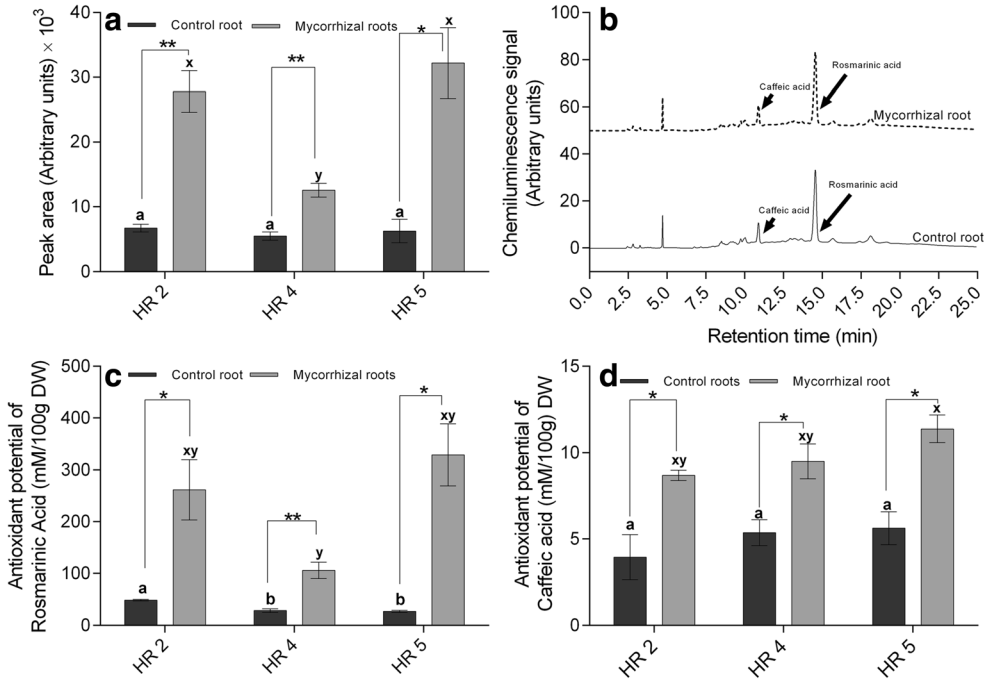

Fig. 2 Effect of *R. irregularis* on the total and individual antioxidant potential of three transformed root lines (HR 2, 4 and 5) of *O. basilicum* after 60 days of co-culture. Comparison between control and mycorrhizal roots **a** total antioxidant potential, **b** chemiluminescence profile, **c** and **d** antioxidant potential of rosmarinic acid and caffeic acid. Data represented as mean ± SD of three replicates. *Bars topped by the same letter* indicate no significant difference between HR 2, 4 and 5 for their control (**a**, **b**) and mycorrhizal roots (*x*, *y*) according to Games Howell's test ($p \leq 0.05$) except for control samples of rosmarinic acid and caffeic acid (analyzed using Tukey's HSD; $p \leq 0.05$). * and ** shows significant difference between control and mycorrrhizal roots at $p \leq 0.01$ and $p \leq 0.001$ by *t* tests based on Holm Sidak's method

successful symbiosis that is transformed root line-specific, (ii) undergoes all the ontogenetic stages in a manner similar to those that occur during natural mycorrhiza development, (iii) shows that mycorrhization has no effect on root morphological traits in vitro, (iv) reveals that mycorrhizal transformed roots produce both polyphenolics (rosmarinic acid and caffeic acid) and antioxidants to a greater level than non-mycorrhizal transformed roots and finally (v) demonstrates that enhancement potential is root line-specific and age dependent.

The extent of mycorrhizal colonization was found to be transformed root line-specific under the in vitro conditions because significant differences in colonization percentage were found among the three transformed root lines obtained from three different cultivars of *O. basilicum*. We also found, as reported by Mrosk et al. (2009), with transformed roots of *Medicago truncatula*, that the stages of mycorrhizal ontogenesis in transformed roots grown under in vitro conditions were similar to those found for naturally developed mycorrhiza. A combination approach was used in our study that involved both basic and advanced microscopy for detailed analysis of the mycorrhizal structures formed in in vitro. CLSM was found to be a very useful technique in comparison to light microscopy (LM) and SEM for detailed structural analysis as minimal sample preparation was required and fungal structures autofluoresced brightly, enabling them to be analyzed at high resolution. We suggest the utilization of CLSM for future in vitro-based mycorrhizal research.

Autofluorescence was exploited in the in vitro developed mycorrhizal roots and equivalent autofluorescence intensities

Table 3 Effect of age, mycorrhizal transformed root line and their interaction on biochemical traits

Factors and their interaction	Biochemical traits								
	Total phenolics			Rosmarinic acid			Caffeic acid		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Age of mycorrhizal transformed root line	59.83	2	<0.0001	162.45	2	<0.0001	2.16	2	0.144
Mycorrhizal transformed root line	72.95	2	<0.0001	59.60	2	<0.0001	49.85	2	<0.0001
Age of co-culture × mycorrhizal transformed root line	4.94	4	0.0001	10.81	4	<0.01	4.30	4	0.01

F variance of the group means, *df* degrees of freedom and *P* significance value

were detected in all mycorrhizal structures similar to that reported for sections of whole roots of palm (Dreyer et al. 2006). Due to the in vitro origin of the transformed roots used in our study, no masking of autofluorescence of hyphae and vesicles was observed as reported for hardened in vivo plant roots (Ames et al. 1982; Gange et al. 1999). In addition to the autofluorescence of fungal structures, there was some autofluorescence detected in the cell wall of the control transformed roots, presumably because of the presence of phenolics in the cell wall (Dreyer et al. 2006). Arbuscules were found to grow to maximum size in the cortical cell and then degrade following formation of septa in the hyphal attachment as discussed by others (Javot et al. 2007). The presence of both active and collapsed arbuscules in the in vitro mycorrhizal roots developed in our study further confirms its similarity with mycorrhizal development in in planta. Thus, we propose CLSM as a very useful technique for the evaluation of the transient nature of arbuscules. Furthermore, it also may be used for live imaging of arbuscule development.

SEM has been used regularly for mycorrhizal research especially with roots of higher plants and liverworts (Glenn et al. 1985; Russell and Bulman 2005; Duckett et al. 2006) but its application to in vitro mycorrhizal roots has, to date, been limited to the characterization of root hairs, vesicles, or intra-radical sporulation stages only (Tiwari and Adholeya 2003; Puri and Adholeya 2013). Herein we report, for the first time, utilization of SEM for studying all mycorrhizal structures formed in in vitro. We propose future utilization of this technique to enable a greater, detailed understanding of mycorrhiza development. Similar to the results obtained with CLSM, SEM also supported the conclusion that mycorrhizal ontogenesis in the non-transformed (derived from an in planta system) and transformed roots is alike.

In the present study, *R. irregularis* was selected for co-culture development and polyphenolic enhancement studies because of its model organism status and high multiplication rate in in vitro (Ceballos et al. 2013). Studies conducted on the effect of mycorrhizal interactions on growth parameters in pot- and/or field-based experiments usually assess the consequences of colonization after a set interval of time (for example: 16 weeks, Scagel and Lee 2012; Lee and Scagel 2009, 5 weeks, Toussaint et al. 2008) thereby underestimating the importance of quantifying the effect with respect to time. The approach used in our study shows the importance of growth related studies as mycorrhizal colonization was observed to affect total phenolics and rosmarinic acid in an age-dependent manner in three different transformed root lines of *O. basilicum*.

Mycorrhizal colonization is known to affect rosmarinic acid production in basil and sage when grown in soil under greenhouse conditions (Toussaint et al. 2007, 2008; Lee and Scagel 2009; Nell et al. 2009; Scagel and Lee 2012). The aforementioned studies are quite different from our report in terms of experimental design, system, plant material and aim, however, similar to the observations made by Nell et al. (2009) our study also reports that mycorrhizal colonization increases rosmarinic acid production in roots. Our study confirms the enhancement potential of *R. irregularis* for rosmarinic acid, caffeic acid and antioxidants in vitro. The increase in the content of polyphenols and other secondary metabolites after mycorrhization is likely the result of the influence of AMF on phytohormone production, stimulation of signaling cascades and ultimately those biosynthetic pathways that lead to the increased production of defense-related compounds (Zhang et al. 2013; Andrade et al. 2013; Zubek et al. 2015). The increase in total phenolics in transformed roots after colonization with *R. irregularis* can be correlated with Krebs cycle activation and plastid metabolism that increase the level of endogenous fatty acids, carotenoids and amino acids such as tyrosine that are parent molecules of phenolics (Lohse et al. 2005). Because phenolics have important medicinal activities, we propose that *R. irregularis* can be used for enhancing their production in transformed roots.

Antioxidants derived from plants are excellent alternatives to those derived chemically (Francis et al. 2010; McDermott et al. 2011). For the high level and standardized production of antioxidants, transformed roots have been recognized as an excellent source (Thiruvengadam et al. 2014; Srivastava et al. 2016a). Through our study, we have shown applicability of mycorrhiza to further enhance the production of antioxidants in transformed roots. Colonized transformed root lines had greater levels of antioxidants than control roots. Rosmarinic acid and caffeic acid were identified as the major antioxidant molecules in the mycorrhizal roots with only a small number of other low activity peaks present in the chemiluminescence profile of mycorrhizal roots. *R. irregularis* significantly affected antioxidant potential of rosmarinic acid in all transformed roots which adds to the fungus' role as a biotic elicitor. The antioxidant measure used in our study is based on an established methodology of acidic potassium permanganate chemiluminescence which has been shown to be a direct measure of a phenolic molecule's ability to quench hydrogen peroxide oxidation in cell culture (Conlan et al. 2010). It is important to note that small phenolic molecules possess some pro-oxidant activity due to their ability to be easily oxidized whereas large phenolics do not possess pro-oxidant activity (Hagerman et al. 1998). This has been highlighted by other researchers (Dai and Mumper 2010) and care was taken in our study so that our work describes the antioxidant potential towards limiting hydrogen peroxide-induced oxidation. In the plant-based systems, pro-oxidation also is possible through other mechanisms including when high amounts of metal ions are present or the pH is sufficiently high both of which need to be taken into account for the design of robust experimental conditions. The rapid assessment of antioxidants enabled through the use of the acidic potassium permanganate-based chemiluminescence assay means that its application to other mycorrhizal systems to identify antioxidant potential would be worthwhile.

In conclusion, our study identified *R. irregularis* as a biotic elicitor in in vitro, and the developed co-culture system (HR 4—*R. irregularis*) as an efficient resource for the production of high levels of rosmarinic acid. This study thus sets up a platform for (i) exploration of diverse mycorrhizal species for their influence on secondary metabolite production in in vitro, (ii) identification of factors that may be induced by *R. irregularis* and responsible for its enhancement properties such as key pathway enzymes and their induced genes, phytohormones (such as cytokinin, gibberellin and jasmonic acid) and signaling molecules (such as hydrogen peroxide, salicylic acid and nitric oxide) and (iii) application of the acidic potassium permanganate-based chemiluminescence assay for identifying the effect of mycorrhization on antioxidant production in planta or in vitro. The current study also amply demonstrates the strong potential for future incorporation of arbuscular mycorrhizal fungi in the commercial production, in vitro, of secondary metabolites derived from transformed roots.

AMF Arbuscular mycorrhizal fungi, *Ar* Argon, *CLSM* Confocal laser scanning microscopy, CO_2 Carbon dioxide, *CPD* Critical point drying, *EC* Electrical conductivity, *EDTA* Ethylene diamine tetra acetic acid, *GAE* Gallic acid equivalent, *HPLC* High performance liquid chromatography, *HR* Hairy root, *HSD* Honestly significance difference, *LM* Light microscopy, *M* Minimal medium, *N* Nitrogen, *P* Phosphorus, *SD* Standard deviation, *SEM* Scanning electron microscopy.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals This article does not contain any studies with human or animal subjects.

Informed consent Not applicable.

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