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Marchantia polymorpha subsp. ruderalis (Bischl. & Boissel.-Dub.) -arbuscular mycorrhizal fungi interaction: beneficial or harmful?

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Abstract
Marchantia polymorpha is a common liverwort whose use as a model plant in physiological and evolutionary processes is increasing in recent years. As far as plant-microorganism interactions are concerned, there are still few studies conducted with *M. polymorpha*. Specifically, in the interaction of *M. polymorpha* with arbuscular mycorrhizal fungi (AMF), it has been described how AMF colonize the *M. polymorpha* tissues, without knowing more about the interaction. In this study, *M. polymorpha* is inoculated with different AMF formulations, analyzing the direct effect on *M. polymorpha*'s growth and the nutritional content, along with stress responses. Moreover, expression levels of defense genes in *M. polymorpha* were analyzed. The results obtained showed how *M. polymorpha*-AMF interaction is detrimental to plant under in vitro conditions. A reduction in its growth and viability of its tissues was observed, in addition to an increase only in nutritional content of those elements related to plant defenses, together with the reactive oxygen species (ROS) content. *Rhizophagus fasciculatus* is only present in the formulation that causes major damage to the plant, including symptoms of tissue damage, and that mostly colonizes the plant. It suggests its possible role as a plant pathogen, due to the inability of *M. polymorpha* to defend it against the AMF by the route of salicylic acid (SA).

Keywords *Marchantia polymorpha* · Liverwort · Arbuscular mycorrhizal fungi · Plant-microbe interactions · Plant defense · Plant colonization

1 Introduction

Marchantia polymorpha (L.) represents the most widely distributed common liverwort in temperate regions. Taxonomically, *M. polymorpha* belongs to the Marchantiales subclass (with most of 400 species), which in turn belongs to the Marchantiopsida class, Marchantiophyta division, and the *Bryophyta* sensu *lato* superdivision. *Marchantia polymorpha* thallus represents a plant tissue up to 15 mm in diameter and 0.6 mm thick, with radial growth through dichotomous divisions at the ends. This body has a clear dorsoventral differentiation, the upper area of assimilation, the middle storage area and the basal area where the rhizoids are formed (Shimamura 2015).

In *M. polymorpha* there are a small number of pathogens known, it having raised as a model plant in understanding the evolutionary mechanisms behind plant-microorganism interactions (Poveda 2020a). Four pathogenic fungal strains: *Irpex lacteus*, *Phaeophlebiopsis peniophoroides*, *Bjerkandera adusta* and *Bjerkandera adusta* have been isolated from diseased *M. polymorpha* (Matsui et al., 2020). Regarding beneficial fungi, the fungal endophytic microbiota of *M. polymorpha* was analyzed, from wild populations of the liverwort, obtaining a range from aggressively pathogenic to strongly growth-promoting, but the majority of isolates caused no detectable change in host growth (Nelson et al. 2018). These results shown that some known pathogens of vascular plants live in *M. polymorpha* and can confer benefits to this nonvascular host (Nelson et al. 2018). Great diversity of different fungal microbiomes that can be found in *M. polymorpha* has been reported from different geographical areas (Nelson and Shaw, 2019).

Arbuscular mycorrhizal fungi (AMFs) can form symbiotic relationship with 80% of plant species (Ferlian et al. 2018). At present, molecular and paleobiological studies have shown how the origin of these fungi and that of terrestrial plants occur simultaneously over time (about 470 million years ago), being necessary for the success of plant terrestrial colonization

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(Feijen et al. 2018; Rimington et al. 2018; Strullu-Derrien et al. 2018).

Development of a mycorrhizal symbiosis requires a continuous signal exchange between the two symbionts, which triggers coordinated differentiation of both partners, to enable their interaction within the root cells, there must be a partial suppression of plant defense responses (Liao et al. 2018). Mycorrhizal hyphae are able to colonize places where plant roots could never reach, in addition, these structures have the ability to absorb nutrients not available to the plant and have active transporters much more closely related to mineral nutrients (Berruti et al. 2016). As a result, the fungus can contribute to the plant phosphorus, especially, but also other nutrients of low mobility, such as ammonium, potassium, copper, iron, sulfur, molybdenum or zinc (Chen et al. 2018). In response, the plant mainly provides carbohydrates to the fungus, which meet their needs, although it does not have a negative impact on the plant, due to photosynthetic compensation for the fungal supply of nutrients and reduced root development (Berruti et al. 2016; Chen et al. 2018). Moreover, it is widely demonstrated that the inoculation of mycorrhizal fungi provides tolerance to host plants against various stressful situations such as heat, salinity, drought, metals and extremes of temperature (Begum et al. 2019) and biotic attacks (Poveda et al. 2020).

Regarding the specific interaction between AMFs and non-angiosperm plants, a study carried out by Ogura-Tsujita et al. (2016) described the diversity of mycorrhizal fungi existing in interaction with various species of ferns (*Osmunda banksiifolia*, *Diplopterygium glaucum*, *Dicranopteris linearis*, *Plagiogyria japonica*, *P. euphlebia*, *Cyathea podophylla* and *C. lepifera*), describing how up to 97% of gametophytes were internally colonized, specifically the multilayered midrib (cushion) tissue (Ogura-Tsujita et al. 2016). In this sense, the emergence of AMFs and land plants strikingly coincided at nearly 450 million years ago, with the earliest known fossils dated to 407 million years ago, suggesting that associations with fungi probably facilitated land colonization and true root development (Kamel et al. 2017).

Relating the AMFs and *Marchantia* there is only a few studies so far. Another species of liverwort, *M. foliacea*, has been observed to form a specialized symbiosis with the genus *Glomus* of AMF (Russell and Bulman, 2005). This symbiosis includes hyphae thalli-colonization and formation of arbuscules, but the benefits of interaction were not studied (Russell and Bulman, 2005). Also, in *M. nepalensis*, it was described how different AMFs are able to internally colonize the liverwort, forming what they call mycothalli (thalli invaded by fungus) (Verma and Langer, 2014). Similar results were observed in *M. pappeana* (Rimington et al. 2018) and in *M. paleacea* (Field et al. 2019). As far as *M. polymorpha* is concerned, Ligrone et al. (2007), exhaustively described the way in which AMFs of the genus *Glomus* are able to colonize liverwort tissues, being the first to find rhizoids colonized by fungi, along with a wide colonization of parenchymal tissues.

The objective of this work is to deepen the knowledge of the *M. polymorpha*-AMFs interaction. The way in which fungi colonize liverwort tissues has already been widely described, but the possible benefits or harms that the plant can obtain from such colonization have not been advocated, nor the defensive responses of the plant that allow such colonization. Since liverworts are at the evolutionary base of vascular plants, the analysis of the *M. polymorpha*-AMF interaction becomes essential for the evolutionary understanding of symbiosis, something fundamental to increase its efficiency in current agricultural and forest production systems.

2 Materials and methods

2.1 Plant and fungal material

Marchantia polymorpha Tak-1 (subsp. *ruderalis*) was kindly provided by the Jasmonate Signalling in Plants Group (National Center for Biotechnology-CSIC, Spain). Plants were always maintained in active asexual growth in Gamborg (GMB) (Duchefa, Haarlem, the Netherlands) solid medium (agar 1%) in a growth chamber at 22 °C, 40% relative humidity (RH) and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}.

As AMFs inoculation the mycorrhizal formulations MT-01 (M1), MT-02 (M2) and MT-03 (M3), was used. Each formulation contained at least 1×10^6 spore kg^{-1} in an inert substrate and included different AMF species. M1: *Rhizophagus irregularis* and *Funneliformis mosseae*. M2: *Glomus microagregatum*, *F. mosseae*, *Claroideoglomus claroideum*, *R. irregularis* and *R. fasciculatus*. M3: *G microagregatum* and *C. caroideum*.

2.2 Interaction assay

In order to put *M. polymorpha* and AMF in direct contact, fragments of approximately 1 cm^2 of *M. polymorpha* thalli were deposited inside Phytatray II boxes (Sigma, St. Louis, MO, USA) (6 fragments per box), prepared as follows. In each Phytatray II box 100 mL of a sterile mixture of peat/vermiculite (3:1) was deposited in the bottom. Subsequently, the culture substrate was irrigated with 15 mL of sterile water and 3 mL of liquid GMB. Six *M. polymorpha*-thalli were transferred to each box. During the life of the plants the boxes were kept inside a growth chamber at 22 °C, 40% RH and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}, always in sterile conditions.

AMF inoculations were done by burying 100 mg of different fungal inoculum (M1, M2 and M3) (1000 spore g^{-1}) per *M. polymorpha*-thalli at 1 cm below the substrate surface, just

before transplanting the liverworts. Each assay was repeated 3 times with 4 boxes per condition in each repetition: *M. polymorpha* without inoculation (Mp), inoculated with M1 (Mp + M1), inoculated with M2 (Mp + M2) and inoculated with M3 (Mp + M3); encompassing a total of 24 plants per condition and repetition. The assay was maintained for 5 weeks, after which samples were collected for each of the subsequent analysis: 2 plants per box for molecular analysis (qPCR and RT-qPCR), 1 plant per box for fungal staining, 1 plant per box for viability and oxidative stress analysis, and 2 plants per box for dry weight and macro and micronutrient analysis.

2.3 Plant growth analysis

The quantification of the plant/above ground biomass of *M. polymorpha* was carried out by two methods. Firstly, five weeks after AMF inoculation the plants were photographed in the inside Phytatray II boxes, and the images obtained were analyzed for the quantification of the percentage of existing plant by the software MulticolorEngine (TinEye, Toronto, Ontario, Canada) (https://labs.tineye.com/color/). In this way, we could measure the quantity of plant according to its occupation in a 2D plan. In the second method dry weight of each plant was determined by keeping the fresh plant for 48 h at 65 °C; using a total of 8 plants per condition and repetition.

2.4 Macro- and micro-nutrient analysis

First, a pool was formed with all the plants of each condition used to calculate the dry weight and minced. The pulverized plant material (3 pools for each condition: one pool for each repetition) was sent to the Ionomics Service of CEBAS-CSIC (Spain), who performed the content analysis in C, N, P, K, S, Ca, Mg and Fe, using the instrument Elemental Analyst model TruSpec CN628 in the case of N, and the ICP THERMO ICAP 6500DUO for the analysis and detection of the remaining macro- and micronutrients.

2.5 Colonization analysis

Both for the molecular analysis of the colonization and for the analysis of gene expression, 2 plants were collected per box (8 per condition) and repetition. Each plant was superficially washed with sterile water and immediately frozen in liquid nitrogen and pulverized with a mortar. The quantification of AMF DNA in *M. polymorpha* tissues was performed by qPCR as previously described Poveda et al. (2019), starting from different types of tissue. DNA was extracted from the tissues with a cetyl-trimethyl-ammonium bromide (CTAB) extraction method, as reported previously (Dellaborta et al., 1983). A mix was prepared in a 10-μL volume using 5 μL of Brilliant SYBR Green QPCR Master Mix (Roche, Penzberg, Germany), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The 18S rRNA gene of AMF and the *Elongation Factor 1* (*EF1*) gene of *M. polymorpha* were used; their corresponding primer pairs are indicated in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) programmed for 40 cycles under the following conditions: denaturation, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from 3 tissue-pools of 8 plants each one for each condition. Cycle threshold values served to calculate the amount of fungal DNA using standard curves. Values of AMFs DNA were referred to the amount of *M. polymorpha* DNA in every corresponding sample.

For visual observation of colonized *M. polymorpha* tissues, AMFs were collected one plant per box (4 plants per condition) and repetition. The methodology of staining AMFs in plant tissues with trypan blue described by Frater et al. (2018) was followed, with some modification. *M. polymorpha* plants were cleared in a 2.5 to 5% KOH solution at 90 °C for 30 min, treated in alkaline H_2O_2 for 15 min, then acidified in 1% HCl for 15 min. After, plant-tissues were soaked in a trypan blue solution made of 1:1:1 (*v:v:v*) glycerol, lactic acid, water and 1% (v) of 0.2 μm filtered trypan blue solution for 20 min on a hot plate set to 90 °C.

2.6 Vitality test

Reduction of triphenyltetrazolium chloride (TTC) by tissue to the red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF (Ruf and Brunner, 2003). From pools formed from 4 halves of plants per condition and repetition, 100 mg were transferred to 1 mL of 1% TTC in triplicate and incubated for 48 h at 37 °C. After incubation, 100 mg of Ballotini Glass Balls 0.15–0.25 mm diameter and others 100 mg of 1 mm diameter were added to each sample in 1,5 mL Eppendorf tubes, shaking vigorously by a pulse of 20 s in Silamat S6 (Ivoclar Vivadent, Madrid, Spain). After centrifuging the samples for 15 min at 10,000 rpm, the supernatant was removed and 1 mL of isopropanol was added per tube. The samples were again agitated in Silamat and centrifuged in the same way, and the supernatant was used to quantify its absorbance at 620 nm, being an indirect measure of the vitality of the *M. polymorpha*-tissues.

2.7 Indirect quantification of ROS in tissues

The indirect quantification of reactive oxygen species (ROS) in *M. polymorph*-tissues was carried out by measuring

Table 1 Primers used in this work

Code	Sequence (5′-3′)	Use	References
AML-F AML-R	CTTTCGATGGTAGGATAGAGG ACAACTTTAATATACGCTATTGGA	18S rRNA to AMFs quantification	Lee et al. (2008)
Mp-EF1-F Mp-EF1-R	AAGCCGTCGAAAAGAAGGAG TTCAGGATCGTCCGTTATCC	Endogenous *M. polmorpha* gene	Yoshikawa et al. (2018)
Mp-ICS-F Mp-ICS-R	GACTATGAGGAGGTTTCTTTCC GCTACATTTACTGCAAGTAGGG	Synthesis gene of SA in *M. polmorpha*	Gimenez-Ibanez et al. (2019)
Mp-PR1-F Mp-PR1-R	TAACAACTGTCAGCTGAAGACC CTTCCAGACAACCTGAGTGTAA	Response gene of SA in *M. polmorpha*	Gimenez-Ibanez et al. (2019)
Mp-LOX1-F Mp-LOX1-R	GGCATATGGATTTACACACAGCGAG CCGGATCCTAGATGGAAATGCTCCAAG	Synthesis gene of JA in *M. polmorpha*	Kanamoto et al. (2012)
Mp-COI1-F Mp-COI1-R	AGGACAGAAGGCACTGAAGTTC CTGCTTCTCAGAAACAGTCATGC	Response gene of JA in *M. polmorpha*	Monte et al. (2018)

electrolyte leakage, similar to the method used by Aguilar et al. (2015) for leaf discs, which really measures cellular oxidative damage related to the production of ROS. From each tissue portion of 4 halves of plants per condition and repetition, 1 cm^2 of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the tissues (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total ions present in the tissues (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

2.8 Gene expression studies

The analysis of the expression of defense-related genes in *M. polymorpha* tissues was carried out in the same qPCR-tissue-pools by RT-qPCR, using the methodology described by Poveda (2020b), with some modifications. The RNA extraction was carried out with the TRI reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain), and then used for reverse transcription with an oligo (dT) primer with the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan), following the manufacturer's protocol. Gene expression was analyzed by RT-qPCR, using an ABI PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All PCR reactions were performed in triplicate in a total volume of 10 μL for 40 cycles under the following conditions: denaturation, 95 °C, 30 s; annealing, 60 °C, 1 min; extension, 72 °C, 1 min. Threshold cycles (CT) were determined using the 7000 SDS System Software (Applied Biosystems, Foster City, CA, USA), and CT values were calculated using the *M. polymorpha EF1* gene as an endogenous control. The primers used are given in Table 1: genes of the isochorismate synthase (*ICS*), pathogenesis-related protein 1 (*PR-1*), synthesis and response genes to salicylic acid (SA), respectively, and lipoxygenase 1 (*LOX1*), and coranatine-insensitive 1 (*COI1*), synthesis and response genes to jasmonic acid (JA), respectively. The choice of a synthesis gene and a response gene for SA and JA has been based on trying to cover the two main plant defensive hormonal pathways against pathogenic and beneficial fungi (Poveda, 2020c).

2.9 Statistical analysis

The statistical analysis of the data was carried out with the Statistix 8.0 software. One-way ANOVA using Tukey's multiple range test at $P < 0.05$ was used for pairwise comparisons; the different letters indicate the significant differences.

3 Results

3.1 Effect of AMFs on the growth and nutritional status of *M. polymorpha*

Negative effect of inoculation of AMF on the growth of *M. polymorpha* was observed after 5 weeks (Fig. 1b-d) compared to the uninoculated plants (Fig. 1a), in in vitro culture with AMF formulation that are not native to the soils from which the plants have been extracted. Furthermore, damaged tissues (pale-brown colors) were observed in the interaction of *M. polymorpha* with the formulation M2 (Fig. 1c). We observed both at the level of percentage of existing plant (as determined by MulticolorEngine method) and dry weight, a

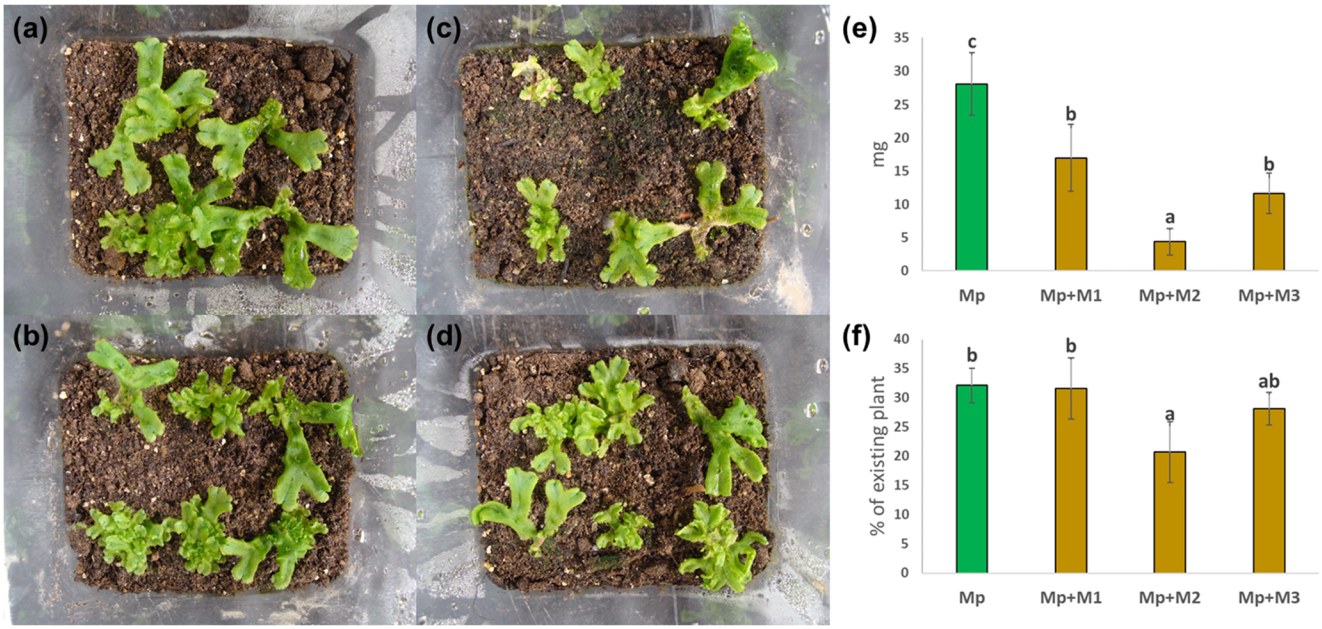

Fig. 1 Effect of AMFs on the growth of *M. polymorpha* 5 weeks after inoculation. Visual appearance of Mp (a), Mp + M1 (b), Mp + M2 (c) and Mp + M3 (d), and plant biomass analyzed by visual quantity of the plant (e) and the dry weight (in mg) (f). Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, eight plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

significant decrease occurred in the plants inoculated with the formulated M2 (Mp + M2) (Fig. 1e-f) compared to *M. polymorpha* without inoculating (Mp). This significant decrease with M2 also occurred in dry weight compared to the application of formulations M1 (Mp + M1) and M3 (Mp + M3) (Fig. 1e). In the case of the percentage of existing plant, there were no significant differences between the application of M2 and M3, nor between the application of M3 or M1 and uninoculated plants (Fig. 1f).

As regards the macro- and micro-nutrient content in *M. polymorpha* tissues (Table 2), the analysis of the total C present in *M. polymorpha* tissues showed how inoculation with the AMFs implies a significant decrease in their content, being even more significant with the M1 and M3 fungal inoculum. In N content, inoculation with AMFs implied an increase in plant tissues, being even more significant with M1 and M3 formulations. Results similar to those quantified in the content of K, except with the M2 formulation, which did not present significant differences to the control without inoculation. As far as P is concerned, inoculation with AMFs implied a significant decrease in their content in *M. polymorpha* tissues. Opposite to what is observed in the content of S and Ca, with a significant increase after inoculation with all AMFs inoculum. Results also quantified in the Fe content, except that being even more significant with the M2 formulation. Finally, there were no significant differences in the Mg contents of plant tissues.

Table 2 Macro- and micro-nutrient content in *M. polymorpha*

	C[1]	N[2]	P[2]	K[2]	Ca[2]	S[2]	Mg[2]	Fe[3]
Mp	48.31 ± 2.03c	28.79 ± 2.65a	2.56 ± 0.39b	18.29 ± 1.58a	1.28 ± 0.17a	2.35 ± 0.26a	10.23 ± 1.26a	86.63 ± 9.75a
Mp + M1	41.65 ± 0.89a	34.89 ± 1.03c	1.74 ± 0.22a	22.56 ± 0.26b	1.64 ± 0.09b	2.83 ± 0.13b	9.87 ± 0.68a	112.36 ± 6.89b
Mp + M2	44.87 ± 1.38b	32.58 ± 1.35b	1.36 ± 0.58a	16.59 ± 2.03a	1.83 ± 0.23b	3.01 ± 0.35b	11.12 ± 1.58a	158.01 ± 15.27c
Mp + M3	40.96 ± 1.13a	35.65 ± 1.24c	1.28 ± 0.69a	23.89 ± 3.23b	1.52 ± 0.02b	2.78 ± 0.08b	10.56 ± 0.98a	107.98 ± 8.59b

Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, pools of eight plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

[1] Nutrient content in %

[2] Nutrient content in mg g^{-1} of plant tissue

[3] Nutrient content in mg kg-1 of plant tissue

Table 3 *M. polymorpha* tissues AMFs colonization

Treatments	*M. polymorpha*			AMFs			Ratio[c]
	Ct	SD	Qty[a]	Ct	SD	Qty[b]	
Mp + M1	20.98	0.03	0.90	30.14	0.19	0.08	0.09 ± 0.01a
Mp + M2	20.69	0.11	1.20	27.53	0.12	0.78	0.65 ± 0.04b
Mp + M3	20.56	0.06	1.25	29.58	0.21	0.25	0.20 ± 0.03a

[a] Quantity of plant DNA (ng) referred to *EF1* gene

[b] Quantity of fungi DNA (ng) referred to 18 s rRNA

[c] Proportion of fungal DNA vs. plant DNA. Values are the means of three *M. polymorpha* pools (eight plants each one) from three independent experiments with the corresponding standard deviations. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

Quantification of AMFs DNA in *M. polymorpha* (5-week-old plants) was performed by qPCR, using the 18S rRNA for AMF

3.2 Colonization of *M. polymorpha* by AMFs

The colonization levels of *M. polymorpha* tissues by the different formulated AMFs is presented in Table 3. With the M2 formulation, the fungal colonization of the tissues was significantly higher than with the M1 and M3 formulations. Visual analysis by microscopy after staining the tissues revealed that in the plants inoculated with the formulates M1 and M3 no AMFs could be found. Inoculation with M2 produced internal colonization of the thalli tissues of *M. polymorpha* (Fig. 2), although no fungal colonization of the rhizoids was observed.

3.3 Vitality and accumulation of ROS in tissues

Regarding the viability of *M. polymorpha* tissues (Fig. 3a), we could see how the application of the different AMFs inoculum significantly reduced this aspect in plants, the reduction being even more significant with the application of the M2 formula. By indirectly accounting for the stress in these tissues, through the indirect accumulation of ROS (Fig. 3b), we could explain how inoculation with different AMF formulations caused a significant leakage ion, being significantly greater with the application of M2.

3.4 Defense gene expression

The analysis of defense gene expression by RT-qPCR (Fig. 4) in *M. polymorpha* showed a significant decrease in the expression of genes related to SA (*ICS* and *PR-1*), compared to uninoculated plants, only with application of formula M2. On the other hand, the inoculation with the formulates M1 and M3 caused a significant increase in the expression of the genes related to JA (*LOX1* and *COI1*), being significantly greater with the application of the formula M2, in comparison with the plants without inoculation. To the *LOX1* gene the expression after inoculation with M1 was significantly greater than with the application of the M3 formulation.

4 Discussion

As reflected in numerous studies over years of research, the symbiosis between the vascular plants and the AMFs proposes important benefits for both: the plant obtains water and diverse nutrients absorbed from the soil by the extensive hypha network of the AMF, while the plant contributes carbohydrates to the fungus resulting from its photosynthetic activity; in addition to many other indirect benefits for the plant, such as greater tolerance to abiotic stresses and/or resistance to pathogens and pests (Berruti et al. 2016; Powell and Rillig, 2018). These benefits enable an increase in the productivity of agricultural crops in interaction with AMFs,

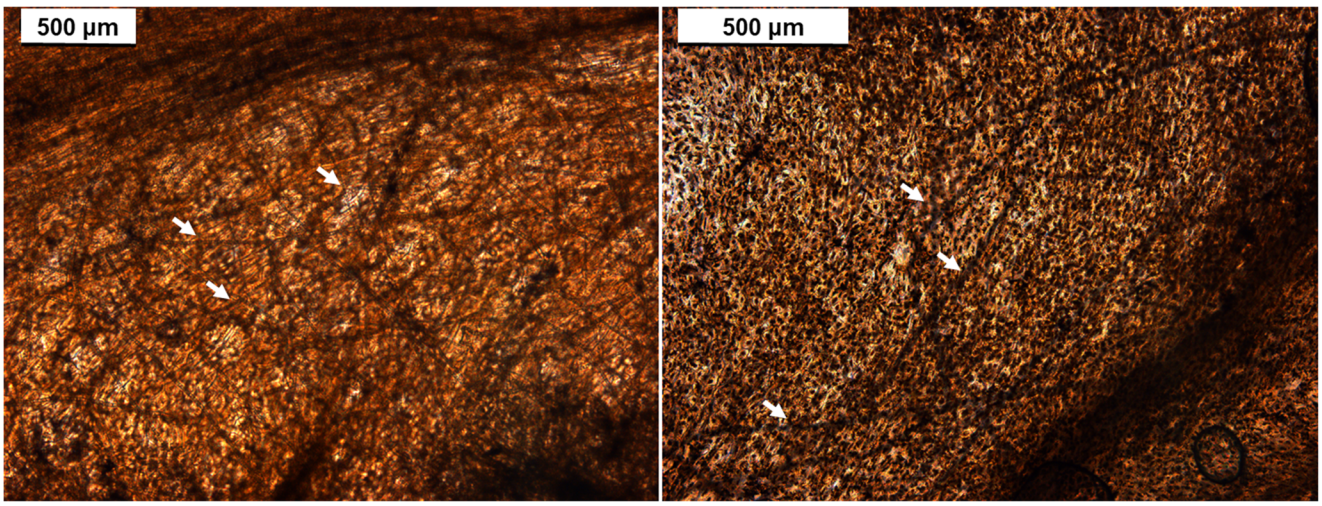

Fig. 2 Microscopic visualization of AMFs colonizing *M. polymorpha*-thalli. Arrows point to intra-thalli hyphae. All photographs have been taken with the inoculation with the M2 formula

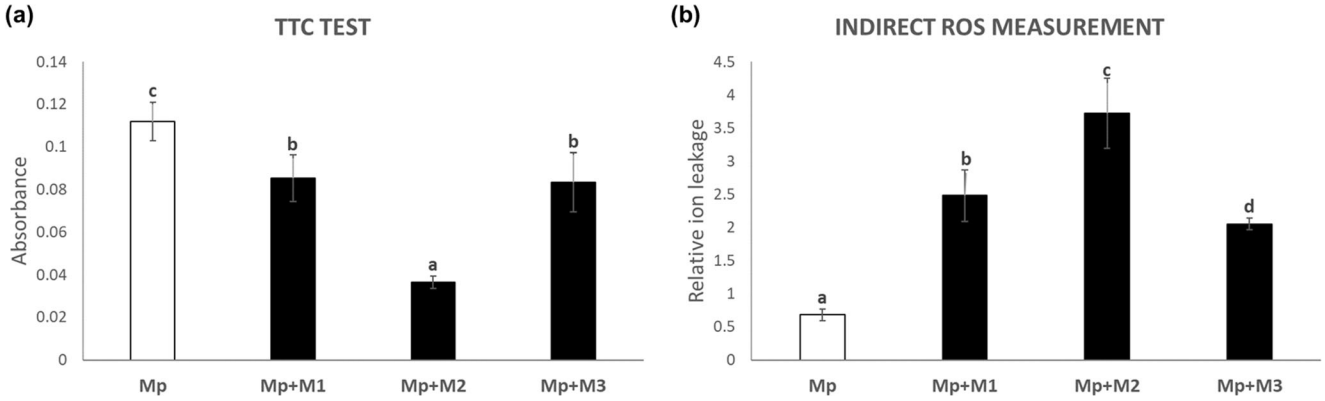

Fig. 3 Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in tissues of *M. polymorpha*. In plants without AMFs-inoculation (Mp) and with the different inoculations (Mp + M1, Mp + M2 and Mp + M3) the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, tissues from four plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

something also observed with formulations such as those used in this study (Poveda et al. 2019).

In the case of liverworts, a study has collected 674 liverworts specimens worldwide (85 species from 35 genera and 3 classes: Haplomitriopsida, Marchantiopsida, and Pelliidae) analyzing the diversity of mycorrhizal fungi present in their tissues (Rimington et al. 2018). The study reported near exclusive colonization of liverworts by the most recently evolved arbuscular mycorrhizal fungal family, Glomeraceae, indicating a recent acquisition of this association from

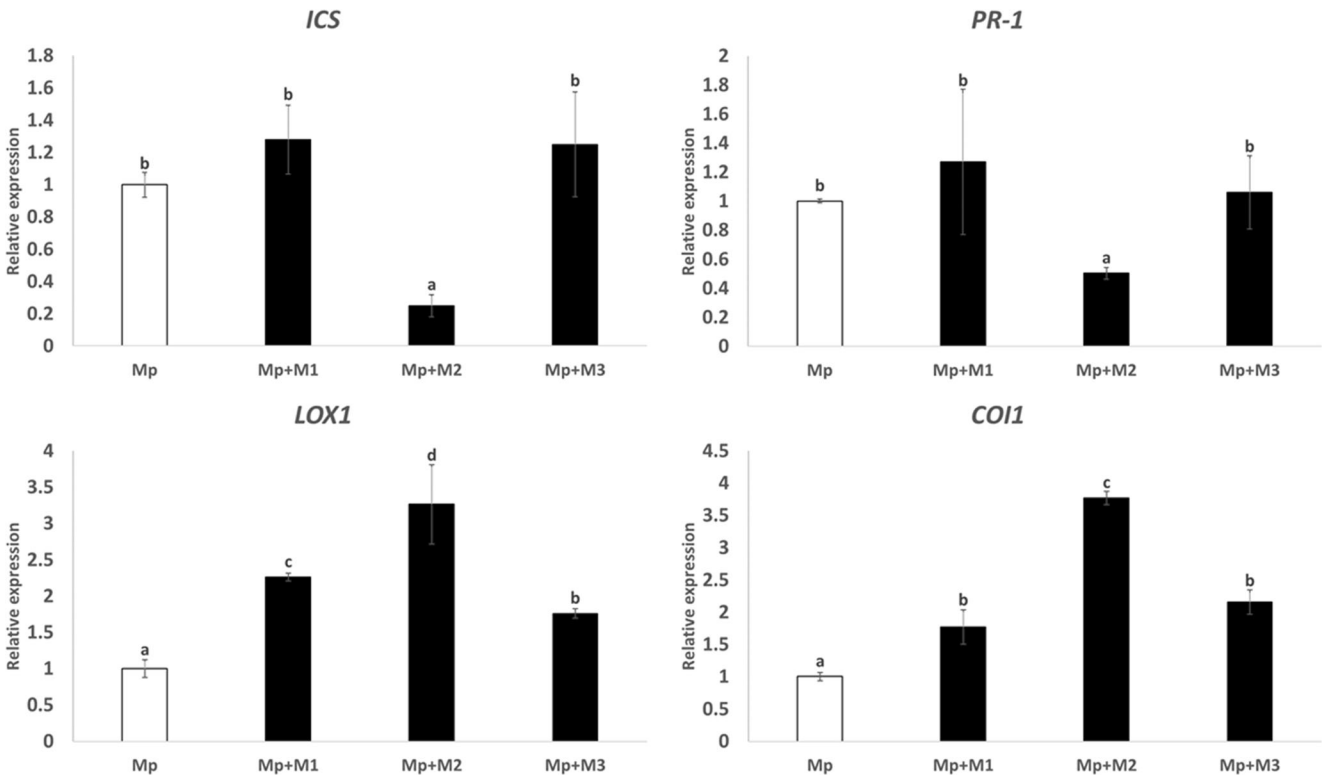

Fig. 4 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in *M. polymorpha*-tissues without AMFs-inoculation (Mp) and with the different inoculations (Mp + M1, Mp + M2 and Mp + M3). The expression of the genes isochorismate synthase (*ICS*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*) and coranatine-insensitive 1 (*COI1*) was quantified. Values correspond to relative measurements against plants without AMFs-inoculation ($2^{-\Delta\Delta Ct} = 1$). The *M. polimorpha EF1* gene was used as an internal reference gene. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, tissues from eight plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$) between the different conditions

flowering plants a different observation from the notion that arbuscular mycorrhizal-like associations in liverworts represent the ancestral symbiotic condition in land plants (Rimington et al. 2018).

As far as *M. polymorpha*-AMFs interaction is concerned, our study shows a negative effect of the interaction of fungi belonging to family Glomeraceae with the plant, under our laboratory conditions, with reference to the parameters analyzed: plant biomass or viability and stress. Until now, this type of negative effect of AMFs on plants, not even on liverworts, has not been described. Most of the studies described how it colonizes the tissues of *M. polymorpha* subsp. *montivagans* in detail (Ligrone et al. 2007) or of other species such as *M. foliacea* (Russell and Bulman, 2005), *M. nepalensis* (Verma & Langer, 2014), *M pappeana* (Rimington et al., 2018) or *M. paleacea* (Field et al. 2019). Nevertheless, sequencing of the entire *M. polymorpha* subsp. *ruderalis* genome indicates that there are gene families missing in the liverwort required for successful arbuscular mycorrhizal colonization, despite their presence in charophytes and closely related *Marchantia* species (Bowman et al. 2017). In this sense, in *M. polymorpha* subsp. *ruderalis* and subsp. *polymorpha* the presence of AMF in their tissues had not been described until now (Ligrone et al. 2007).

With the application of the M2 formulation, it has even been possible to observe damage in the tissues of *M. polymorpha*. Since the only AMF in M2 that is not present in the rest of the formulations is *R. fasciculatus*, we could think that this fungal species, alone or in combination with some of the other species, can behave as a plant-pathogen. Despite the results obtained, there are no other studies that raise the possibility of pathogenic behavior of AMFs, neither specifically for the species *R. fasciculatus*. Pathogenic behavior could not only be verified visually and with the negative effect on plant biomass but also with a reduction in the viability of *M. polymorpha* tissues and an increase in ROS accumulation. In this sense, although we quantified fungal colonization of plant-tissues with the application of M1 and M3, it has been significantly less than with the M2 formulation, without having been able to observe the fungi by microscopy. It could indicate that of the 5 different Glomeraceae species used, only *R. fasciculatus* is capable of effectively colonizing the tissues of *M. polymorpha*. Results totally contrary to those reported in interactions with other plants, such as *Capsicum annuum*, where colonization by *R. fasciculatus* is always lower compared to the rest of AMF (Kumar et al. 2018).

Analysis of the macro- and micro-nutrient content in the tissues of *M. polymorpha* indicated the absence of a truly effective symbiosis with AMFs. We quantified a lower content of C in the tissues of *M. polymorpha* colonized with the AMFs, indicating that the plant to be delivering photoassimilates to the fungi (Bago et al. 2000), however, the benefit to the plant as conferred by the fungus was not quantified. The increase in C content in plants inoculated with M2, compared to M1 and M3, could be due to the observed tissue damage, because tissue damage promotes the synthesis and accumulation of defense carbohydrates locally in the attacked places (Schultz et al. 2013). In the absence of benefits for the plant from the association, we observed a lower P content, something contrary to an effective mycorrhization (Campos et al. 2018), and no effect on the Mg content, directly related to symbiosis (Zare-Maivan et al. 2017). On the other hand, we quantified an increase in S and Ca content when *M. polymorpha* interacts with AMFs, being a direct symptom of an increase in plant defenses against fungi (Hocking et al., 2017; Gruhlke 2019), also quantified in N, related to defense protein synthesis (Mur et al. 2017), K, related to defensive responses (Gao et al. 2018), and Fe, in direct relation to an increase in ROS accumulation in tissues by the Fenton reaction (Krohling et al. 2016), obtaining the same trend of results than with the indirect quantification of ROS in these plant tissues. In this sense, the significant reduction in the accumulation of N and K in the application of the M2 formula would indicate a lower defense capacity of the plants, in line with the damage results obtained. These results support the possibility that the *M. polymorpha*-AMFs interaction is not really beneficial under in vitro conditions, at least for the plant, resulting in lower growth and greater defense.

Regarding gene expression levels related to plant defense responses, our study has reported how the interaction between AMFs and *M. polymorpha* results in an increase in the expression of the JA-related genes, but either the absence of changes or a decrease in the expression of the genes related to SA. In flowering plants, it has been verified that for the association between mycorrhizae and plants to occur, the partial suppression of SA-dependent responses is required (Pozo & Azcón-Aguilar 2007; López-Ráez et al. 2010). Something similar to that observed during the beneficial interaction between the endophytic fungus *Serendipita indica* (or *Piriformospora indica*) and *Arabidopsis thaliana* (Jacobs et al. 2011). Therefore, SA would be the key hormone in controlling these symbiotic fungal-plant relationships. Despite this, we observe how *M. polymorpha* responds to colonization of AMFs by activating its defensive response by JA. These results agree with those observed by Alonso-Ramírez et al. (2014) in the interaction between the beneficial fungus *Trichoderma* and *A. thaliana*. In this study it could be verified, through the use of plants incapable of synthesizing SA, how the plant tries to defend itself against the root colonization of *Trichoderma* by JA-response, which is not effective and causes the vascular colonization of the fungus, going from a beneficial microbe to a systemic pathogen (Alonso-Ramírez et al., 2014). Something similar we could be observing in the interaction AMFs-*M. polymorpha*, or at least with *R. fasciculatus*, where an absence of response by SA triggers the response by JA, without preventing the fungus from causing tissue damage.

At this point, we could consider the evolutionary role that SA could have had in the development of a correct fungus-plant symbiosis, for which studies with other groups of fungi in interaction with *M. polymorpha* and other cryptogamic plants should be carried out. On the other hand, as regards defense against pathogens, it has been demonstrated how in *M. polymorpha* occurs antagonistic interactions between SA and JA pathways during plant-fungus interactions with the pathogens *Irpex lacteus*, *Phaeophlebiopsis peniophoroides*, *Bjerkandera adusta* and *Bjerkandera adusta* (Matsui et al., 2020).

As final conclusions, our work has reported that, at least with the Glomeraceae species used, the *M. polymorpha*-AMFs interaction is harmful to the plant, and even causes tissue damage. Of the different species used, *R. fasciculatus* could be the only one with the capacity to effectively colonize the tissues of *M. polymorpha* subsp. *ruderalis*, causing damage to the plant. In this sense, defense gene expression analysis indicates that SA could mediate the key defensive response to avoid this damage and regulate a correct establishment of the AMFs-*M. polymorpha* symbiosis, since the JA-mediated response is present and not effective. This represents a further approximation to the concept of plant parasitism-symbiosis continuum, of great importance in the case of *M. polymorpha* (Poveda, 2020a). Furthermore, it is important to note that this work has been carried out with inoculums that are not native to the soils where *M. polymorpha* is developed, so that, under natural conditions, the results could be different.

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