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Regulation of mycorrhizal colonization under stress in tomato depends on symbiotic efficiency

Javier Lidoy [a,*], Álvaro López-García [a,b], Clara Amate [a], Juan Manuel García [a], Victor Flors [c], José Manuel García-Garrido [a], Concepción Azcón-Aguilar [a], Juan Antonio López-Raez [a], María José Pozo [a]

[a] Dept. of Soil and Plant Microbiology, Estación Experimental del Zaidín, CSIC, Granada, Spain
[b] Instituto Interuniversitario de Investigación del Sistema Tierra en Andalucía, University of Jaen, Jaén, Spain
[c] Plant Immunity and Biochemistry Laboratory, Department of Biología, Bioquímica y Ciencias Naturales, Universitat Jaume I, Castelló, Spain

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ABSTRACT

The mutualistic symbiosis between plants and arbuscular mycorrhizal (AM) fungi is based on a balanced nutrient exchange between both partners, with the plant achieving improved nutrition and stress tolerance. The symbiosis is finely-tuned according to plant's needs and surrounding conditions, usually through phytohormonal signaling. Thus, environmental conditions or stress factors modulating phytohormone signaling may influence the symbiosis. This study compares the colonization abilities of 2 AM fungal species, *Funneliformis mosseae* and *Rhizophagus irregularis*, independently or in combination, in tomato plants subjected to different stress conditions. These included salt stress and systemic defense activation by aboveground application of the defense-related hormones methyl jasmonate, abscisic acid and salicylic acid. The results show that root colonization by the two fungal species differs depending on the stress treatment. Nutrient and transcriptional analyses revealed that changes in colonization correlated with differential regulation of nutrient exchange, plant defensive responses, and symbiosis regulatory genes. Specifically, under salt stress *R. irregularis* colonization decreased, while *F. mosseae* colonization was promoted. These differential regulation of colonization under stress positively correlated with changes in the functionality of the symbiosis. Overall, the results support that the benefits provided by each AM fungi influence carbon reward and determines the control of root colonization by the host plant.

1. Introduction

Fungi are important components of plant microbiota and fulfill multiple functions in plant health and ecosystem functioning (Pozo et al., 2021). Among plant-associated fungi, arbuscular mycorrhizal (AM) fungi are of special interest since they are widespread in very diverse environments and establish the most ancient plant-microbe symbiosis with more than 70% of land plant species (Brundrett and Tedersoo, 2018; Genre et al., 2020). The AM symbiosis is a mutualistic association having important benefits for both partners, the plant and the fungus (Bennett and Groten, 2022). It can improve plant nutrition, mainly increasing phosphorus (Pi), nitrogen and water uptake (Parniske, 2008; Bonfante and Genre, 2010; Xie et al., 2022). It can also enhance plant fitness by boosting plant resistance/tolerance against diverse stress conditions, including biotic and abiotic challenges (Parniske, 2008; Pozo et al., 2015; Lenoir et al., 2016; Santander et al., 2017; Ruiz-Lozano et al., 2018).

The term "arbuscular" comes from the characteristic highly branched tree-like structures formed by these AM fungi within the root cortical cells to increase the fungal-plant contact surface (Parniske, 2008). It is in the arbuscules where the exchange of nutrients between the two partners takes place. Pi is taken up from soil by the fungal extraradical mycelium and translocated to the plant cell in the arbuscule. From there, it is taken up by the plant via specific plant Pi transporters of the Pht1 family, such as the PT4 (Balestrini et al., 2007; Hijikata et al., 2010; Ezawa and Saito, 2018; Ferrol et al., 2019). In return, the plant provides the fungal partner with photosynthates in the form of carbohydrates and lipids. Due to the obligate biotrophic nature of AM fungi, the fungus is

* Corresponding author.
E-mail address: javier.lidoy@eez.csic.es (J. Lidoy).

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completely dependent on this carbon input from the plant (Salmeron-Santiago et al., 2021). In fact, up to 20% of the carbon fixed by photosynthesis is directed to the fungus (Bago et al., 2000; Keymer et al., 2017). According to the high cost of the symbiosis for the plant and the extreme dependence of the fungus, the plant controls fungal colonization according to the nutrient demand, growing conditions and/or fungal efficiency (Hammer et al., 2011; Kiers et al., 2011; Werner and Kiers, 2015). Remarkably, the regulation of the symbiosis is not a simple control process of the carbon sink towards the fungus, but it seems to be regulated by more specific control mechanisms at different levels (Vierheilig et al., 2000; MacLean et al., 2017; Ho-Plágaro and García-Garrido, 2022).

Two differentiated stages can be considered in symbiosis establishment: the pre-symbiotic and the symbiotic stage. During the pre-symbiotic stage, a complex molecular dialogue occurs between the two partners in the rhizosphere before contact. This molecular communication starts when the plant roots exude signaling compounds into the rhizosphere to attract and activate the AM fungus and promote the symbiosis, specially under nutrient deficient conditions (Akiyama et al., 2005; Bouwmeester et al., 2007; López-Ráez et al., 2011, 2017). Among these "cry for help" signals compounds, strigolactones (SLs) play an important role in this stage, inducing AM fungus spore germination, activating fungal metabolism and hyphal branching of germinating spores to promote the contact with plant roots (Besserer et al., 2006; Waters et al., 2017). On the other hand, the AM fungus releases Myc factors [short-chain chitin oligomers (COs) and lipochitooligosaccharides (LCOs)] that activate a set of genes belonging to the common symbiotic signaling pathway in the plant to facilitate fungal accommodation within the roots (Maillet et al., 2011; Genre et al., 2013; MacLean et al., 2017). The establishment and development of the symbiosis (symbiotic stage) also requires a high degree of coordination between the two partners (MacLean et al., 2017). During root colonization, a transcriptional reprogramming is activated in cells of the epidermis and the root cortex related to transcriptional regulation, cell wall modification and modulation of the defensive response to accommodate the fungus and control fungal development (López-Ráez et al., 2010; Sugimura and Saito, 2017; Pimprikar and Gutjahr, 2018). The attenuation of the plant defensive response is essential for the establishment of the symbiosis. In fact, the AM fungus actively promotes the suppression of plant defenses by secreting peptidic effectors (Kloppholz et al., 2011; Schmitz et al., 2019; Zeng et al., 2020). The recognition and establishment of a functional symbiosis requires a very precise and fine-tuned regulation of plant responses, mainly orchestrated by phytohormones and other signaling molecules (Pozo et al., 2015; Bedini et al., 2018; Martínez-Medina et al., 2019). Indeed, almost all phytohormones studied to date are involved, to some extent, in the control of fungal colonization, arbuscular development and/or symbiotic functioning (Pozo et al., 2015; Bedini et al., 2018; Ho-Plágaro and García-Garrido, 2022). Phytohormones allow the integration of environmental and internal cues to generate specific plant responses modulating plant growth and development, defense responses, and plant adaptation to different abiotic and biotic contexts, such as salinity or drought, or the interaction with different (micro)organisms (Pieterse et al., 2012; Lenoir et al., 2016; Bedini et al., 2018). Therefore, the environmental influence on hormone levels may have an impact on the plant interaction with AM fungi (Pozo et al., 2015). The most studied example is the effect of Pi availability. Under low Pi conditions there is a promotion of rhizosphere signaling and root transcriptional reprogramming giving rise to an increased symbiotic development, while under high Pi conditions the symbiosis is repressed (Breuillin et al., 2010; Balzergue et al., 2011, 2013). Mechanical wounding in Medicago leaves also leads to changes in mycorrhizal colonization (Landgraf et al., 2012), whether other stresses may actively promote symbiotic establishment is still controversial (Aroca et al., 2013; López-Ráez, 2016).

It has been shown that the plant has systemic control mechanisms to prevent excessive colonization, the so-called mycorrhizal autoregulation. The autoregulation of mycorrhiza shares several mechanisms with the autoregulation of nodulation in legumes, although the precise molecular mechanisms are not fully understood (Catford et al., 2003; Foo et al., 2016). Recently, a few genes with a putative role in the AM autoregulation process, such as *CLV2* and CLE peptides, have been described (Wang et al., 2018; Müller et al., 2019; Karlo et al., 2020; Ho-Plágaro and García-Garrido, 2022; Wulf et al., 2023). In addition to the regulation of the colonization rates, the plant also controls arbuscule formation, functionality and lifespan through the action of different transcription factors that regulate AM fungal accommodation in root cells, arbuscule formation, nutrient exchange and arbuscule senescence (Ho-Plágaro and García-Garrido, 2022). Some apocarotenoids (mycorradicin and α-ionols), known as the 'yellow pigment complex', are able to maintain the functionality of the AM symbiosis by regulating arbuscular turnover (Fester et al., 1999, 2002; Walter et al., 2010).

It is well known that some AM fungi are more efficient colonizers than others, and their benefits to the plant may also vary (Powell et al., 2009; Chagnon et al., 2013). In this sense, it has been proposed that some fungi are more efficient in improving plant nutrition, while others are better at enhancing stress tolerance (Powell et al., 2009; Chagnon et al., 2013; Rivero et al., 2018; Marro et al., 2022). Moreover, metabolic changes in the host plant during the symbiotic establishment may vary depending on the colonizing fungus both in absence of stress (Fernández et al., 2014; Rivero et al., 2015) or under stressful conditions (Rivero et al., 2018).

Due to the multiple benefits that AM symbioses can provide to plants in agro- and ecosystems, bioinoculants based on AM fungi have already been commercialized as biofertilizers and bioprotection agents (Chen et al., 2018; Szczałba et al., 2019). However, the variability of results under field conditions limits their use and potential applications nowadays. This variability is related to the high context dependency of mycorrhizal effectivity, as multiple environmental conditions may impact the symbiosis and its functionality (Hart et al., 2018; Holland et al., 2018; Kokkoris et al., 2019; Orine et al., 2022). Therefore, it is important to understand the effects of the environmental context on the plant-AM fungus interaction to improve AM applications, thus increasing its implementation in agricultural and ecological settings (Lenoir et al., 2016; Hartman and Tringe, 2019; Orine et al., 2022). To achieve these goals, we need to understand how the symbiosis is regulated under different stresses, and how different AM fungi may behave under different plant stresses.

In the present study, we compare two different AM fungi, *Funneliformis mosseae* and *Rhizophagus irregularis* -commonly used as agro-inoculants- alone or in combination, in their ability to colonize tomato roots under different stress conditions. We explore how different 'environmental' conditions impact AM symbiosis establishment and functioning, including abiotic stress (salinity) or activating biotic stress related signaling pathways. We achieved this by exogenous application of defense-related hormones in the shoots -avoiding direct contact with the fungus-: abscisic acid (ABA), which is a central regulator of plant responses to osmotic stress and modulator of biotic stress responses; jasmonates (JA), key regulator of plant responses to herbivorous insects and necrotrophic pathogens; and salicylic acid (SA), which mainly orchestrates responses against biotrophic pathogens (Pieterse et al., 2012). We hypothesize that the plant regulates AM symbiosis establishment and functionality depending on the stress faced, but its impact depends on the colonizing fungus. We also test whether inoculation with a combination of AM fungi leads to an improved symbiotic establishment and enhanced benefits for the plant under the different conditions. To explore the mechanisms underlying such effects, we analyzed the transcriptional regulation of different pathways. We specifically test for changes in: i) pre-symbiotic signaling, ii) the defensive status of the plant, iii) the regulation of nutrient exchange and iv) the control and autoregulation of the symbiosis. Our results show that transcriptional regulation of plant defenses and carbon supply (lipids and sugars) to the AM fungus are the most important contributors to the regulation of

mycorrhizal colonization in our system, and this regulation seem to be adjusted by the symbiotic efficiency.

2. Materials and methods

2.1. Biological material and growing conditions

Isolates of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler 2010 (DAOM 197198) and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (BEG12, International Bank of Glomeromycota) are continuously maintained in greenhouse pot cultures with *Trifolium repens* and *Sorghum vulgare*. The inoculum consisted of root fragments, mycelia and spores in a vermiculite-sepiolite (1:1, v/v) substrate. Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were surface sterilized by immersion in 50% commercial bleach solution containing 0.02% (v/v) Tween20 for 10 min. Then, the seeds were rinsed thoroughly with sterile water and incubated for 14 days in sterile vermiculite at 25º C. Tomato plantlets were then transferred to 300 mL pots filled with sand, loamy soil and vermiculite (1:1:1, v/v/v), supplemented or not with mycorrhizal inoculum as described below. The soil was collected at the grounds of IFAPA (Granada, Spain) (Quiroga et al., 2017). The soil was sieved (<2 mm) and steam-sterilized (100º C, 1 h for 3 days consecutively), and the sand and vermiculite were autoclaved (121º C, 20 min). Four AMF inoculation methods were performed: control plants without AM fungal inoculation (Non mycorrhizal, Nm); inoculated with *R. irregularis* (5% v/v) (Ri); with *F. mosseae* (5% v/v) (Fm); and with a mix of both, *R. irregularis* (5% v/v) and *F. mosseae* (5% v/v) (FmRi). The Nm received the same amount (5% v/v) of sterilized vermiculite-sepiolite as the other inoculants. All plants received an aliquot of a filtrate (<20 μm) of the 2 AM fungal inocula in order to provide the microbial populations accompanying the AM fungi. Plants were grown in a glasshouse under controlled conditions (24 – 18º C; 16: 8 h, light: dark), watered when necessary with tap water and brought to field capacity once a week. Each week, plants were watered with Long Ashton nutrient solution (Hewitt, 1953) with reduced phosphorus concentration (0.335 mM) to promote symbiosis establishment. Plants were harvested after 6 weeks of growth, and the fresh weight of shoots was determined. Root and shoot material were immediately frozen in liquid nitrogen and stored at − 80º C. A homogeneous aliquot of each individual root system was taken for mycorrhizal assessment and quantification.

2.2. Phytohormone and salt treatments

The different stress treatments were applied since transplantation to act on mycorrhizal establishment and were maintained along the experiment. For the salt stress treatment, plants were treated with 150 mM of NaCl two weeks after AM fungal inoculation. Any irrigation drainage from the substrate was carefully avoided in any subsequent irrigation. In order to test the effect of defense signaling activation and mimic both biotic and abiotic stresses, plants were treated aboveground with three different stress-related hormones. Prior to each phytohormone treatment the substrate was covered with a plastic to avoid any direct contact of the belowground tissues with the hormones. The stress treatments were applied by spraying the shoots until run off once a week from the second week upon AM fungal inoculation (four weeks in total). The hormone treatments were: i) Abscisic acid (ABA) 50 μM; ii) Methyl jasmonate (JA) 50 μM; and iii) Salicylic acid (SA) 100 μM. Hormone stocks were prepared in ethanol and then diluted in sterile demi-water containing 0.02% (v/v) tween20 before application. For the control treatment, plants were sprayed with a mock solution with the same ethanol concentration. Seven independent replicates were used for the Nm, Fm and Ri inoculations, and 11 replicates for the FmRi inoculation. Plants from the different treatments were distributed in the greenhouse following a fully randomized design.

2.3. Mycorrhizal quantification

Mycorrhizal quantification was determined as described in García et al. (2020) by root histochemical staining after clearing the roots in 10% KOH and staining the fungal structures with 5% black ink in 2% acetic acid solution (Vierheilig et al., 2005). Mycorrhizal colonization was determined following the gridline intersection method (Giovannetti and Mosse, 1980) using a Nikon SMZ1000 stereomicroscope. Quantification of AM fungal structures (arbuscules and vesicles) within the mycorrhizal roots was performed as described in Trouvelot et al. (1986).

2.4. Analysis of gene expression by qPCR

RNA extraction from roots, purification, synthesis of the corresponding cDNA and qPCR was performed as described in Gamir et al. (2020). We analyzed the expression of marker genes related to pre-symbiotic signaling, hormonal signaling and defense responses, nutrient exchange between the symbiotic partners coding for plant Pi transporters, sugar and lipid metabolism and transport and, those related to the control of the symbiosis by qPCR using gene-specific primers (Table S1). Relative quantification of specific mRNA levels were performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen, 2001). Expression values were normalized using the normalizer gene *SlEF-1α* encoding the tomato translation elongation factor-1α (López-Ráez et al., 2010). Four independent biological replicates per treatment were analyzed.

2.5. Determination of mineral nutrients in roots

Nutrient content of roots was measured at the Ionomic Laboratory of the Technical Services of the Estación Experimental del Zaidín (EEZ-CSIC) in Granada, Spain. Frozen roots were ground to a fine powder and lyophilized. Three or four biological replicates were analyzed for each treatment. Element concentrations were analyzed after acid digestion of the samples (50 mg dry weight), by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720–ES). Mineral nutrient data is shown in Tables S2 and S3.

2.6. Hormone quantification

OPDA, JA, ABA, and SA were analyzed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) from samples previously frozen, grounded and lyophilized (~50 mg) as described previously by Flors et al. (2008). The UPLC was interfaced into a triple quadrupole tandem mass spectrometer (TQD, Waters). LC separation was performed using an Acquity UPLC BEH C18 analytical column (Waters) at a flow rate of 300 ll min1. Quantifications were performed with MassLynx 4.1 software (Waters) using the internal standards as a reference for extraction recovery and the standard curves as quantifiers.

2.7. Statistical analyses

The effect of stress (factor levels: Control, NaCl, ABA, JA, SA), mycorrhizal inoculation (factor levels: Nm, Fm, Ri, FmRi) and their interaction on mycorrhizal colonization and shoot fresh weight was tested via linear modeling and Tukey's HSD post hoc test ($p < 0.05$) (the Nm treatment was not included in the analyses for mycorrhizal colonization). For a better fitting of models, mycorrhizal colonization was subjected to arcsine transformation of square root Correctness of model fitting was checked by using DHARMa R package (simulateResiduals function, Hartig and Lohse, 2022).

The effect of the experimental treatments on gene expression was analyzed by permutational analysis of variance (PERMANOVA, adonis function vegan R package, Oksanen, 2008). In this case, inoculation with *R. irregularis* and *F. mosseae* were treated as different variables in a crossing design (values 1/0). Because the genes were quantified in

different runs of qPCR for the different stress treatments, their expression values were normalized by calculating the standardized effect size of each gene expression relative to the non-mycorrhizal control without hormone treatment per run ($Value_{sample}$-$Mean_{control}$/$Desvest_{control}$). The matrix of gene expression was used as response variable in the PERMANOVA and the chemical treatment, *R. irregularis* inoculation, *F. mosseae* inoculation and their interactions as explanatory factors (using euclidean distance as measure of dissimilarity and 999 permutations). To illustrate the found effects in PERMANOVA, the expression levels of selected marker genes of the main defense pathways were checked across treatment levels (unpaired t-test analysis using Statgraphics), and a principal component analysis (PCA) was arranged for the whole gene expression matrix and the distribution of experimental factors plotted against their first two axes. Similarly, to better reveal patterns for the colonization of each fungus separately, two PCAs were arranged only for the set of samples inoculated with either *R. irregularis* or *F. mosseae*.

The effect of gene expression on colonization was studied via linear modeling. Due to the lack of records for the non-mycorrhizal treatment (i.e. no colonization), they were excluded from this analysis. In a first instance, the number of genes included in the analyses was reduced by variance inflation factor analysis (VIF) (vif function, car R package, Fox and Weisberg, 2011). Genes were removed progressively until VIF value was below 5. The remaining set of genes was subjected to stepwise model selection where variables were added-removed until the model reached the lowest Akaike Information Criterion (AIC) value (ols_step_both_aic function, olsrr R package, Hebbali, 2020). A final model was built with the selected set of genes, i.e. the least number of genes that better explained mycorrhizal colonization. Correctness of model fitting was checked by using DHARMa R package (simulateResiduals function, Harti and Lohse, 2022).

3. Results

3.1. Activation of plant stress signaling differentially impacts mycorrhizal colonization by different AM fungi

The exogenous application of defense phytohormones - ABA, JA and SA -, mimicking plant stress, had a low impact on plant growth. Salinity was the only treatment leading to a significant reduction of shoot biomass, evident in non-mycorrhizal plants (Nm) and in plants colonized by *F. mosseae* (Fm) or *R. irregularis* (Ri) as compared with non-stressed (Control) plants (Table 1). The interaction between stress and AM fungal inoculant significantly affected shoot biomass ($F_{12,138} = 5.84$, $P < 0.0001$), while the stress impact was lower in Fm or Ri plants, although only the combined inoculation with the two fungi (FmRi) completely suppressed the reduction in shoot biomass due to salinity. None of the hormonal treatments (ABA, JA and SA) had an effect on shoot biomass. Mycorrhizal inoculation per se did not significantly affect plant biomass ($F_{3,138} = 0.82$, $P = 0.4846$).

AM colonization of tomato plants was well established after 6 weeks of inoculation in all mycorrhizal inoculants, showing well-developed arbuscules, intraradical hyphae and vesicles. Absence of colonization was confirmed in non-inoculated (Nm) plants (data not shown). Mycorrhizal levels varied in response to the different AM fungal inoculants ($F_{2,110} = 5.44$, $P = 0.0056$), the different types of stresses ($F_{4,110} = 3.91$, $P = 0.0052$) and their combination ($F_{8,110} = 4.38$, $P = 0.0001$) (Fig. 1). Under control conditions, the lowest colonization levels (14.8%) were found in plants inoculated with *F. mosseae* (Fm), while *R. irregularis* (Ri) and the combined AM fungal inoculant (FmRi) showed greater colonization rates, 26.9% and 28.5%, respectively (Fig. 1). Remarkably, the double inoculation with both AM fungi (FmRi), containing the same amount of each inoculant, thus, containing double amount of total inoculant, did not result in a higher mycorrhizal colonization. Interestingly, the colonization level in the combination treatment (FmRi) was similar to that observed in Fm plants in the rest of treatments. Salt stress significantly increased mycorrhizal colonization in Fm plants, while it was reduced in Ri plants as compared with control conditions. ABA treatment only increased mycorrhization in the case of Fm plants, as observed under salt stress. No significant effects for the other phytohormones applied (JA and SA) were detected in Fm nor Ri plants. However, all the three hormonal treatments - ABA, JA and SA - reduced mycorrhizal levels when the combination of the 2 AM fungi FmRi was used. Noteworthy, colonization seems to have a maximum threshold under our system and experimental conditions close to 30% of the root length (Fig. 1, yellow line).

Table 1
Effect of the different treatments on shoot fresh weight (SFW) of tomato plants. Plants inoculated with *F. mosseae* (Fm), *R. irregularis* (Ri) or the double inoculation of Fm and Ri (FmRi) were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 μM), methyl jasmonate (JA 50 μM) and salicylic acid (SA 100 μM). Data represents means (n = 7 for Nm, Fm, Ri; n = 11 for FmRi). Data followed by an asterisk (*) are significantly different from the Nm Control by the HSD Tukey post hoc test ($p < 0.05$).

SFW (g)	C	NaCl	ABA	JA	SA
Nm	10.0 ± 0.2	8.0 ± 0.1 *	10.1 ± 0.2	9.9 ± 0.1	10.2 ± 0.1
Fm	9.4 ± 0.2	8.4 ± 0.1 *	10.3 ± 0.2	9.4 ± 0.2	10.0 ± 0.1
Ri	9.5 ± 0.2	8.5 ± 0.2 *	10.3 ± 0.1	10.0 ± 0.1	10.3 ± 0.1
FmRi	9.7 ± 0.1	9.6 ± 0.1	10.4 ± 0.1	10.0 ± 0.1	10.4 ± 0.1

3.2. Shoot hormonal treatments and salinity impact signaling pathways belowground

We performed an extensive transcriptional analysis of well characterized marker genes involved in hormonal signaling and defense responses, presymbiotic signaling, nutrient exchange between the partners -P, lipids and carbohydrate metabolism and transport- or control of the symbiosis (see Table S1) to explore the mechanisms underlying the observed changes in mycorrhizal colonization. First, we checked the activation of plant defense signaling pathways in the roots by the treatments analyzing the expression levels of selected marker genes of the main defense pathways (ABA, SA, JA) in non-mycorrhizal plants. Salt stress strongly induced ABA metabolism compared to the control (Table 2). It induced more than 6 times the expression of *NCED1*, encoding for a 9-*cis*-epoxycarotenoid 1 -a key enzymatic step in ABA biosynthesis- (Thompson et al., 2000). Salt stress also induced the expression of *Le4*, encoding an ABA-inducible dehydrin (Kahn et al., 1993), while it down-regulated the expression of an ABA-8'-hydroxylase (*ABA-hydrox*), involved in ABA catabolism (Nitsch et al., 2009). The results agree with a significant increase of ABA content in salt treated plants (Fig. S1). Salinity also induced SA-related pathogenesis related (PR) proteins as *P14c* and *Pr1b1* (Niderman et al., 1995; Tornero et al., 1997), while no significant changes were detected for marker genes associated with JA signaling. Regarding the hormonal treatments applied in the leaves, moderated changes were observed in roots. The periodic application of hormones in shoots did not alter endogenous root hormone levels in the roots (Fig. S1), but the hormonal signaling pathways were transcriptionally regulated (Table 2). ABA application reduced both the expression of *Le4* and *ABA-hydrox* with respect to the control plants. The ABA treatment also triggered the expression of *LapA*, a JA-dependent peptidase co-regulated by ABA (Chao et al., 1999). The JA application also significantly induced the expression of *LapA* (Table 2). Finally, the SA treatment reduced the expression of the ABA markers *Le4* and *ABA-hydrox*, and that of *LoxD*, encoding a lipoxygenase D involved in JA biosynthesis (Wasternack and Song, 2017). These results agree with the well-known negative crosstalk between the JA-SA signaling pathways (van der Does et al., 2013; Wasternack and Song, 2017). The expression of another lipoxygenase (*LoxA*) was also regulated by SA treatment. *LoxA* encodes a lipoxygenase from the 9-LOX branch of oxylipins, largely root specific (Itoh et al., 2002), and

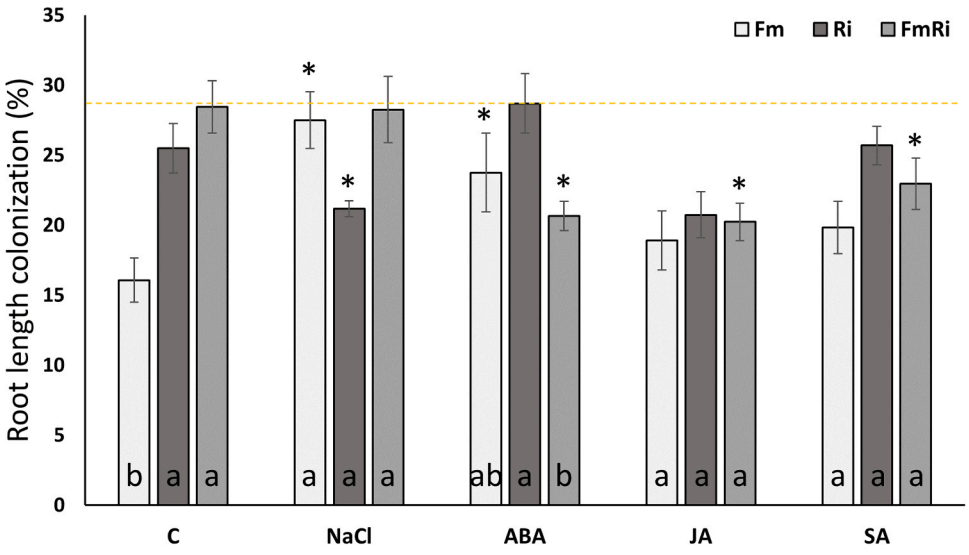

Fig. 1. Mycorrhizal colonization of tomato roots. Plants inoculated with *F. mosseae* (Fm), *R. irregularis* (Ri) or a double inoculation of Fm and Ri (FmRi) were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 μM), methyl jasmonate (JA 50 μM) and salicylic acid (SA 100 μM). Data represents the means ± SEM (n = 7 for Nm, Fm, Ri; n = 11 for FmRi). Yellow dotted line: hypothetical colonization threshold. Data from the different AMF inoculants within a given treatment not sharing a letter in common are significantly different according to the HSD Tukey post hoc test ($p < 0.05$). Columns marked by an asterisk (*) denote significantly different colonization levels of the treatments as compared to the untreated controls for each AM fungus (t-test, $p < 0.05$).

Table 2
Regulation of hormone marker gene expression by the different treatments. Plants were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or a weekly treatment on shoot of abscisic acid (ABA 50 μM), methyl jasmonate (JA 50 μM) and salicylic acid (SA 100 μM). Data correspond to fold change in the averaged gene expression in treated non mycorrhizal plants as compared to the untreated control (n = 4). Color indicates fold changes over 2 fold (up; red) and below 0.5 (down; blue). Bold values indicate significantly different to the control (t-test, $p < 0.05$).

Pathway	Gene	C	NaCl	ABA	JA	SA
ABA	*NCED1*	1.00	**6.02**	0.88	1.95	1.18
	Le4	1.00	**4.17**	**0.50**	0.69	**0.43**
	ABA-hydrox	1.00	**0.21**	**0.38**	1.21	**0.19**
JA/Oxylipins	*LoxA*	1.00	2.20	1.24	2.34	**3.54**
	LoxD	1.00	1.86	1.35	0.99	**0.29**
	Jar1	1.00	1.32	**0.67**	**0.71**	**1.61**
	PinII	1.00	3.09	1.48	0.58	0.92
	LapA	1.00	1.90	3.81	**3.38**	1.40
SA	*PAL*	1.00	1.09	0.94	0.81	0.74
	P14c	1.00	3.45	1.96	0.80	1.81
	PR1b1	1.00	**11.73**	1.00	1.03	2.74

generally antagonistic with the 13-LOX pathway responsible for JA biosynthesis. LOXA is involved in controlling the spread of the AM fungus within the roots and it is under the control of JA levels (León-Morcillo et al., 2012). Overall, the expression analysis revealed a moderate activation of the defense pathways in roots upon foliar application of the defense stress treatments.

Transcriptional data in the roots showed a significant effect of both mycorrhizal fungi and their interaction and the stress treatments on the gene expression profiles according to the PERMANOVA (Table 3). Noteworthy, only the interaction of the stress treatments with *F. mosseae* inoculation significantly impacted transcription, while *R. irregularis* did not induce significant changes in interaction with the stress treatments. When repeating the PERMANOVA excluding the salt treatment, the Fm x stress effect disappeared (data not shown), indicating that the modulation of gene expression by the presence of *F. mosseae* across the stress treatments was mainly due to the salt treatment. Considering all genes analyzed, the PCA analysis also illustrates the impact of the stress treatments on root transcriptional profiling, and a good correlation of the hormone-related marker genes was observed across the treatments

Table 3
PERMANOVA analysis of the effect of mycorrhizal inoculants, stress treatment and their interactions on the gene expression profile.

	Df	F	R^2	P
R. irregularis (Ri)	1	21.124	0.133	0.001
F. mosseae (Fm)	1	18.369	0.116	0.001
Stress treatment	4	9.092	0.229	0.001
Ri x Fm	1	13.868	0.087	0.001
Ri x Stress	4	0.955	0.024	0.470
Fm x Stress	4	1.938	0.049	0.010
Ri x Fm x Stress	3	0.875	0.017	0.586
Residuals	55		0.346	
Total	73		1.000	

(Fig. 2A). Noteworthy, the effect of mycorrhiza outperformed the effect of the stress treatments for the genes analyzed, completely separating them from the non-mycorrhizal treatment - explaining 30% of the variance - (Table 3, Fig. 2B). Interestingly, there was a common pattern associated with mycorrhizal colonization, as we observed a general

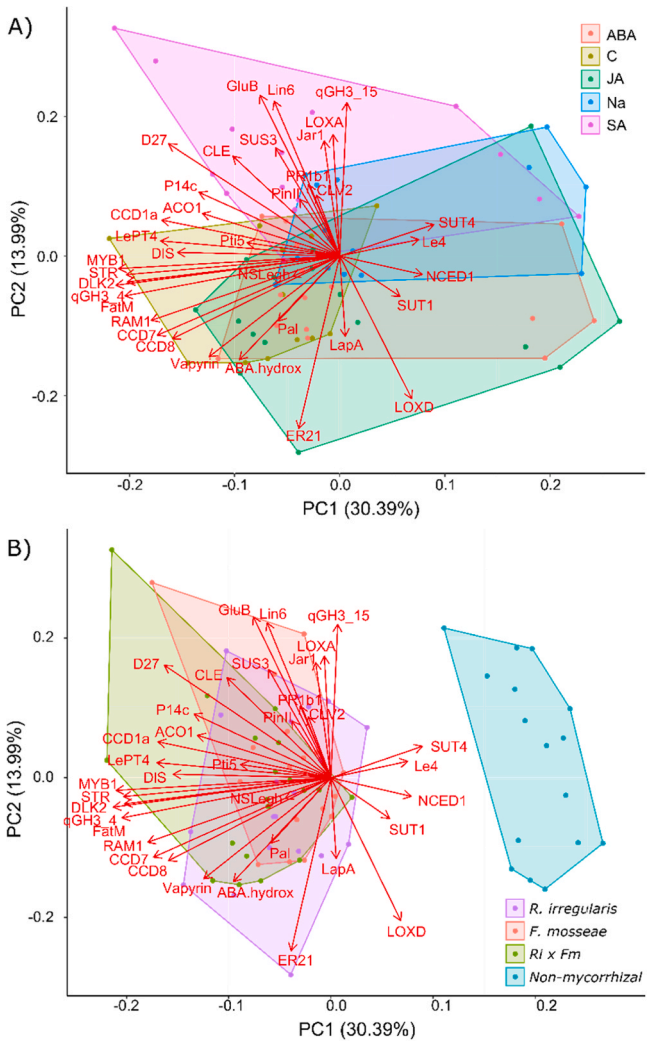

Fig. 2. Impact of the treatments in gene expression in roots. PCA ordination of gene expression profiles, according to stress treatment applied (A) or to mycorrhizal inoculation (B). Gene data were transformed into standardized effect sizes. Non-mycorrhizal (Nm) or mycorrhizal plants inoculated with *F. mosseae* (Fm), *R. irregularis* (Ri) or a combination of Fm and Ri (FmRi) were subjected to different treatments two weeks after inoculation: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 μM), methyl jasmonate (JA 50 μM) and salicylic acid (SA 100 μM).

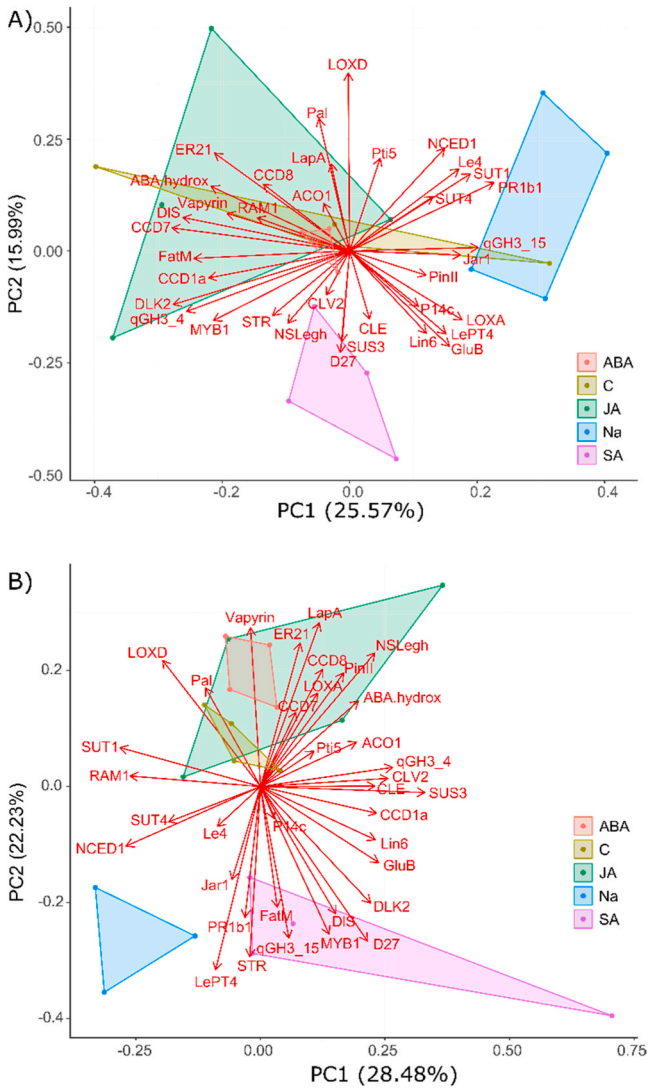

Fig. 3. Impact of the treatments in root gene expression. PCA ordination of gene expression profile in plants colonized by *R. irregularis* (A) or *F. mosseae* (B). Plants were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 μM), methyl jasmonate (JA 50 μM) and salicylic acid (SA 100 μM).

mycorrhizal fingerprint where all mycorrhizal inoculants clustered together (Fig. 2B). The full set of expression data including non-mycorrhizal and mycorrhizal plants are presented in Table S4. Finally, a more detailed analysis focused on the impact of the stress treatments on root transcriptional profiling separately in Fm and Ri plants revealed that the salt stress and the SA treatments had the strongest impact on the transcriptional profiles (Fig. 3A,B).

3.3. Correlation of root gene expression profiles and mycorrhizal colonization

Once the effect of stress treatments in root colonization and the transcriptional regulation of the corresponding marker genes was confirmed, we explored the potential correlation between gene expression and mycorrhizal colonization levels. Aiming to identify those genes whose expression pattern better explained the variation in AM colonization, we arranged a stepwise model selection until a model showing the lowest AIC value was obtained. The final model, built with the least number of genes explaining the mycorrhizal colonization data, included six genes: *LePT4*, *FatM*, *SUS3*, *P14c*, *GluB* and *PAL* (Fig. 4A). *LePT4* and *FatM* showed a positive correlation with colonization. They are both involved in nutrient exchange between the AM fungus and the plant. *LePT4* encodes for a plant Pi transporter active in arbuscocytes (arbuscule-containing cells) and linked to the Pi uptake through mycorrhiza (Balestrini et al., 2007). *FatM* is involved in the supply of lipids from the host plant to the fungus (Bravo et al., 2017). On the other hand, a negative correlation was found between colonization and the gene encoding the sucrose synthase *SUS3*, related to carbohydrate metabolism, and with three genes related to plant defense, *PAL*, *P14c* and *GluB*, coding for a phenylalanine ammonia lyase, a basic PR1 protein and a basic b-1,3 glucanase, respectively (van Kan et al., 1992; Gamir et al., 2017; Lefevere et al., 2020). Thus, the analysis pointed to major correlation of the colonization levels with genes related to nutrient exchange (positive correlation) and defense (negative correlation), but no changes in genes associated with pre-symbiotic signaling.

When a linear model was fitted, only *FatM*, *SUS3* and *P14c* significantly correlated with AM fungal colonization (Fig. 4A). Although these genes were those better explaining colonization levels, they also

A

	Estimate	SS	$F_{1,51}$	P
LePT4	0.007	0.006	1.511	0.225
FatM	0.035	0.023	5.755	**0.020**
SUS3	-0.011	0.022	5.697	**0.021**
P14c	-0.027	0.020	5.103	**0.029**
GluB	0.019	0.009	2.395	0.128
PAL	-0.005	0.009	2.239	0.141
Residuals		0.201		

B

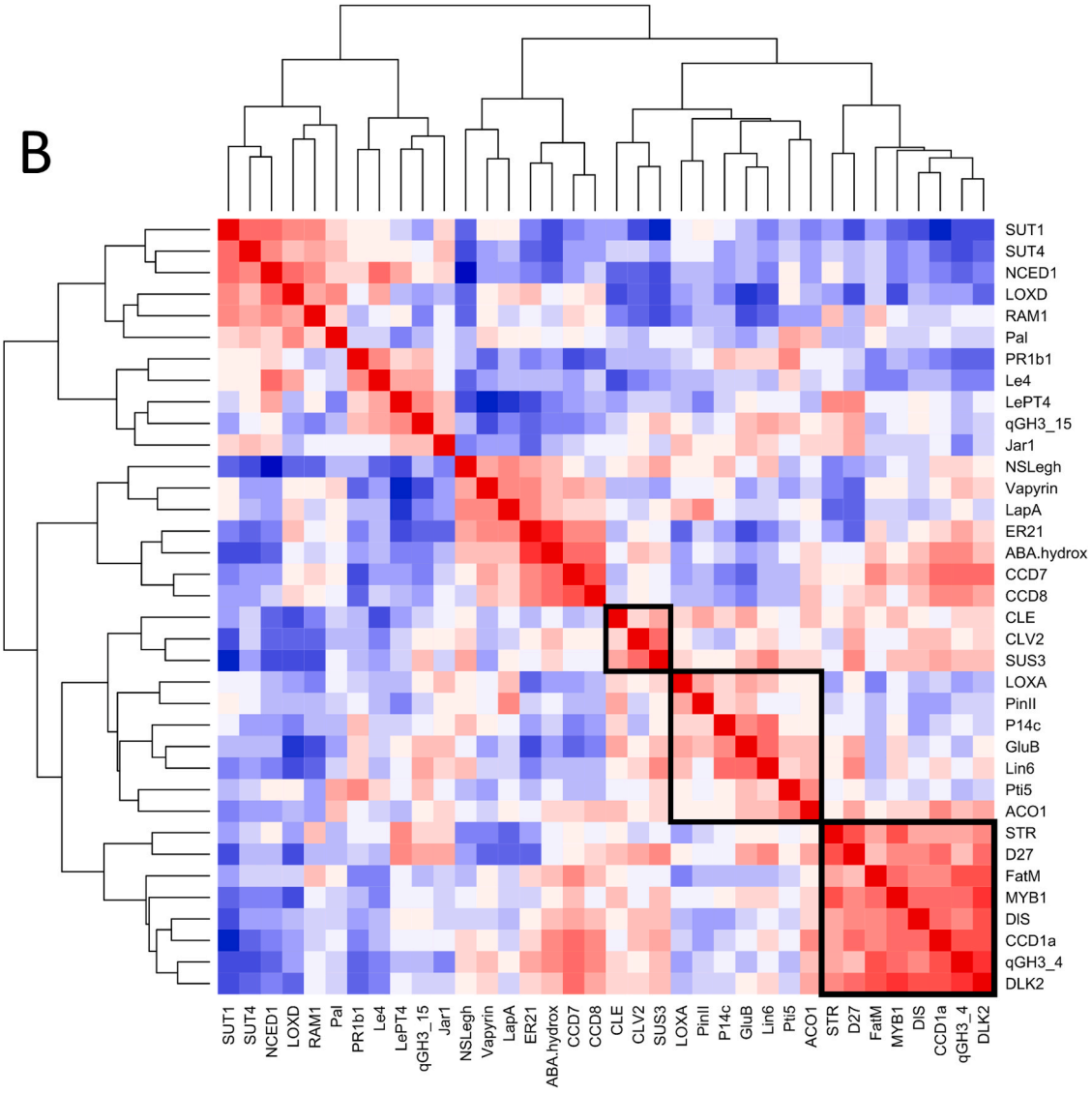

Fig. 4. Correlation matrix of gene expression. (A) Linear model showing the effect of the expression of selected genes on arbuscular mycorrhizal fungal colonization in roots (arcsine transformed). Model estimate, SS – Sum of squares, F values and degrees of freedom (as subscript) and associated P values. (B) Correlations across gene expression. Red colors denote positive correlations, blue denotes negative correlations. Color intensity indicates the correlation strength.

correlated with other genes that, due to their co-variation, were not retained in the statistical selection. Plotting the whole correlation matrix (Fig. 4B) allowed to reveal sets of genes that correlated with *FatM*, *SUS3* and *P14c* in explaining colonization levels. *FatM* positively correlated with *STR* and *DIS*, both involved in lipid transfer to the AM fungus (Keymer et al., 2017). *FatM* also matched with *MYB1-putative*, *DLK2*, *GH3.4*, *D27* and *CCD1a*, that are genes related to the control of the symbiosis (Fig. 4B) (Walter et al., 2015; López-Ráez et al., 2015; Floss et al., 2017; Ho-Plágaro et al., 2021). *SUS3* correlated with the genes associated with the autoregulation of AM symbiosis, with the regulatory

peptide *CLE* and its receptor *CLV2* (Wang et al., 2018; Müller et al., 2019; Karlo et al., 2020; Wulf et al., 2023). Finally, *P14c* matched with other defense-related genes, such as *GluB*, *Pti5*, *ACO1*, *PinII* and *LOXA*, and with the gene coding for the invertase LIN6 (Fig. 4B).

3.4. Salt stress differentially impacts expression of mycorrhizal-related genes depending on the colonizing AM fungus

Since the salt stress treatment had an opposite impact on root colonization by *F. mosseae* and *R. irregularis*, we further analyze the effect of this treatment in more detail. Salinity increased *F. mosseae* colonization and repressed that of *R. irregularis*, while no effect when using the combination of the 2 AM fungi was observed (Fig. 1). We compared the expression profiles of the genes selected from the linear model as significantly affecting AM fungal colonization - *FatM*, *SUS3* and *P14c* -, and those correlating with these genes, according to the correlation matrix and according to their function. The different functional groups showed differential regulation patterns depending on the colonizing fungus. For instance, under salt stress, the genes related to lipid transfer (*FatM*, *DlS* and *STR*) were repressed in Ri plants, while they did not change in Fm plants (Fig. 5). A similar pattern was found for *MYB1-putative* and *DLK2*, associated with the control of the symbiosis. In contrast, genes related to sugar metabolism (*SUS3*, *Lin6*) were repressed by salinity in Fm plants but induced in Ri. Regarding the genes associated with the autoregulation of the symbiosis *CLE* and *CLV2*, and those related with the biosynthesis of the 'yellow pigment', related to arbuscule control and turnover (*D27* and *CCD1a*), were repressed in Fm plants, whereas they did not change in Ri plants. Finally, the defense-related genes were generally induced in Ri upon salinity, while they did not change or were even partially repressed in Fm plants. Interestingly, plants with double inoculation (FmRi) showed intermediate patterns, generally with less pronounced changes than those occurring in the individual inoculations.

3.5. The impact of salt stress on symbiosis functionality depends on the colonizing AM fungus

The growth promotion effects in salt treated mycorrhizal plants were independent of the Na ion concentration. Salinity-treated plants had increased Na levels in both roots and leaves. The symbiosis did not significantly affect Na concentration nor the K/Na ratio (Tables S2 and S3). However, salt stress had a strong impact on the plant's acquisition of Pi. Under salinity P content was significantly reduced in roots in non-mycorrhizal plants and in Ri plants. In contrast, plants colonized by *F. mosseae* alone (Fm), or combined with *R. irregularis* (FmRi) did not show such reduction in P levels under salinity (Fig. 6A). P content in shoots followed the same trend, with a reduction in salt stressed plants except for Fm plants that even showed a significant increase in P levels (Fig. 6B). Pi uptake is a major benefit that the plant receives from the AM fungus, and the induction of plant Pi transporters in arbuscocytes is considered a hallmark of symbiosis functionality. We evaluated the expression of the mycorrhiza-specific Pi transporter *LePT4* tomato gene (Balestrini et al., 2007; López-Ráez et al., 2015). *LePT4* expression levels were significantly induced under salt stress in plants colonized by *F. mosseae*, up-regulated in Fm (4-fold) and FmRi (3-fold) but not in Ri plants (Fig. 6C). Thus, marker gene transcription and the P levels analyses support an increase in the efficiency of the symbiosis under salt stress conditions in plants colonized by *F. mosseae* (Fig. 6A,B).

We further assess the impact of salt treatment on the mycorrhizal symbiosis. Specifically, we analyzed the colonization levels of different inoculants, as shown in Fig. 1 and Fig. 7A. In addition, we assessed the abundance of intraradical fungal symbiotic structures with a particular focus on arbuscule and vesicle abundance. The abundance of arbuscules decreased significantly in Ri and FmRi plants under salt stress, whereas the abundance of arbuscules remained unchanged in Fm plants (Fig. 7B). On the other hand, under salt stress, vesicles, lipid containing reservoir structures from the fungus were reduced in Ri plants, alone (2-fold) or in combination (FmRi, 3-fold) (Fig. 7C). These results suggest that salt stress has a differential effect on the symbiotic structures depending on the colonizing fungus. The reduced expression of genes coding for the Pi transporter and lipid transfer in Ri plants - not observed in Fm plants - may underlie this reduction in arbuscules and vesicles in Ri plants.

The discrimination of individual fungal structures in the double inoculated FmRi treatment by microscopy is challenging, thus a molecular approach was employed to distinguish the colonization patterns of the two fungi. This was achieved through the use of AM fungi general (*HgEF*) or the species-specific primers for *F. mosseae* and *R. irregularis*, as described by Thonar et al. (2012). The analysis revealed that the AM colonization in Fm or FmRi increases in the presence of salt (Fig. S2), and this increase is enhanced when *F. mosseae* is inoculated in the double inoculation (Fig. 7D). Conversely, the colonization of *R. irregularis* decreases marginally with increasing salt concentration (Fig. S2), but in salt treatment the levels remain unchanged when *R. irregularis* is inoculated in combination with *F. mosseae*, as compared to its single inoculation (Fig. 7E). Thus, the molecular quantification of AM fungi confirm that the combined inoculation behave more like *F. mosseae* inoculation, with the salt concentration stimulating the growth of *F. mosseae*, while having little to no effect on the colonization pattern of *R. irregularis*.

4. Discussion

The use of AM fungi as biostimulants in agricultural and ecological settings is receiving increased interests for sustainable plant management. However, despite the well characterized benefits of the symbiosis, their application is still challenging because of the variability of the results when applied into production systems (Duhamel and Vandenkoornhuyse, 2013; Tkacz and Poole, 2015). This variability mainly relies on the impact of environmental conditions on the development and functionality of the symbiosis and on the functional diversity of the interaction between different plant-fungal genotype combinations (Hart et al., 2018; Holland et al., 2018; Kokkoris et al., 2019; Orine et al., 2022). Here, we hypothesized that the plant is able to regulate the development and the extension of mycorrhizal colonization according to the plant needs and its environmental context, and that this effect varies depending on the fungal partner. We found significant differences in mycorrhizal colonization between two different AM fungi (*F. mosseae* and *R. irregularis*), that vary depending on the treatments applied. We explored whether changes in colonization levels correlated with changes at the transcriptional level related to different plant signaling pathways. Interestingly, fungal colonization rates correlated with the modulation of the plant defensive responses, especially related to the SA-dependent pathway, changes related to carbon -lipids and sugars- supply from the plant to the fungus, and changes in the control and autoregulation of the symbiosis. Particularly, salt stress impacted differently on *F. mosseae* and *R. irregularis*, promoting the colonization by the first one and restricting the latest. This differential regulation seems to depend on the benefits provided by each fungus, with *F. mosseae*, but not *R. irregularis*, compensating the negative effect of salt stress on Pi acquisition. The plant restricted lipid supply, and enhanced defenses and symbiosis control in the interaction with *R. irregularis* under salt stress. In contrast, defenses and symbiosis control were reduced and lipid supply maintained in the interaction with *F. mosseae*. These results support the Kiers' free market hypothesis (Kiers et al., 2011), where greater benefits provided by the AM fungus is rewarded with a higher carbon input by the host plant.

It is known that the environmental context impacts organism homeostasis and may trigger systemic changes that modify its interactions with other organisms (Gruden et al., 2020). Phytohormones and their crosstalk play major roles in plant responses to the environmental context (Pozo et al., 2015). In the present work, we show that the exogenous application of stress related hormones to mimic stressful

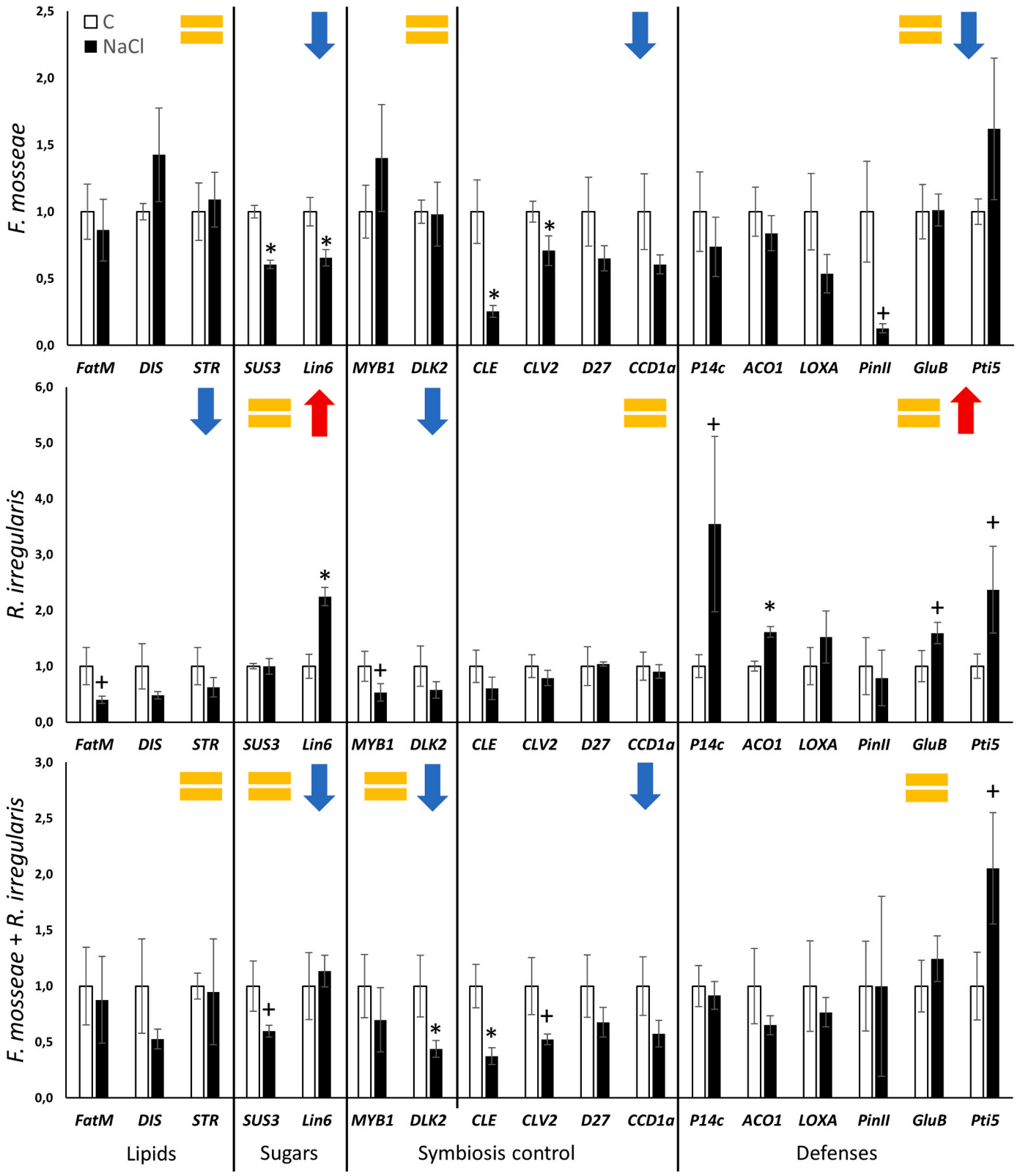

Fig. 5. Impact of salt stress on gene expression in plants inoculated with the different AM fungi. Mycorrhizal plants inoculated with *F. mosseae*, *R. irregularis* or a combination of Fm and Ri (FmRi) were subjected to salt stress (150 mM of NaCl solution) (NaCl) or left untreated (C). Bars represent relative gene expression values in salt treated plants normalized to the values in the control treatment (set to 1). Expression values were normalized in each sample using the normalizer gene *SlEF*. Data shown are mean ± SEM of four independent biological replicates. Statistical analysis was performed with unpaired t-test analysis between each respective control (C); + $p < 0.1$, * $p < 0.05$. Colored symbols represent the general trend (=, no changes; red arrow up, induction; blue arrow down, repression) in the regulation by salt for each given mycorrhizal treatment.

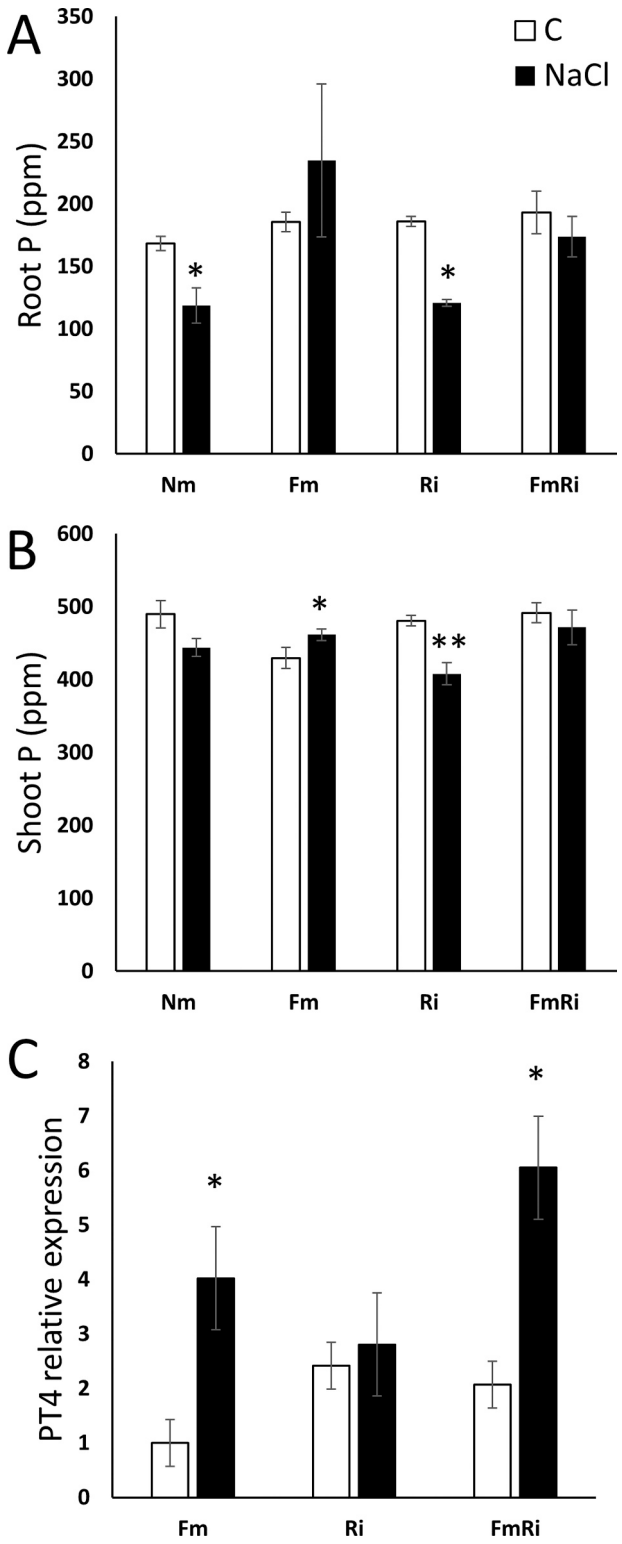

Fig. 6. Impact of salt stress on P acquisition by the plant. Non-mycorrhizal (Nm) or mycorrhizal plants inoculated with *F. mosseae* (Fm), *R. irregularis* (Ri) or a combination of Fm and Ri (FmRi) were subjected to salt stress by adding 150 mM of NaCl solution (NaCl) or left untreated (C). (A) Phosphorus concentration in (A) roots and in (B) shoot. (C) Relative expression of the plant phosphate transporter 4 (*LePT4*) normalized using the normalizer gene *SlEF*. Data shown are mean ± SEM of 4 biological replicates. Columns noted with an asterisk (*) are significantly different to their untreated controls (t-test, $p < 0.05$).

environments impacted mycorrhizal colonization. Our results support an active regulation of mycorrhizal colonization levels by the plant, that maintains the colonization within defined margins avoiding excessive colonization. This process is likely to be orchestrated by phytohormones: indeed, phytohormones have an effect on the establishment and maintenance of the AM symbiosis (Pozo et al., 2015; Ho-Plágaro and García-Garrido, 2022). However, promoting or repressing effects in mycorrhizal colonization for the same hormone have been sometimes reported, probably depending on the partners genotypes and the environmental/experimental conditions determining plant needs (Pozo et al., 2015; Bedini et al., 2018). This observation fits with our results showing that the impact of the stress conditions on colonization is dependent on the AM fungal genotype. The initial colonization rates seemed to be determinant, as the plant promoted colonization of the lower colonizer (*F. mosseae*) to reach the hypothetical maximum threshold, while it restricted the colonization by the most efficient colonizer *R. irregularis*. The hypothesis of a maximum threshold is supported by the fact that the double inoculation, containing the sum of both inocula, did not reach higher levels than those achieved by *R. irregularis* alone. The results support that mycorrhizal colonization, once established, is well-controlled in a delimited margin probably adjusted depending on the context through a process known as autoregulation (Wang et al., 2018). *R. irregularis* is a very efficient colonizer, usually reaching higher levels than other AM fungi including *F. mosseae* (López-Ráez et al., 2010; Voříšková et al., 2019; Liu et al., 2022). In fact, *R. irregularis* is usually one of the most abundant fungi within roots in natural soils despite multiple AM fungal species being present in the soil (Varela-Cervero et al., 2015). The mechanisms underlying such success are under scrutiny, but several effectors with immunomodulatory properties have been described in *R. irregularis*, and to what extent are conserved among different AM fungi is yet to be explored (Zeng et al., 2018).

We aimed to investigate the mechanisms contributing to the regulation of mycorrhizal colonization under stress conditions. Following a transcriptomic approach with well characterized marker genes we analyzed the contribution of different signaling pathways to the changes in colonization observed in our system. The changes, both at the colonization and transcriptional levels, were more evident under salt treatment -the only treatment actually reducing plant biomass- and that can affect not only the symbiosis but also the AM fungi directly. Indeed, salinity affected the colonization by the 2 AM fungi differentially. Salt stress led to a reduced P content in roots and shoots of non-mycorrhizal and *R. irregularis* plants, but *F. mosseae* plants did not show such reduction. A protective effect of AM fungi against salinity has been shown in several plant species, such as lettuce, maize and tomato (Aroca et al., 2013; Estrada et al., 2013; Rivero et al., 2018). The protection is usually related to enhanced plant tolerance by increasing water and nutrient uptake, photosynthesis capacity and a better ionic balance and homeostasis (Ruiz-Lozano et al., 2012; Evelin et al., 2019). Indeed, it is described that the main benefit of AM symbiosis in salt stress is the mitigation of the reduced Pi uptake (Porcel et al., 2012). Here, while *F. mosseae* protected the plant against Pi depletion by salinity, no protection was observed in *R. irregularis* colonized plants. These changes correlated with the promotion of colonization by *F. mosseae* and with a reduced colonization by *R. irregularis* although we cannot exclude the effect of salinity on the fungus itself (Yamato et al., 2008) and on its colonization capacity. However, the transcriptional regulation in the plant to promote the most efficient AM symbiont (*F. mosseae*) and restrict the most demanding one (*R. irregularis*) is likely.

We explored different potential mechanisms contributing the regulation of colonization through the analysis of marker genes related to pre-symbiotic signaling, plant defenses, control of nutrient exchange or specific control/autoregulation of the AM symbiosis. Rhizospheric signaling, mainly orchestrated by strigolactones, is involved in the *de novo* recruitment of mycorrhizal fungi and it is supposed to be stimulated under environmental stresses (López-Ráez et al., 2008; Aroca et al.,

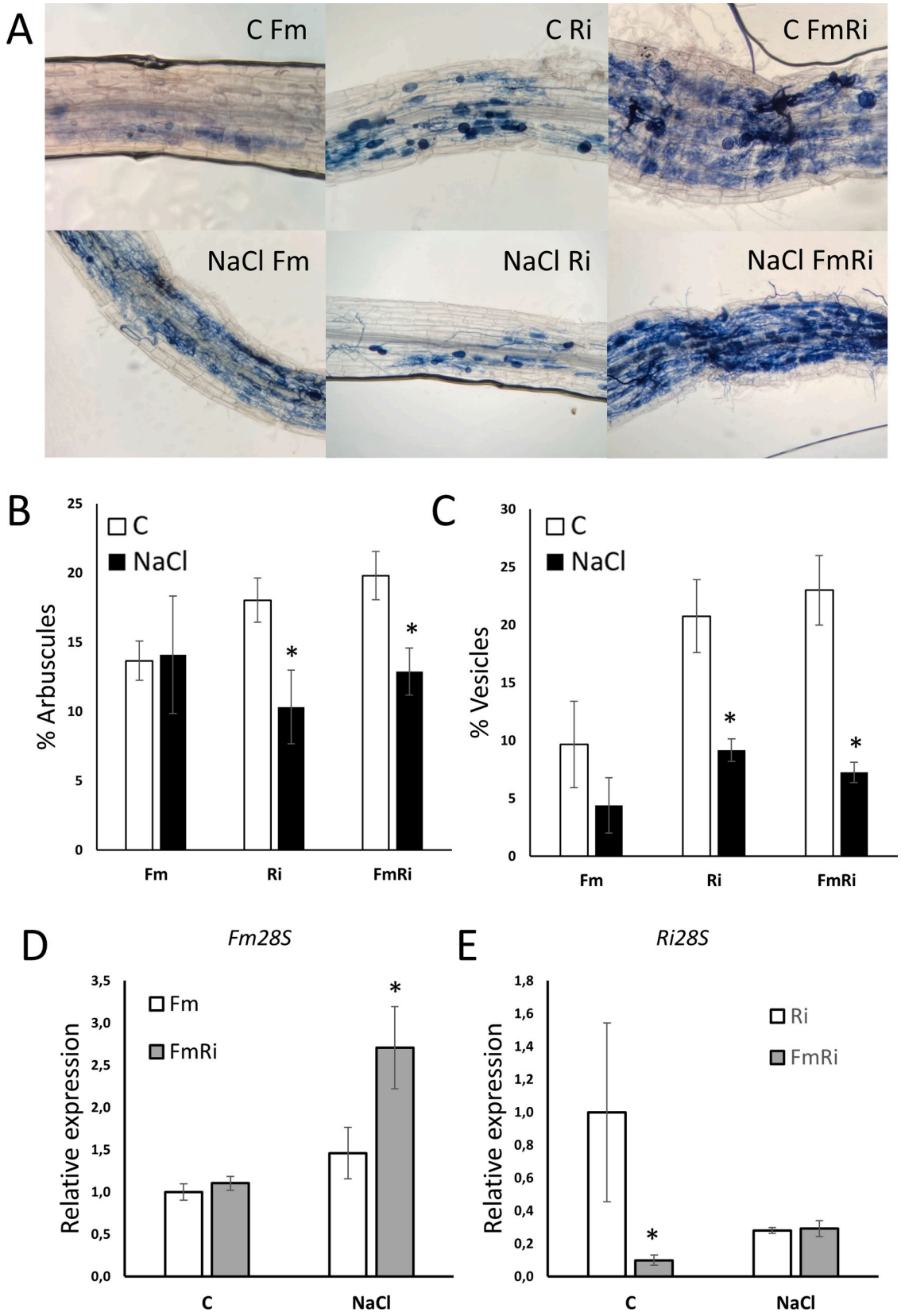

Fig. 7. Mycorrhizal colonization and DNA fungal quantification in salt stress treatment. Non-mycorrhizal (Nm) or mycorrhizal plants inoculated with *F. mosseae* (Fm), *R. irregularis* (Ri) or a combination of Fm and Ri (FmRi) were subjected to salt stress by adding 150 mM of NaCl solution (NaCl) or left untreated (C). (A) Representative images of mycorrhizal colonization in the different treatments. Abundance of fungal (B) arbuscules or (C) vesicles within the colonized areas. (D) Relative expression of *F. mosseae* specific primer *Fm28S*. (E) Relative expression of *R. irregularis* specific primer *Ri28S*. Expression values were normalized using the tomato reference gene *SlEF*. Data shown are mean ± SEM of 5 (B,C) or 4 (D,E) independent biological replicates. Statistical analysis was performed with unpaired t-test analysis; * $p < 0.05$, * * $p < 0.01$.

2013; Ruiz-Lozano et al., 2016). Analysis of genes involved in strigolactone biosynthesis do not support a role of early signaling during the pre-symbiotic stage in the changes observed. Thus, we did not find support for a relevant "cry for help" under our experimental conditions, likely because, pre-symbiotic contact was already established as the treatments started two weeks upon inoculation (Al-Babili and Bouwmeester, 2015). In contrast, overall changes in the colonization levels correlated with the regulation of plant defenses, nutrient exchange and control of the symbiosis. Our global analysis points out that the tradeoff between nutrients provided by the fungus (P) and by the plant (C) plays a key role in the differential regulation of colonization. Under salt stress, *F. mosseae* colonization increased in the single (Fm) and the double inoculated (FmRi) plants. This enhanced colonization correlated with higher expression of the mycorrhiza-specific plant Pi transporter *PT4*. This transporter is required for symbiotic Pi uptake, being a well-documented marker of mycorrhizal levels and symbiosis functionality (Harrison et al., 2002; Balestrini et al., 2007). Similarly, under drought conditions, plants actively promote the colonization of *F. mosseae,* that showed the greater symbiotic benefits, by preferential carbon allocation (Forczek et al., 2022). It has been shown that the host plant regulates carbon supply to the fungus based on the Pi input received (Helber et al., 2011), and that enhanced lipid allocation towards the fungus promotes arbuscule formation (Feng et al., 2020). Colonization by *R. irregularis* -not providing benefits against the stress in terms of P uptake- was reduced under these stress conditions. The reduction correlated with lower expression of the genes involved in lipid synthesis and delivery to the AM fungus in the arbuscocytes, including *FatM* (encoding for an ACP-thioesterase) and *DIS* (disorganized arbuscules, encoding a β-keto-acyl ACP synthase I) and *STR2* (Stunted Arbuscule 2, encoding an heterodimeric Adenosine Triphosphate (ATP)-Binding Cassette (ABC) transporter) (Keymer et al., 2017). Remarkably, these genes were not inhibited by salt in the interaction with *F. mosseae*, supporting the idea of an active control of the plant over fungal colonization depending on the benefits obtained. Regarding the control of the arbuscule itself, salt reduced the expression of the negative regulator of arbuscule branching (*DLK2*) (Ho-Plágaro et al., 2021) and its senescence (*MYB1*) (Floss et al., 2017) in plants colonized by *R. irregularis* and the dual inoculation, likely promoting arbuscule formation to enhance symbiosis functionality.

Colonization levels by *F. mosseae* and *R. irregularis* also correlated with the modulation of plant defense responses. It is known that plants require a precise finetuning of their immune system in order to contain potential attackers while promoting mutualistic interactions (Zamioudis and Pieterse, 2012; Zipfel and Oldroyd, 2017; Plett and Martin, 2018; Martínez-Medina et al., 2019). Under salt stress, a higher induction of defensive genes was observed in plants inoculated with *R. irregularis* as compared with those colonized by *F. mosseae*. This suggests that the plant is more actively trying to control this fungus, likely very demanding as it is a very good colonizer and displays a high number of vesicles -fungal energy reservoirs-. This enhanced defense response correlated with a reduction in mycorrhizal levels and vesicles abundance, indicating that the plant is indeed controlling colonization rates and nutrient flux to the fungus, likely favoring those AM fungi that are more efficient in nutrient supply. In agreement with this idea, a reduction in the expression of genes associated to symbiosis autoregulation -*CLE* and *CLV2* - was observed in plants inoculated with *F. mosseae* as compared with those colonized by *R. irregularis*. The same behavior was observed for the gene *CCD1a*, involved in the biosynthesis of mycorradicin and α-ionols that regulate the arbuscule lifespan (Walter et al., 2015). Taken together, our results support the idea that the tradeoff between mineral nutrients and carbon between the AM fungus and the host plant drives symbiotic levels and efficiency, and that it affects the modulation of plant defenses and the autoregulation of the symbiosis.

Overall, we show here that different AM fungi have distinct colonization strategies, and that host stress may differently affect the symbiosis. Our results points to a modulation of colonization extension according to nutritional tradeoffs, following the motto 'more for the better'. Moreover, this study support the resilience of mycorrhizal interactions, as despite the activation of different defensive pathways, the system buffers the changes, and overall, mycorrhizal colonization is maintained within a given range, likely balancing the interaction for mutual benefit. Finally, the results suggest that the inoculant combining AM fungal strains was the most efficient in stress alleviation, and seems to be more stable across treatments. This point the interest of using AM fungal consortia as commercial biostimulants in order to obtain improved benefits and enhanced stability in the ever-changing conditions in agrosystems, especially in the current context of climate crisis.

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CRediT authorship contribution statement

Javier Lidoy: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Álvaro López-García:** Formal analysis, Data curation, Writing – review & editing. Clara Amate: Investigation, Formal analysis. **Juan Manuel García:** Methodology, Supervision, Resources, Writing – review & editing. **Victor Flors:** Formal analysis. **José Manuel Garcia-Garrido:** Investigation. **Concepción Azcón-Aguilar:** Conceptualization, Supervision, Validation, Project administration, Writing – review & editing. **Juan Antonio López-Raez:** Validation, Funding acquisition, Writing – review & editing. **María José Pozo:** Conceptualization, Methodology, Supervision, Validation, Project administration, Funding acquisition, Writing – review & editing.

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.

Data Availability

Data will be made available on request. All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105479.

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