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RESEARCH ARTICLE

Exploring the yeast-mycorrhiza-plant interaction: *Saccharomyces eubayanus* negative effects on arbuscular mycorrhizal formation in tomato plants

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Abstract

Aims Many studies have reported beneficial effects of yeasts on the colonization and development of arbuscular mycorrhizae, thought a few studies have also shown neutral effects. All these studies have in common that the mechanism, by which yeasts and mycorrhizae interact, is little understood. Here, we explore how plant growth-promoting yeasts affect the colonization of tomato plants by beneficial mycorrhizal fungi.

Methods We tested the influence of the soil yeasts *Candida saitoana, Tausonia pullulans,* and *Saccharomyces eubayanus* on colonization of tomato roots by the mycorrhizal fungus *Rhizophagus irregularis*. We analyzed mycorrhizal parameters and the expression pattern of mycorrhiza-specific genes. In plants co-inoculated with *S. eubayanus* and *R. irregularis*, we measured the root accumulation pattern of jasmonic acid, oxo-phytodienoic acid, abscisic acid and salicylic acid, and the expression of genes related to plant hormone signaling and metabolism.

Results The three yeasts had distinct effects on mycorrhizal colonization: *C. saitoana* had no effect on mycorrhizal parameters, *T. pullulans* delayed mycorrhizal colonization at an early stage, and *S. eubayanus* slowed colonization down throughout the entire trial. In plants co-inoculated with *S. eubayanus* and *R. irregularis*, we observed a sustained increase in jasmonic acid and up-regulation of the JA biosynthesis related genes *LOXD*, *OPR3,* and *AOS1*.

Conclusion Co-inoculation with yeast affected mycorrhizal colonization and altered the expression pattern of mycorrhizal and plant defense-related genes. In particular, the yeast *S. eubayanus* modified plant defense hormones such as jasmonic acid, which is linked to mycorrhizal-induced resistance in tomato plants.

Keywords *Rhizophagus irregularis* · Jasmonic acid · Plant-defense hormones · Priming effect

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Introduction

Arbuscular mycorrhizal (AM) mutualistic association with roots is the most widespread symbiosis in the plant kingdom (Smith and Read 2008). Root colonization by AM fungi (AMF) triggers a complex sequence of events which determine physical and physiological changes in the roots (Bonfante and Genre 2010). Mycorrhizal symbiosis also produce changes in genes expression in the host plants (Bonfante and Genre 2010; Ho-Plágaro et al. 2021). These transcriptional changes could be track to different processes: synthesis of signaling molecules important in the plant-fungi recognition, such as apocarotenoids (i.e. gene *D27*, coding for beta carotene isomerase); nutrient exchanges between plant and fungi, which depends upon transporters present only in the peri-arbuscular membrane of plant cells (i.e. gene *PT4*, coding for inorganic P transporter); the formation and function of specialized fungal structures inside the root (i.e. gene *RAM1,* involved in arbuscule formation); or altering plant hormones biosynthesis pathway (Bonfante and Genre 2010; Fiorilli et al. 2019, Ho-Plágaro et al. 2021). It has been proposed that plant hormones are key components in regulation of AMF colonization and development (Bedini et al. 2018; Liao et al. 2018; Pozo et al. 2015). Strigolactones secreted by plants are especially important at the initial stages of AMF colonization (Lanfranco et al. 2018), while ethylene, salicylic acid (SA), and cytokinins inhibit AMF colonization (Foo et al. 2013). Other plant hormones affect late stages of mycorrhizal development by regulating arbuscular development and lifespan: biologically active gibberellins suppress arbuscule formation (Floss et al. 2013); abscisic acid (ABA) and auxins positively regulate arbuscule development and functionality (Etemadi et al. 2014; Martín-Rodríguez et al. 2011); and jasmonic acid (JA) and its derivatives, known as jasmonates, have both positive and negative effects (Hause and Schaarschmidt 2009). It has been suggested that JA induces the expression of defense-related genes which negatively regulate AMF colonization, and that JA-insensitive tomato mutant plants (*jai-1*) are highly susceptible to AMF colonization (Herrera-Medina et al. 2008).

Many microorganisms are also known to alter plant hormonal "programs", and it was suggested that AMF and nitrogen fixing bacteria (NFB) share some steps in symbiotic dialogue (Hause et al. 2007; Hause and Schaarschmidt 2009). Nevertheless, AMF and NFB are not the only microorganisms in direct contact with plant roots, and the joint action of soil organisms will determine plant fitness and growth. The direct interaction with plants, affecting nutrient cycling and availability, pathogenic activity, microbial competition and others, could be particularly relevant to understand the soil microorganisms interactions. Both bacteria and yeast might promote or suppress AM colonization and development in roots; however, the associated mechanisms are still little understood.

Soil yeasts are able to promote plant growth directly through production of plant hormones and other growth regulators (Amprayn et al. 2012; Cloete et al. 2009; Nassar et al. 2005; Nutaratat et al. 2014; Xin et al. 2009), and indirectly as result of interaction with symbiotic microorganisms such as NFB and AMF (Boby et al. 2008; Fracchia et al. 2003; Mohamed 2015; Singh et al. 1991). Some researchers have also described direct beneficial effects of yeasts on mycorrhizal fungi development, including enhanced mycelial production, hyphal length, and spore germination *in-vitro* (Fracchia et al. 2003; Sampedro et al. 2004). Most studies reported these beneficial effects through dual inoculation of mycorrhizal fungi and yeasts (Fracchia et al. 2003; Sarabia et al. 2017; Singh et al. 1991). As a contrasting result, Gollner et al (2006) reported that yeasts did not affect mycorrhizal colonization of maize and significantly decreased the extraradical mycelial production. The above mentioned reveal the need for further investigate the yeast-mycorrhiza-plant interaction.

Environmental characteristics should be considered when setting plant production including the cultivation condition (greenhouse or open field), soil properties and the fertilization methods (biological, chemical or mixed). In cold-temperate regions region such as Northwest-Patagonia the used of cold adapted or psichrotolerant microorganisms in agricultural practice could be an attractive strategy to promote sustainable agriculture. For the last several years we have been studying the potential used of psichrotolerant yeast from pristine native forests in Patagonia as potential tool to enhance plant production in cold-temperate environments. A large number of yeast strains were isolated from the soil and rhizosphere of *Nothofagus* trees in natural forest in Patagonia (Mestre et al. 2011, 2014). These yeasts were characterized

as potential plant growth promoters due to their ability to produce auxin-like compounds and to solubilize inorganic phosphates (Mestre et al. 2016, 2021); some of these yeasts had positive effects on the growth of poplar cuttings and tomato plants (Mestre et al. 2017 and unpublished data). The aim of the present work was to explore whether Patagonian native yeasts affect mycorrhizal colonization of tomato plants, to quantify and characterize the meaning of these interaction and to propose potential mechanisms for such yeast-mycorrhiza-plant interaction. Tomato was selected as model plant for the present study due to the economical importance of tomato worldwide (and in Patagonia) as well as the great body of knowledge on mycorrhiza-tomato interaction and the previous experience of the authors working with the *Rhizophagus irregularis-Solanum lycopersicum* mycorrhizal association (Ho-Plágaro et al 2021, Martín-Rodríguez et al. 2011, 2016). The present manuscript represents an initial step into deciphering the mechanism behind the interaction between plant growth-promoting yeast and mycorrhizae when they are applied together, focusing on the putative role of plant hormones mediating the interaction in co-inoculated plants.

Material and methods

Yeast strains

The yeast *Tausonia pullulans* CRUB 1772 (Basidiomycota) was isolated from rhizospheric soil in *Nothofagus pumilio* and produced auxin-like compounds (Mestre et al. 2016). *Candida saitoana* CRUB1770 (Ascomycota), isolated from bulk-soil in *Nothofagus antarctica* forest, produced weak auxin-like compounds and solubilized inorganic P (Mestre et al. 2016). *Saccharomyces eubayanus* CRUB2014 (Ascomycota), isolated from rhizospheric soil in *Nothofagus pumilio,* produced auxin-like compounds (unpublished data) and solubilized inorganic P (Mestre et al. 2021).

Plant trial and inoculation

Solanum lycopersicum L. (Mill.) cv. Moneymaker (accession LA0575) seeds were surface disinfected by immersion in 2.35% w/v sodium hypochlorite for 5 min, followed by shaking in sterile water at room temperature for 1 day in the dark; they were then germinated on a sterilized moistened filter paper for 4 days at 25 °C in the dark. Germinated seeds were transferred to pots with steam-sterilized (40 min at 120° C) vermiculite and placed in a growth chamber for 15 days. Seedlings were then transferred to 300 mL pots containing a steam-sterilized (40 min at 120° C) mixture of expanded clay, vermiculite, and coconut fibre (3:3:2, by volume). At this time (0 days post-inoculation with mycorrhiza = 0 dpi), half the seedlings were inoculated with a piece of monoxenic culture in Gel-Gro medium produced according to Chabot et al. (1992), containing infected carrot roots and around 100 spores of *Rhizophagus irregularis* (DAOM 197,198) as mycorrhizal treatments (Myc). The remaining seedlings were not inoculated with *R. irregularis*, thus constituting non-mycorrhizal treatments.

Yeast strains were grown in liquid MYP medium (% w/vol, malt extract 0.7, yeast extract 0.05, peptone 0.25, agar 1.5) for 72 h at 20 °C. The yeast cells were harvested by centrifugation at 5000 rpm for 15 min and re-suspended with sterile half-strength Long Ashton nutrient solution (Hewitt 1966) containing 25% of the standard P concentration (% w/vol, NO_3K 3.03; $(NO_3)_2Ca.4H_2O$ 10.19, $SO_4Mg.7H_2O$ 1.84, EDTAFe 0.25, $SO_4Mn.4H_2O$ 0.22; $SO_4Cu.5H_2O$ 0.29, BO_3H_3 1.86, $Na_26MoO_4.2H_2O$ 0.03, $PO_4HNa_2.2H_2O$ 0.58) at a concentration of ca. 10^6 cells/mL. For plant inoculation, 15 mL of yeast suspension was applied at the base of the seedling 5 days after transplanting (and after mycorrhizal inoculation = 5 dpi). Each yeast strain was used separately to inoculate mycorrhizal plants (co-inoculation treatments) or non-mycorrhizal plants (single inoculation treatments); plants with no inoculation (neither AM nor yeasts) were considered the Control treatment. Each of the eight treatments had 16 replicates, with a total of 128 plants in the trial. Plants were cultivated in a growth chamber (day:night cycle: 16:8 h, 24:19 °C; relative humidity 50%). All plants were regularly watered with 20 mL of a modified Long Ashton nutrient solution containing 25% of the standard P concentration to prevent mycorrhizal inhibition caused by excess P.

The inclusion of multiple time points in experimental designs should be a standard procedure when describing AM fungal colonization in order to quantify the dynamic of the association. Monitoring weekly the root length colonization on control (Myc)

plants we considered suitable a first harvest times when the colonization was moderately established (around 30%) colonization and a second harvest two weeks later when mycorrhizal colonization was fully established. Accordingly, plants were harvested at 47 and 63 days after mycorrhizal inoculation (47 dpi and 63 dpi, respectively). Each root system was washed and rinsed several times with water, weighed, and cut into pieces of about 1 cm. Root fragments were mixed to obtain a homogeneous sample of the entire root system, which was used for different determinations: one portion of the sample was used to estimate root colonization, and the remaining sample was immediately frozen in liquid nitrogen and stored at − 80 °C until use for RNA extraction and hormone quantification. All roots systems were processed equally regarding time and manipulation procedures. Fresh root and shoot weight was recorded at harvest and dry shoot biomass was measured after dried for 1 week at 70 °C. Water content in aerial part of the plant was used to estimate root and total dry biomass.

Estimation of root colonization

The non-vital Trypan blue histochemical staining procedure was used according to Phillips and Hayman (1970). Stained roots were observed under a light microscope, and the intensity of root cortex colonization by the AMF was determined according to the procedure described by Trouvelot et al. (1986). The parameters measured were frequency of colonization (% F), intensity of colonization (% M), and arbuscule abundance in the entire root (% A). Three microscope slides containing 30 root pieces of 1 cm were analysed per root system.

RNA extraction and gene expression quantification

The procedures for RNA isolation and gene expression analysis by real-time quantitative PCR used in the present manuscript were previously validated and published (León Morcillo et al. 2012; Ho-Plágaro et al. 2021). Frozen roots (0.2 g) were ground in a mortar with liquid N and transferred to a plastic tube to be treated with TRI reagent (Bioline). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden) and treated with RNase-Free DNAse I (Qiagen, Hilden) following the manufacturer's instructions. The DNAse-treated RNA was cleaned using NucleoSpin RNA clean-up (Machery-Nagel) according to manufacturer's instructions. cDNA was synthesised by reverse transcription using PrimeScript RT Master Mix, Perfect Real Time, (Takara Bio) following the supplier's protocol. qPCRs were prepared in a 20-µl reaction volume containing 1 µl of diluted cDNA (1:2), 10 µl 2 × TB Green Premix Ex Taq (Tli RNaseH Plus) (Takara Bio), and 200 nM of each primer (Supplementary Table 1). The PCR program consisted of a 3-min incubation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 58–63 °C, and 30 s at 72 °C. The specificity of the PCR amplification procedure was checked using a melting curve after the final PCR cycle (70 steps of 30 s from 60 to 95 °C, at a heating rate of 0.5 °C). Experiments were carried out on three biological replicates. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The Ct values of all genes were normalized to the Ct value of the tomato *EF-1α* (Solyc06g005060.2.1) and *Actine* (Solyc11g005330.1.1) housekeeping genes. To study genes induced by mycorrhizal colonization, we reported the qPCR data for each gene as a relative expression with respect to the single inoculation treatment with *R. irregularis* (Myc). To study genes linked to hormonal jasmonic acid and salicylic acid pathways, we reported the data for each gene as a relative expression with respect to the Control treatment (with no inoculation).

Hormone quantification

Jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), abscisic acid (ABA), and salicylic acid (SA) were analysed by ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS) as described by Flors et al. (2008). A 50 mg aliquot of dry tomato root tissue was homogenized in 2.5 ml of ultra-pure water and centrifuged at 5000 g for 40 min. The supernatant was then acidified and partitioned against diethyl-ether, dried in a nitrogen atmosphere, and resuspended in 1 ml of water/methanol (90:10, v/v). The extraction method used here was non-selective and yield a mixture of ABA and other ABA-derivate. Aliquots of 10 µl of this solution were injected into a UPLC system. The UPLC equipment was an ultra-high efficiency liquid chromatograph (UPLC, H-Class, Waters, Mildford), and was coupled with a triple quadrupole mass spectrometer

(Quattro-Micro, Waters, Mildford). Instrument control, data collection, analysis, and management were controlled by the MassLynx 4.1 software package. LC separation was performed using an A BEH column (2.1 * 50 mm, 1.7 µm, Waters). The composition of the mobile phase (Fm) was H_2O + 0.1% Formic Ac (Fm A) and MeOH + 0.1% Formic Ac (Fm B), at a flow of 0.25 mL / min. A 7 min linear gradient elution method was applied 60, 10, 60 (Fm A) in 2.5 min. The effluents from the UPLC were introduced into the mass spectrometer. The ionization source temperature was 120ºC and the desolvation gas temperature 350ºC. The cone gas and desolvation gas flow rates were 600 and 0 L/h, respectively. The capillary voltage was 3.0 kV, and cone voltage 15 V. Argon gas (2.83 10^{-3} mbar) was in the collision cell. Quantifications were carried out with MassLynx 4.1 software (Waters) using the internal standards as a reference for extraction recovery and the standard calibration curves as quantifiers. The mixture of internal standards added to each sample during tissue homogenization contained 100 ng of [2H6]ABA, 100 ng of dihydrojasmonic acid, 100 ng of prostaglandin B1, and 100 ng of [2H5]SA.

Statistical analysis

A one-way ANOVA was performed, followed by the Tukey test; when the homogeneity of variances assumption could not be met, the Kruskal–Wallis test was performed followed by Tukey's rank test. All statical analyses were performed with *agricolae* (de Mendiburu 2020) and *pgirmess* (Giraudoux et al. 2018) packages on R software version 4.0.3 (2020–10-10); differences at $p < 0.05$ were considered significant. Data representation as dotplot was performed with *ggplot2* (Wickham 2016) package on R software.

Results

Effect of yeast inoculation on the mycorrhizal colonization pattern in tomato plants

Mycorrhizal colonization was observed in all plants inoculated with *R. irregularis*. Plants inoculated solely with mycorrhizal fungus (Myc) presented 35% colonization at 47 dpi and 44% colonization at 63 dpi (Fig. 1). A similar pattern was observed in plants co-inoculated with *R. irregularis* and the yeast *C. saitona (*Myc + C.s.). Mycorrhizal plants co-inoculated with *T. pullulans* (Myc + T.p.) presented lower colonization values at 47 dpi than the mycorrhizal control plants, but colonization values at 63 dpi were similar to control Myc plants (Fig. 1). For plants co-inoculated with *R. irregularis* and *S. eubayanus*, colonization values remained lower than control plants at both harvest times (Fig. 1).

Mycorrhizal colonization parameters such as frequency, intensity, and arbuscule abundance revealed significant differences at harvest. At 47 dpi, all colonization parameters were lower in plants co-inoculated with *T. pullulans* than those inoculated solely with *R. irregularis* (Myc + T.p. *vs*. Myc) (Fig. 1). At the final harvest times, 63 dpi, all parameters were lower for plants co-inoculated with *S. eubayanus.* Estimated dry plant root and total biomass showed no significant differences compare with the control of each harvest time (Fig. 1).

To obtain additional evidence of the effect of yeasts on mycorrhizal development, we studied mycorrhizal colonization at transcriptional level at last harvest time by analyzing fungal *GinEF*, and plant mycorrhizal-related genes *D27*, *PT4* and *RAM1*. Gene expression at 63 dpi showed significant differences, which related with the colonization changes measured in the different co-inoculation treatments (Fig. 2). Plants co-inoculated with *C. saitonana* and *T. pullulans* (Myc + C.s and Myc + T.p) and those from single mycorrhizal inoculation treatment (Myc) presented similar values, while plants co-inoculated with *S. eubayanus* showed the lowest expression value for all genes examined (Fig. 2).

As a whole, the results of AM colonization and mycorrhizal-related gene expression suggest that co-inoculation with *C. saitonana* and *T. pullulans* had little effect on AM development, even considering the negative effect of *T. pullulans* inoculation on AM colonization early in the interaction. However, co-inoculation with *S. eubanayus* had a significant negative impact on mycorrhizal frequency and intensity, as well as on arbuscular frequency and mycorrhiza-related gene expression. In view of these results, we decided to further explore potential mechanisms of AM regulation mediated by *S. eubayanus.*

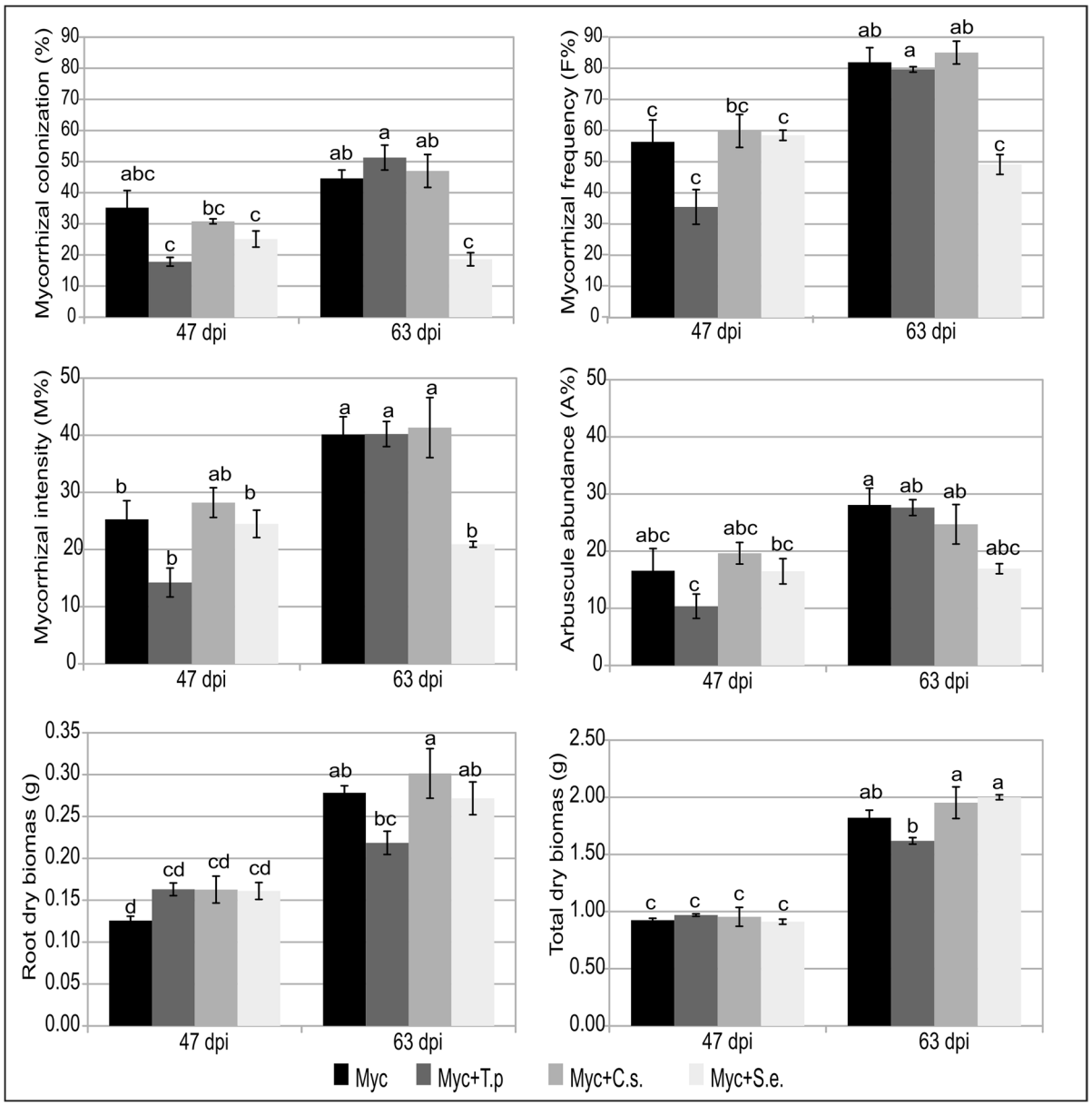

Fig. 1 Mycorrhizal colonization measured as percentage of root length colonization and mycorrhizal parameters, and plant biomass for co-inoculated plants at 47 and 63 dpi harvests (n = 3). Statistical significance was determined with $\alpha = 0.05$ using one-way ANOVA. Bars correspond to standard error. Different letters indicate different homogenous groups calculated by Tukey test. Myc: *Rhizophagus irregularis*; C.s.: *Candida saitoana*; T.p.: *Tausonia pullulans*; S.e.: S*accharomyces eubayanusm*

Plant hormone-related pathway in plants co-inoculated with *S. eubayanus*

In order to evaluate whether the *S. eubayanus*-induced defense against mycorrhizal colonization was caused by alteration of the hormonal homeostasis of the roots, we determined the SA, JA, and ABA content in the roots of non-mycorrhizal and mycorrhizal tomato plants co-inoculated or not with *S. eubayanus*.

The concentration of ABA was higher in non-mycorrhizal treatments (Control and S.e.) than in mycorrhizal treatments (Myc and Myc + S.e.) at

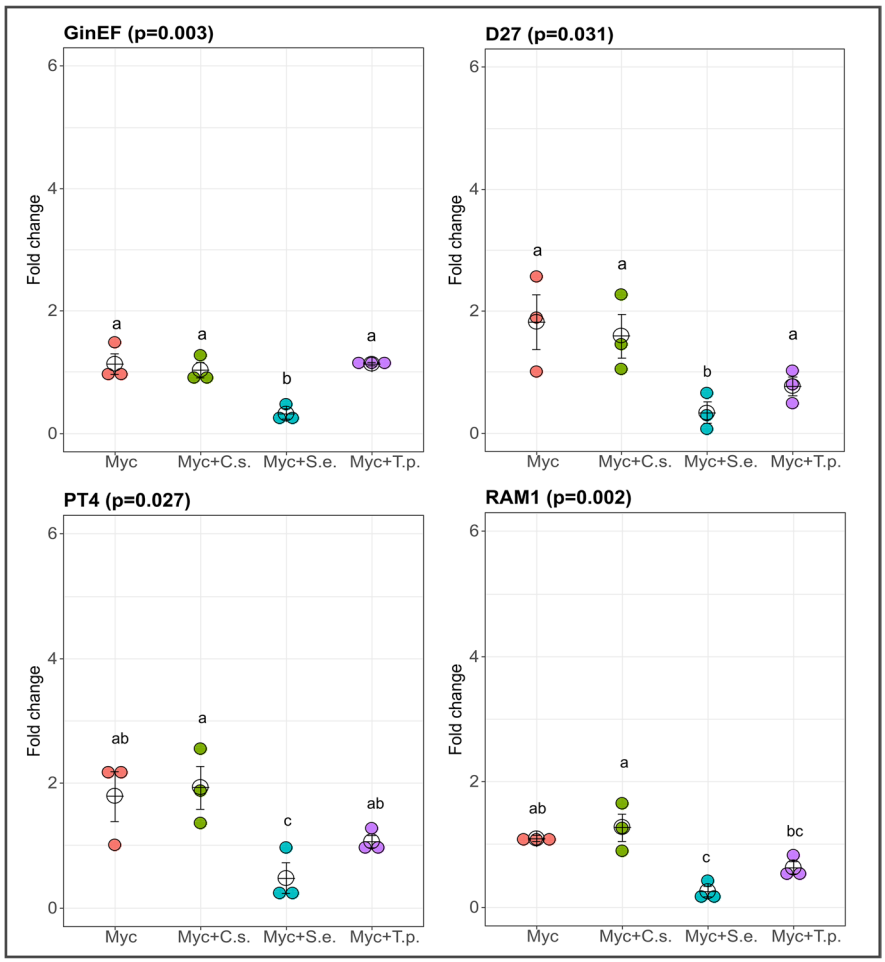

Fig. 2 Relative gene expression measured by RT-qPCR for mycorrhiza-related genes in roots of co-inoculated plants at 63 days post-inoculation (dpi) harvests. Each gene as a relative expression with respect to the single inoculation treatment with *R. irregularis* (Myc). Statistical significance was determined with $\alpha = 0.05$ using one-way ANOVA. *: Kruskal–Wallis analysis. Different letters indicate different homogenous groups calculated by Tukey test for each variable. Myc: *Rhizophagus irregularis*; C.s.: *Candida saitoana*; T.p.: *Tausonia pullulans*, S.e.: S*accharomyces eubayanus.* Studied genes: *GinEF* = *R. irregularis* elongation factor; *D27* = tomato beta carotene isomerase d27; *RAM1* = tomato GRAS transcription factor *RAM1*; *PT4* = tomato mycorrhiza-inducible inorganic phosphate transporter 4

47 dpi (Fig. 3). The single *S. eubayanus* inoculation treatment showed the highest ABA level, while the co-inoculation treatment (Myc + Se) showed the lowest ABA level at 47 dpi. ABA concentration was similar for all treatments at 63 dpi, which was lower than ABA concentrations at 47 dpi. The pattern of SA concentration was similar at 47 and 63 dpi: SA was higher in non-mycorrhizal than in mycorrhizal treatments, the control treatment had the highest SA concentration, and the co-inoculation treatment had the lowest SA concentration (Fig. 3). The accumulation pattern of 12-OPDA, which is a precursor of JA pathway, was similar at 47 and 63 dpi; the lowest concentration was determined for the co-inoculated treatment, whereas the other treatments had similar concentrations (Fig. 3). At 47 dpi, JA concentration was similar in both mycorrhizal treatments and was much higher than in non-mycorrhizal treatments. At 63 dpi there was greater accumulation of JA in the roots of plants co-inoculated with *R. irregularis* and *S. eubayanus* than in the roots of the other treatments that showed similar concentrations (Fig. 3).

We then examined the time course of gene transcripts involved in SA (*PR1a*) and JA defense (*PinII*) in response to *R. irregularis* and *S. eubayanus* inoculation. Although changes in expression level of *PR1a* at 47 dpi showed no significant differences, we observed a tendency of increased expression of this gene for single inoculated treatments (Myc and S.e.; Fig. 4). At final harvest time the level of *PR1a* gene expression was similar for all treatments (Fig. 4). The transcriptional analysis of the gene *PinII* at 47 dpi showed higher expression in mycorrhizal treatments than in non-mycorrhizal treatments (Fig. 4), while at final harvest time higher *PinII* expression was observed in the *R.*

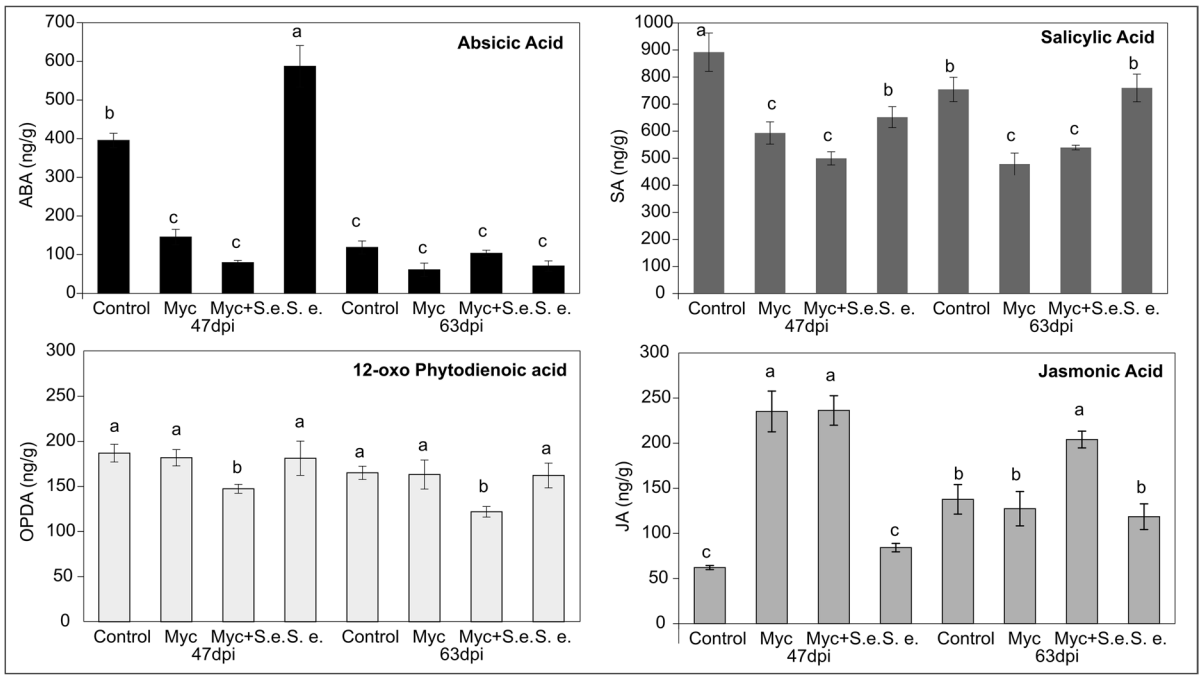

Fig. 3 Endogenous abscisic acid (ABA), salicylic acid (SA), 12-OPDA and jasmonic acid (JA) contents in the roots of tomato plants with different inoculation treatment, harvested at 47 and 63 dpi (n = 3). Different letters indicate different homogenous groups calculated by Tukey test. Control: non-inoculated; Myc: *Rhizophagus irregularis*; S.e.: S*accharomyces eubayanus*

irregularis and *S. eubayanus* co-inoculation treatment (Fig. 4).

Time course of gene transcripts involved in the JA biosynthesis pathway

To determine whether the observed JA accumulation pattern was linked to the up-regulation of biosynthesis related genes, we analysed transcripts of *LOXD*, *AOS1,* and *OPR3*. Co-inoculation with *R. irregularis* and *S. eubayanus* significantly increased transcription of the three genes in the roots at 47 dpi with respect to the control or single inoculation treatments, which all showed similar expression values (Fig. 5). At final harvest, transcript levels of *LOXD* and *OPR3* tended to be similar in all treatments, but we observed a higher level of *AOS1* expression in both single inoculation treatments (Myc and S.e.) than in the control (Fig. 5). The results obtained reveal a conserved pattern of expression of the JA biosynthesis-related genes analysed here, and suggest the activation of their expression under co-inoculation conditions.

Discussion

The study of the interaction of microorganisms and plants has the potential of positively impact crop (food) production. Due to the complexity of the system, it is usually difficult to translate the findings produce in the laboratory (with *in-vitro* or *in-vivo* artificial systems) to field production. Although the present study is not looking to directly link our experiment to real-world agronomy, we take into consideration some tomato production practices. We used a suspension of yeasts as inoculum, which has been proposed by other author as the best inoculation methods (Fracchia et al. 2003; Sampedro et al. 2004) and has also the advantage to be easily included in irrigation practices. We inoculated the yeasts suspension 20 days after seed germination, which coincided with the setting of the first true leaves and is usually the first application of fertilizer through irrigation system (fertigation). Another aspect to be considered when applying multiple microorganisms is the order of inoculation and the direct interaction between microorganisms. The application of yeasts prior to AMF has shown positive

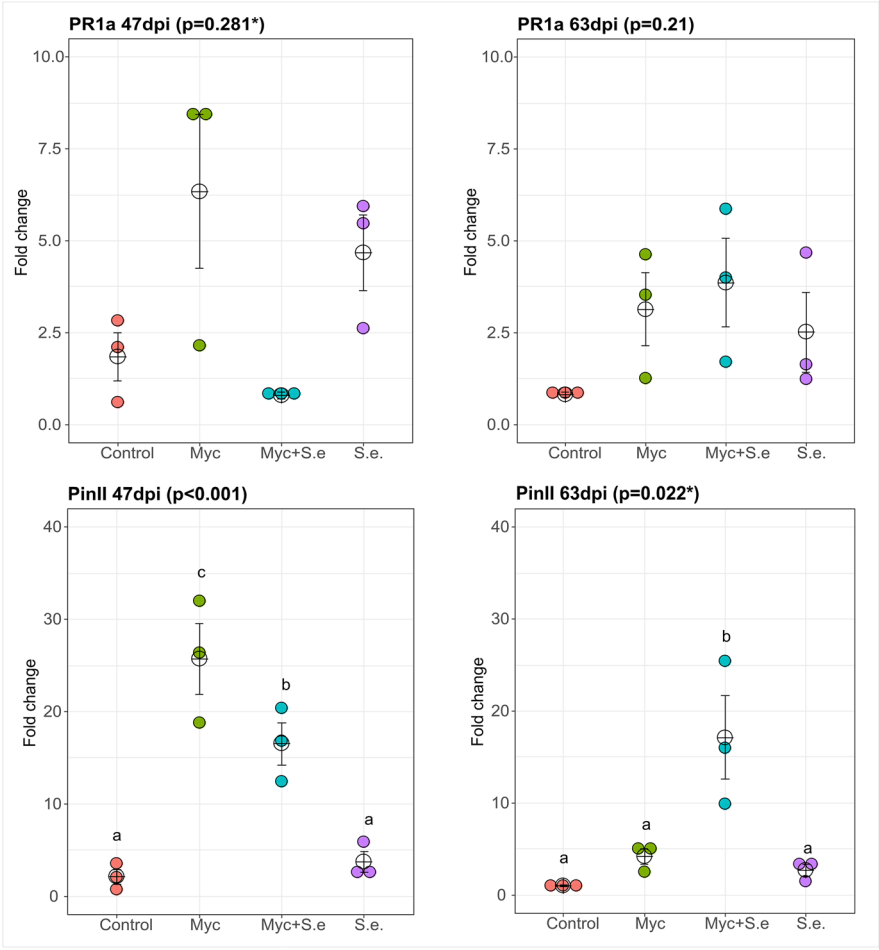

Fig. 4 Expression of defense-related genes *PR1a* and *PinII* in the roots of tomato plants with different inoculation treatment, harvested at 47 and 63 dpi. Each gene as a relative expression with respect to the single inoculation treatment with *R. irregularis* (Myc). Transcript levels were determined using RT-qPCR. Mean value (empty dot) and standard error (bar) for each treatment are shown. Expression was normalized with respect to the control non-inoculated plants, in which expression was designated as 1. *: Kruskal–Wallis analysis. Different letters indicate different homogenous groups calculated by Tukey test. Control: non-inoculated; Myc: *Rhizophagus irregularis*; S.e.: S*accharomyces eubayanus.* Studied genes: *PR1a* = pathogenesis-related protein 1a; *PinII* = proteinase inhibitor II

effects on mycorrhizal colonization of red clover and soybean (Fracchia et al. 2003; Sampedro et al. 2004) and no effect on maize (Gollner et al 2006). Yeast inoculation before the AMF would allow to characterize a putative direct effect of the yeast on presymbiotic stages of the fungus (i.e. stimulation of spore germination and mycelial development). Here, we focused the study on characterizing effects stimulated in the root by the presence of yeast that directly or indirectly could affect the intradical development of the AM fungus.

As mentioned earlier, some researchers have described a direct beneficial effect of yeasts on AMF development (Fracchia et al. 2003; Sampedro et al. 2004), while other authors have also reported negative effect of yeasts on the extraradical mycelium growth (Gollner et al. 2006). In previous studies it has been suggested that the three yeast strains studied here could act as mycorrhizal helpers for native mycorrhizae (Mestre et al. 2017 and unpublished data). Here, we analysed the pattern of AM development in tomato plants which were inoculated with the AM fungus *R. irregularis* and three soil yeasts: *C. Saitoana, T. pullulans* or *S. eubayanus*. Our findings suggested that each yeast had different effects on mycorrhizal colonization by *R. irregularis.*

Co-inoculation of *R. irregularis* and *C. saitoana* (Myc + C.s.) resulted in a pattern of AM development similar to that of control plants inoculated only with *R. irregularis* (Myc). However, in plants co-inoculated with *R. irregularis* and *T. pullulans* or *S. eubayanus,* we observed altered mycorrhization patterns: *T. pullulans* delayed colonization, but the final outcome was similar (or slightly higher) to that of control plants; *S. eubanayus* slowed down mycorrhizal colonization, this impairment being most evident at final harvest time. These results suggest that *T. pullulans* and *S. eubayanus* affect different steps of the

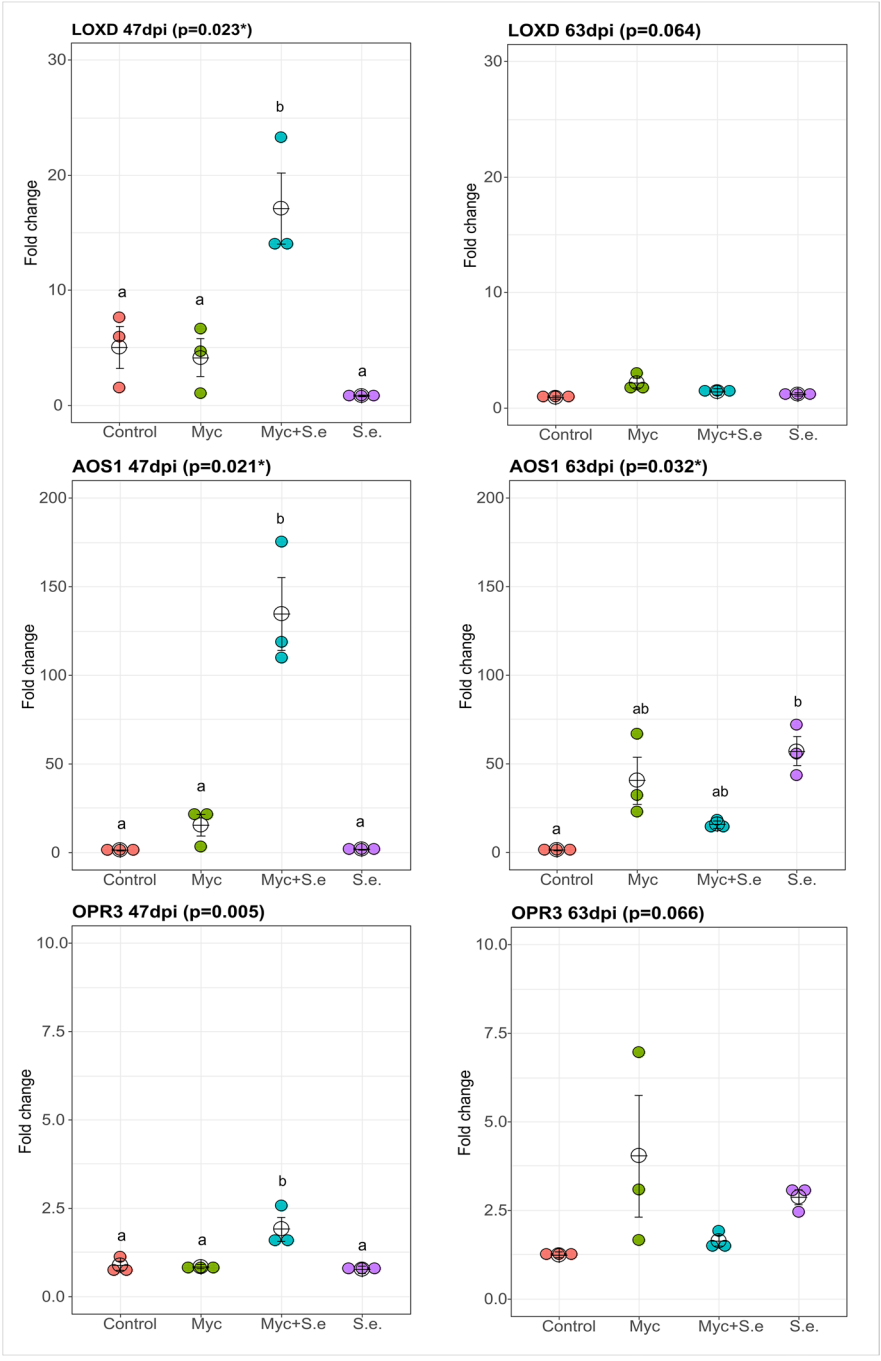

Fig. 5 Expression of genes related to jasmonic acid biosynthesis pathways in the roots of tomato plants with different inoculation treatment, harvested at 47 and 63 dpi. Each gene as a relative expression with respect to the single inoculation treatment with *R. irregularis* (Myc). Transcript levels were determined using RT-qPCR. Mean value (empty dot) and standard error (bar) for each treatment are shown. Expression is normalized with respect to the control non-inoculated plants, in which expression was designated as 1. *: Kruskal–Wallis analysis. Different letters indicate different homogenous groups calculated by the Tukey test. Control: non-inoculated; Myc: *Rhizophagus irregularis*; S.e.: S*accharomyces eubayanus*- Myc: *Rhizophagus irregularis*; S.e.: *Saccharomyces eubayanus.* Studied genes: *LOXD* = Lipoxygenase D; *AOS1* = Allene oxide synthase 1; *OPR3* = oxo-phytodienoic acid reductase 3

development of mycorrhizal colonization. All mycorrhizal parameters studied (colonization frequency, colonization intensity, and arbuscule abundance) followed the same pattern as the colonization rate, determined as percentage of root length colonization. Total plant and root biomass were similar in all treatments, which shows that yeasts did not stimulate root growth and the colonization rate was not affected by differences in the size of the root system. Our results suggest that yeast inoculation affected the global process of mycorrhization rather than specific processes, which means that the alteration of parameters such as

arbuscule formation (arbuscule abundance) or mycorrhizal dispersion within the root (mycorrhizal intensity) was a consequence of alteration in total colonization rate.

The transcriptional analysis of genes related to AM development showed significant lower gene expression for *R. irregularis* and *S. eubayanus* co-inoculation treatments (Myc + S.e.) than for single *R. irregularis* treatment (Myc). The greatest differences in mycorrhization parameters were observed at 63 dpi. *GinEF* expression has been used to evaluate the presence and abundance of *R. irregularis* active mycelium in roots (Martín-Rodríguez et al. 2011). The low expression of *GinEF* in Myc + S.e. treatment at 63 dpi could be attributed to low fungal presence inside the roots, which is compatible with the colonization stalemate observed. Expression of the *RAM1* gene is induced in mycorrhizal plants, and has been linked to the active formation of arbuscules in roots (Rich et al. 2017). The Myc + S.e. treatment showed fewer arbuscules at 63 dpi than the single *R. irregularis* treatment, which could be associated with the low *RAM1* gene expression observed. The *PT4* gene has been associated with active nutrient transport across the plant periarbuscular membrane, and has been used as an arbuscule-related gene marker (Ho-Plágaro et al. 2021). The low expression of the *PT4* gene in Myc + S.e treatment could also be a consequence of the low abundance of arbuscules at 63 dpi in these plants. The *D27* gene is also induced by mycorrhizal colonization, and has been associated with the apocarotenoid pathway in plants (Liao et al. 2018). Apocarotenoids have been linked with several plant growth regulators that mediate mycorrhizal colonization (Fiorilli et al. 2019). The Myc + S.e treatment showed low *D27* expression at 63 dpi, which could also be linked to impairment of mycorrhizal colonization. Taken together, the results show that co-inoculation with *S. eubanayus* negatively impacted AM development. Therefore, we further explored a possible mechanism of AM regulation mediated by a plant defense hormone in the *R. irregularis* and *S. eubayanus* co-inoculation treatment.

The involvement of several plant hormones in mycorrhiza formation and functioning has been proposed, as they modulate plant response to AM fungi (Bedini et al. 2018; Liao et al. 2018; Pozo et al. 2015). Plant stress-related hormones seem to have different effects on AM formation: ABA was shown to be necessary for arbuscule functioning, SA and ethylene negatively affected fungal penetration and colonization, and jasmonates had both positive and negative effects on mycorrhization (Bedini et al. 2018; Liao et al. 2018; Pozo et al. 2015). ABA has been reported as one of the main phytohormones regulating AMF colonization and arbuscule functionality in tomato plants (Herrera-Medina et al. 2007). The ABA level in the Myc + S.e. treatment was not significantly different to that in the Myc treatment, which indicates that the plant defense process affecting AM colonization in co-inoculated plants was not mediated by ABA in our experiments. Concentrations of free ABA in non-mycorrhizal treatments (Control and S.e.) were significantly higher than in mycorrhizal treatments (Myc and Myc + S.e.) at 47 dpi. Sánchez-Romera et al. (2018) also reported a higher content of ABA in non-mycorrhizal than in mycorrhizal plants. Martín-Rodríguez et al. (2016) reported slightly higher free ABA levels and much lower ABA-GE (abscisic acid glucosyl ester) levels (five times lower) in mycorrhizal than in non-mycorrhizal tomato roots. This discrepancy could be attributed to the different quantification methods used. The extraction and purification method by Martín-Rodríguez et al. (2016) was selective and specific for free ABA and its catabolites, whereas the method used here was non-selective and the increase in ABA observed could be attributed to a mixture of ABA and ABA-GE.

Salicylic acid has been proposed as the main hormone associated with defense against microorganisms with a biotrophic lifestyle (Fernández Merlos et al. 2014; Martinez-Medina et al. 2017), which could be the case of mycorrhiza, and SA has been implicated in the early stages of AM fungal penetration (Herrera Medina et al. 2003). Mycorrhizal treatments (Myc and Myc + S.e.) seemed to reduce SA levels in roots at 47 and 63 dpi. Interestingly, the reduction in SA level observed in mycorhizal treatment could be, at least in part, related to the known JA/SA antagonist relationship (Thaler et al. 2002); thus, the treatments with the highest amount of JA at 47 dpi were those with the least amount of SA. Although a tendency for increased expression of *PR1a* was seen in the single inoculation treatment, no statistical difference was observed for *PR1a* gene expression, a pathogenesis-related SA-inducible gene (Torres-Vera et al. 2016).

Therefore, *S. eubayanus* is not directly involved in SA-mediated regulation, and *PR1a* expression may be modulated by factors other than SA level.

In the present study, the JA content of roots was increased in mycorrhizal treatments (Myc and Myc + S.e.) at 47 dpi, and JA levels remained high in the co-inoculated treatment Myc + S.e. at 63 dpi compared to the control and single inoculation treatments. In accordance with this, the expression pattern of *PinII*, which is a typical marker gene for JA wound-regulated response (Torres-Vera et al. 2016), was similar to the pattern of JA accumulation. Our results showing a temporal link between the rise in JA and the decline in free OPDA in co-inoculated Myc + S.e. plant roots suggest that OPDA metabolism may be a control point for JA production in co-inoculated roots. In this sense, a temporal link between the rise in wound-induced systemic production of JA-Ile and the decline in free OPDA has been also observed in Arabidopsis plants (Koo et al. 2009). Nevertheless, a precursor-product relationship between the decline pool of free OPDA and increased JA observed in our study remains to be demonstrated. Quantification of the JA levels was complemented with expression data of genes related to its biosynthesis. At the transcriptional level, some of these genes increased in expression in the co-inoculation treatment, mainly at 47 dpi. LOXD and AOS1 enzymes are involved in the early steps of the pathway, and OPR3 is active in the conversion of 12-OPDA to JA. Although the JA level at 47 dpi was similar in Myc and Myc + S.e. plants, only co-inoculated Myc + S.e. plants showed higher levels of expression for genes related to JA biosynthesis. JA level remained high in the co-inoculation treatment at 63 dpi, which could suggest that the increase in gene transcription at 47 dpi was followed by an increase in JA levels at 63 dpi. All these findings indicate that mycorrhization induces the JA pathway in tomato, as previously reported (López-Ráez et al. 2010), but inoculation with *S. eubayanus* steps up this response, which was measurable even at transcription level. We cannot rule out that in addition to JA, other oxylipins may be increased in tomato roots by the action of *S. eubayanus.* As previously described, both the 9-LOX and 13-LOX pathways of oxylipin production regulate the formation of AM in tomato (León Morcillo et al. 2012).

In conclusion, activation of the JA pathway is part of the plant strategy to control AMF development within the roots; the evidence gathered in the present study suggests that *S. eubayanus* might enhance this plant defense mechanism. Hopefully, the present work will prompt further research to fully understand the effect of *S. eubayanus* in jasmonic acid and other plant-defense hormones biosynthetic pathways, which may include the use of tomato mutants affected in biosynthesis or perception of jasmonic acid. Interestingly, it has been proposed that defense mechanisms mediated by JA also mediate the more rapid or efficient activation of defense responses against pathogen attack on mycorrhization, the so-called priming effect. The priming phenomenon is part of mycorrhizal induced resistance (MIR), a specific mechanism of the induced systemic resistance mechanism (ISR) of mycorrhizal plants (Jung et al. 2012). This raises the question of whether *S.eubayanus* could be a good biocontrol agent if applied as pretreatment, and whether inoculation order may modify the effect of *S. eubayanus* on mycorrhizal colonization.

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Author contribution MCMestre and JMGarcía-Garrido contributed to the study conception and design. Material preparation and data collection was performed by MCMestre and MI Tamayo Navarrete; data analysis was performed by MCMestre and JM García-Garrido. The first draft of the manuscript was written by MCMestre and all authors commented on previous version of the manuscript. All author read and approved the final manuscript.

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Declarations

Declaration of competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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