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Effect of volatile and non-volatile metabolites from *Leptosphaeria maculans* on tomato calli under abiotic stresses

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ABSTRACT

Drought and salinity can be serious problems for agricultural productivity in certain planet areas. *Leptosphaeria maculans* is the causative agent of the blackleg in crucifer plants. In this work, a novel methodology for studying the effects of fungal metabolites (volatile and non-volatile) on plant calli in the presence of abiotic stresses is presented, by using *L. maculans*, tomato calli, and drought and salinity stresses. In this way, this study has reported how, under salinity and drought stresses, the growth and vitality of tomato calli is inhibited, increasing its tissues-oxidation and accumulation of ROS. By applying metabolites from *L. maculans*, the growth of calli treated with non-volatile metabolites showed and increment under salinity and drought conditions. On the other hand, calli treated with volatile metabolites showed an increment in tissues-vitality under salinity and drought conditions. A series of gene expression analysis was also conducted in order to determine the performance of related genes. Results of this study showed that growth related gene expression was induced, together with abiotic stress tolerance gene in response to abscisic acid, *AREB1*. In addition, the application of volatile metabolites from *L. maculans* on tomato calli without abiotic stresses increases its growth and vitality, and reduces its oxidation and accumulation of ROS, in accordance with the results of gene expression obtained. The ability of *L. maculans* metabolites to increase plant tolerance to abiotic stresses could be related to their ability to produce volatile and non-volatile-metabolites, which induce the antioxidant enzyme activity or accumulation of antioxidant compounds, or their ability to increase the expression of ABA-dependent response genes to abiotic stresses.

1. Introduction

Agricultural productivity is continuously reduced as a consequence of abiotic stresses action (*i.e.* drought, salinity, extreme temperatures), which are accentuated due to climate change (Dresselhaus and Hückelhoven, 2018). This group of stresses causes a significant reduction in plant growth and, as a consequence, of agricultural productivity, with considerable losses (Gilliham et al., 2017). In this regard, plants have to adapt to the changing conditions in the environment in which they grow to survive (Pereira, 2016). Therefore, improving plant abiotic-stress tolerance is key for agricultural productivity and also for environmental sustainability (Zhu, 2016).

Drought is the abiotic stress with the greatest implication in reducing agricultural productivity. More than 40% of the earth's surface is considered dry land, increasing continuously as a consequence of the higher temperature that causes global warming (Thiry et al., 2016). Therefore, water represents the most important abiotic factor affecting agricultural productivity (Nuccio et al., 2018). Agricultural losses caused by drought can reach reductions in crop productivity up to 36% (Daryanto et al., 2015), although, depending on the intensity and duration of the stress, losses can reach up to 94% (Bulgari et al., 2019).

On the other hand, unsustainable irrigation practices and land clearing have increased soil salinity, representing a serious problem in crop productivity. Plants must expend a large amount of energy to avoid osmotic damage caused by salt in the soil and to be able to absorb water through the roots. This situation causes reductions in agricultural productivity up to 33%, reaching levels in soils where agriculture is impossible (Munns and Gilliham, 2015).

In this context, microbial biostimulants are capable of reducing the damage caused by these abiotic stresses in crops by improving plant

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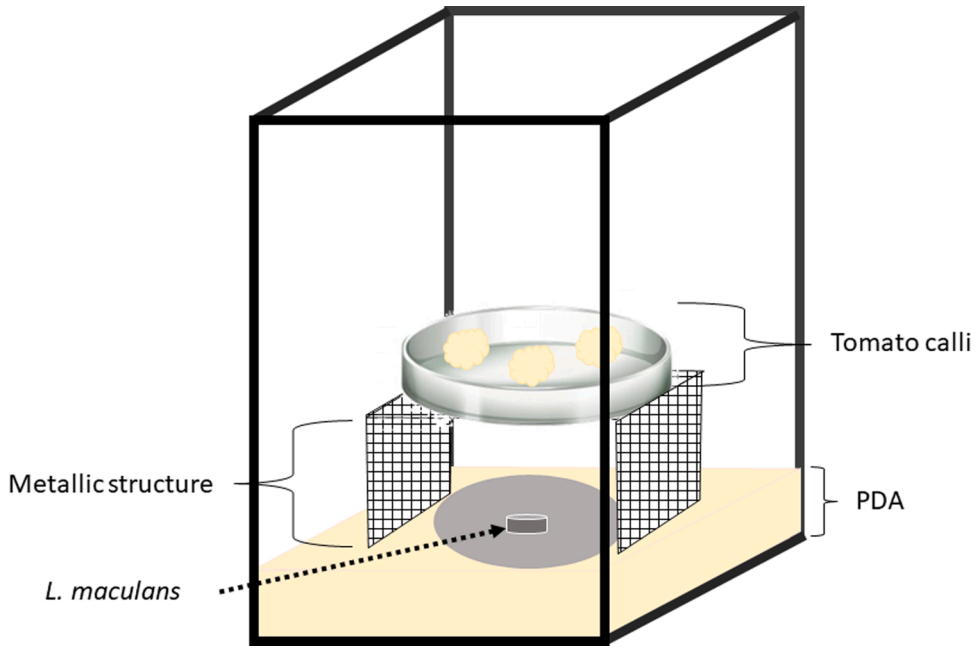

Fig. 1. Methodology carried out for the exposure assays of tomato calli to volatile metabolites from *L. maculans*.

tolerance. These microorganisms promote plant growth and agricultural productivity in drought and/or salinity conditions, improving plant responses to stress, for example, through the accumulation of antioxidant compounds in their tissues (Poveda, 2020a, 2020b). The ability to produce volatile and non-volatile secondary metabolites by these microorganisms has been identified as an important mechanism of action to achieve these positive effects in plants (Poveda, 2021a).

Leptosphaeria maculans (anamorph *Phoma lingam*) is a known filamentous fungus as the causal agent of blackleg or stem canker in crucifers (Howlett et al., 2001; Rouxel and Balesdent, 2005). Its possible hosts include cultivated *Brassica* crops such as *Brassica napus, B. oleraea, B, rapa* and *B. juncea*, in addition to many other wild crucifers species, such as *Arabidopsis thaliana*. The symptoms of the disease are linked to the phases of its life cycle. From the formation of the cotyledons until the end of the growing season, the observable symptoms are black spots on the leaves. During this period *L. maculans* colonizes from leaf tissues to stem and collar, causing at the end of the growing season dry necroses of the crown tissues with occasional stem canker or blackleg, causing lodging of the plants (Rouxel and Balesdent, 2005). *L. maculans* can cause significant yield losses annually in crops such as canola or rapeseed (*B. napus*) in Canada, its largest oilseed crop (Rashid et al., 2021).

In situations of abiotic stresses, *L. maculans* modifies its metabolome in order to resist effectively. Under salt stress the fungus synthesizes in a directed way two metabolites associated with melanin biosynthesis and cell melanization, a self-protection mechanism against the abiotic stress (Pedras and Yu, 2009).

On the other hand, the culture of plant cells, tissues and organs arises as an experimental need to solve important questions in plant biology, currently representing an important biotechnological tool. It is a widely used laboratory technique for the commercial propagation of numerous plant species, the production of haploid and double-haploid plants, the generation of disease-free plant materials, the induction of epigenetic or genetic variation for the isolation of variant plants, or the conservation of valuable plant germplasm; moreover, it is the keystone for genetic engineering of plants (Loyola-Vargas and Ochoa-Alejo, 2012). In this way, *in vitro* plant cultures can be developed in order to produce compounds of industrial and sanitary interest (Efferth, 2019), to test toxic compounds and the possibilities of removing them (bioremediation) (Doran, 2009), or in studies on plant stress physiology, representing plant calli very important *in vitro* study models in understanding physiological processes (Gaspar et al., 2002).

Therefore, the objective of this work is to use these biological resources to determine the effect of secondary metabolites produced by L. *maculans* in tomato calli when subjected to abiotic stresses related to drought and salinity.

2. Materials and methods

2.1. Fungal material

L. maculans CRD13/125/99, facilitated by the Regional Diagnostic Center of the Regional Government of Castilla y León (Salamanca, Spain) and isolated from a rapeseed field from Palencia (Spain), was used. *L. maculans* was routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain) in the dark at 28 °C.

2.2. Tomato calli development

To obtain tomato calli, seeds of the commercial variety San Pedro (Vilmorin, Limagrain, France) were used, by the methodology described by Gerszberg et al. (2016), with some modifications.

The seeds were superficially sterilized by washing with 70% (v/v) ethanol for 15 min, and another washing with 20% (v/v) sodium hypochlorite for 10 min, followed by 3 washes with sterile distilled water. Seeds were placed on Petri dishes with MS basic medium (4.4% MS, 1% agar, 1% sucrose, and pH 5.8); keeping the plates in dark at 23 °C and a relative humidity of 40–50%, for 10 days.

The hypocotyls obtained were cut into 2 cm fragments and placed in Petri dishes with 4.4% DM, 1% agar, 3% sucrose, 1 mg/L benzyladenine, 0.5 mg/L 2,4-dichlorophenoxyacetic, and pH 5.7; keeping the plates in dark at 23 °C and a relative humidity of 40–50%, for 4 weeks. Every four weeks the calli formed were transferred to new Petri dishes with the same culture medium, or were used for the different assays.

2.3. Exposure of tomato calli to L. maculans non-volatile metabolites

The non-volatile metabolites from *L. maculans* were obtained by liquid culture. From Petri dishes with *L. maculans* growing in PDA medium for 10 days, spores were used as inoculant of liquid cultures. Flasks of 100 ml with 50 ml of potato-dextrose-broth (PDB, Sigma-Aldrich,

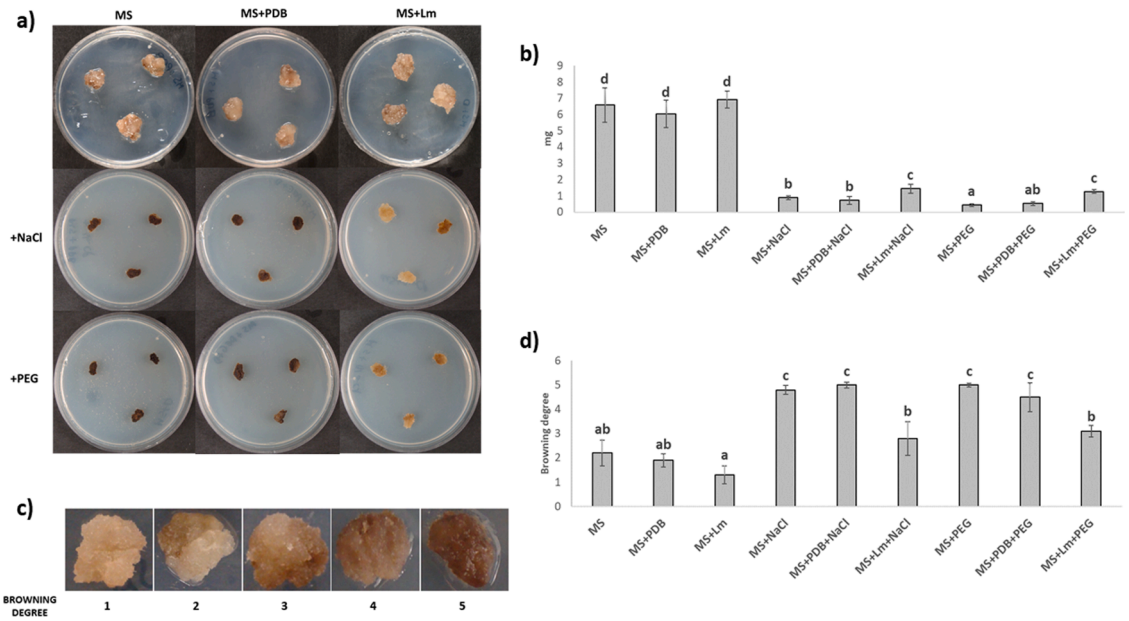

Fig. 2. Effect of non-volatile metabolites from *L. maculans* (+Lm) on tomato calli in MS basic medium (MS), with PDB (+PDB), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. (a) Photographs of the Petri dishes with tomato calli after two weeks under the indicated conditions. (b) Dry weight per tomato calli. (c) Scale of browning degree of tomato calli used. (d) Browning degree by tomato calli. Data are the mean of 15 calli (b) or 42 calli (d) for each condition with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

Madrid, Spain) were inoculated with 1 ml of spore solution diluted to a 2.5×10^6 spores mL^{-1}, determined using a hemocytometer. Flasks were left for 48 h on an orbital shaker at 28 °C and 200 rpm. Subsequently, the liquid culture was centrifuged and passaged through a sterile 0.22 μm filter.

To put the non-volatile metabolites from *L. maculans* in contact with the tomato calli, Petri dishes were prepared with MS basic medium, adding the fungi-filtrate at 10% (v/v). In all the assays, two controls were used: Petri dishes with MS basic medium and Petri dishes with MS basic medium and PDB at 10% (v/v), in order to determine possible effects on tomato calli derived from the liquid fungi-culture medium.

For each condition, 14 Petri dishes were made, where three tomato calli of 100 mg were deposited (42 calli per condition). The dishes were placed in a light chamber (Fitotron AGP-1400-HR, Radiber SA, Barcelona, Spain) with a photoperiod of 16 h of light (80–100 E/m^2/s) and 8 h of darkness at a temperature of 22 °C and a relative humidity of 40–50%, for two weeks.

2.4. Exposure of tomato calli to L. maculans volatile metabolites

To put the tomato calli in contact with the volatile metabolites from *L. maculans*, the Phytatray I boxes (Sigma, St. Louis, MO, USA) was used, following the methodology represented in Fig. 1. PDA medium (30 ml) were deposited in each box, and a metal structure in the form of a table was introduced, sterilizing the assembly at 120 °C for 20 min. Subsequently, the PDA medium from each box was inoculated with an agar disk containing L. *maculans* mycelium from a 10-day-old Petri dish. The controls were boxes without inoculating with the fungus. Finally, a Petri dish with three tomato calli of 100 mg was deposited on each metallic structure. 14 Petri dishes and boxes were used per condition (42 calli per condition).

The boxes were placed in a light chamber (Fitotron AGP-1400-HR, Radiber SA, Barcelona, Spain) with a photoperiod of 16 h of light (80–100 E/m^2/s) and 8 h of darkness at a temperature of 22 °C and a relative humidity of 40–50%, for two weeks.

2.5. Abiotic stress assays

The conditions of abiotic stresses by salinity and drought were elaborated in the Petri dishes where the tomato calli of 100 mg were deposited. The salinity stress was caused by adding NaCl 200 mM to the MS basic medium. For drought stress, polyethylene glycol (PEG 1500 [w/v], Sigma-Aldrich, Madrid, Spain) was added to the MS basic medium at an osmotic potential of −1.5 MPa. PEG is a compound widely used for the induction of drought stress *in vitro* plant cultures (Ghassemi-Golezani et al., 2018).

2.6. Tomato calli analysis

2.6.1. Dry weight

At the end of each test, 15 tomato calli were collected per condition, which were dried at 60 °C for 48 h. The data of dry weight per callus were taken.

2.6.2. Browning

In each of the trials all tomato calli (42 calli per condition) were photographed. Using the browning scale, included in Figs. 2 and 4, a value was assigned to each of the tomato calli individually.

2.6.3. Vitality test

The vitality test was carried out as described by Poveda (2020c). Due to the activity of the mitochondrial respiratory chain of living plant cells, the reduction of triphenyltetrazolium chloride (TTC) to the red-colored insoluble triphenylformazan (TF) occurs (Ruf and Brunner, 2003). Therefore, only living cells should reduce TTC to TF. From each condition, nine tomato calli were used, of which 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. Subsequently, the samples were shaken

Table 1.
Primers used in this work.

CODE	SEQUENCE (5′−3′)	USE	Refs.
Sl-Act-F	CACCACTGCTGAACGGGAA	Endogenous tomato gene	De Palma et al. (2016)
Sl-Act-R	GGAGCTGCTCCTGGCAGTTT		
Sl-ARF1-F	TCTCCTTCATCATTCTCATACTG	Auxin response gene	Zouine et al. (2014)
Sl-ARF1-R	GAACCATTCTCACCATAACC		
Sl-EXP-F	TGGACCAACCACCATTCTCG	Cytokinin response gene	Cheng et al. (2016)
Sl-EXP-R	AGTTGCACTCCACTCGTTTGTA		
Sl-DREB3-F	ATGAATTCCCAAATCTTTTCAACT	ABA-independent abiotic stress tolerance gene	Islam and Wang (2009)
Sl-DREB3-R	TTATAGAGAGGCCCAATCAATTTC		
Sl-AREB1-F	ACAGGAGGGAGTGGTAAGGA	ABA-dependent abiotic stress tolerance gene	Almutairi (2016)
Sl-AREB1-R	AGTCAAAGAGCCTTGCCTCT		
Sl-GRX1-F	TTCCCGAAGGAATCTGGTGTATATGC	Antioxidant activity related gene	Alsadon et al. (2013)
Sl-GRX1-R	TGATTTCCAAGATTCAGTTAAGGCGG		
Sl- PHYT4-F	GCTTAACAATGCACCATGGCA	Cell death-related proteinase gene	Reichardt et al. (2018)
Sl- PHYT4-R	TCGGCATGAATGACTTGTCCA		

again in Silamat and centrifuged. The supernatant was used to quantify its absorbance at 620 nm, being an indirect measure of the vitality of the tomato calli.

2.6.4. *Indirect quantification of ROS in calli*

The indirect quantification of reactive oxygen species (ROS) in tomato calli was carried out by measuring electrolyte leakage, similar to the method used by Poveda (2021b) in *Arabidopsis thaliana* tissues, which really measures cellular oxidative damage, related process to ROS production. From each condition, nine tomato calli were used, of which 100 mg were 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 cell-calli (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 cell-calli (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

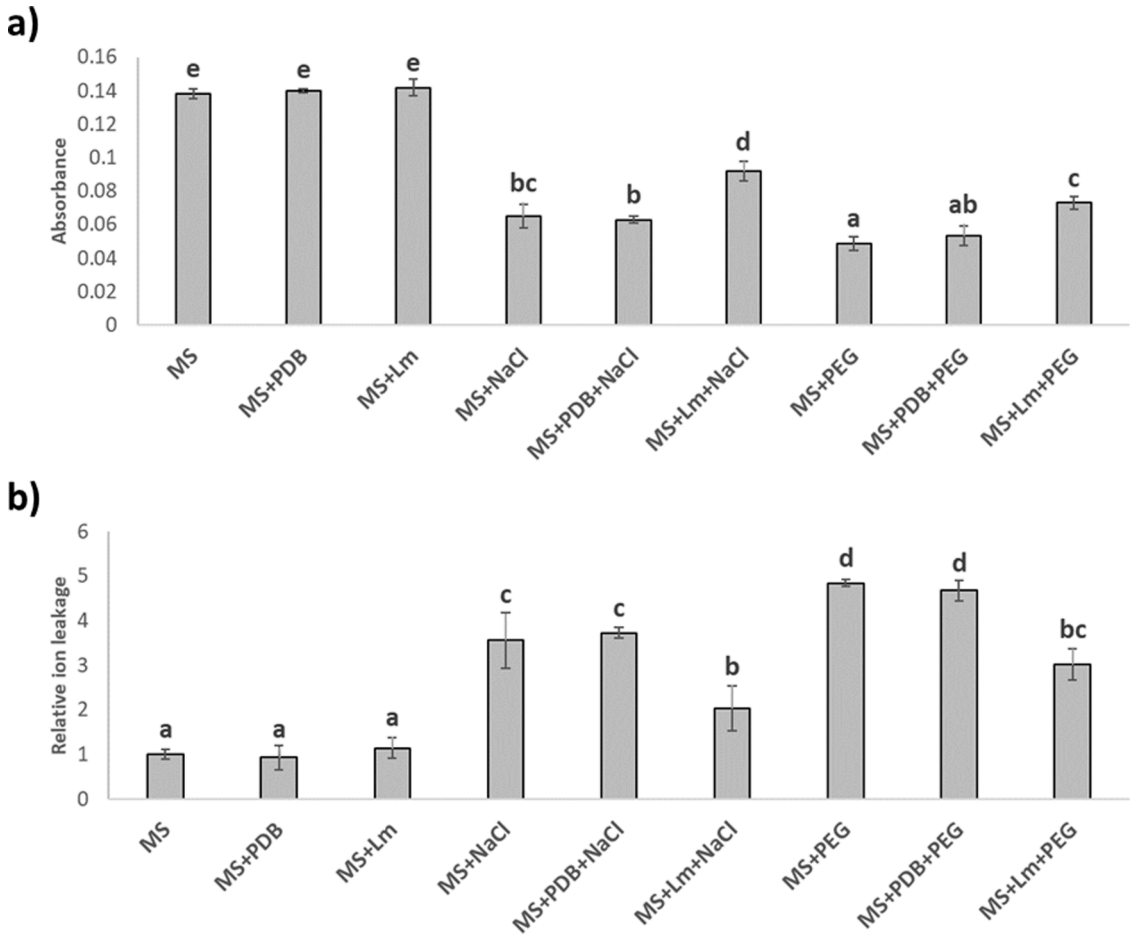

Fig. 3. Tomato calli vitality by TTC test (a) and indirect quantification of ROS (b). In tomato calli with non-volatile metabolites from *L. maculans* (+Lm), in MS basic medium (MS), with PDB (+PDB), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. Data are the mean of 9 calli for each condition with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

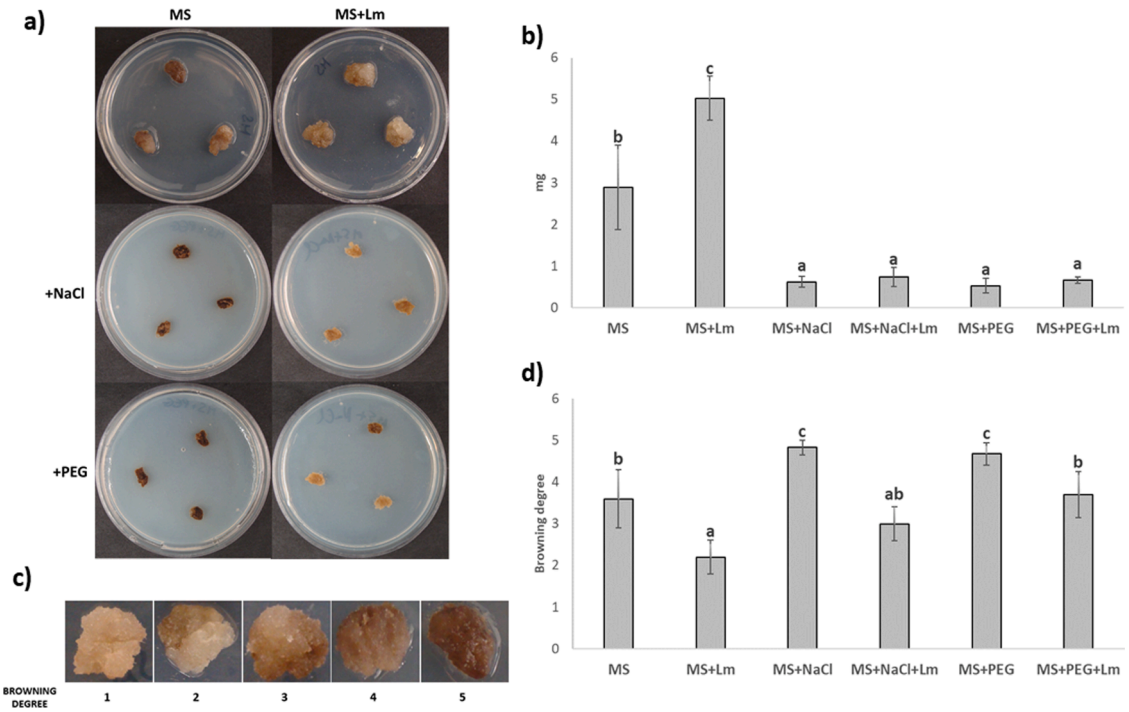

Fig. 4. Effect of volatile metabolites from *L. maculans* (+Lm) on tomato calli in MS basic medium (MS), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. a) Photographs of the Petri dishes with tomato calli after two weeks under the indicated conditions. b) Dry weight per tomato calli. c) Scale of browning degree of tomato calli used. d) Browning degree by tomato calli. Data are the mean of 15 calli (b) or 42 calli (d) for each condition with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

2.7. Gene expression studies

The analysis of the expression of different tomato genes in tomato calli was carried out in three pools of three tomato calli per condition by RT-qPCR, using the methodology described by Poveda (2021c), 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 tomato *actin* gene as an endogenous control. The primers used are given in Table 1: genes of the auxin reponse factor 1 (*ARF1*), cytokinin-responsive expansin (*EXP*), dehydration responsive element-binding protein-3 (*DREB3*), abscisic acid (ABA)-response element binding factor 1 (*AREB1*), glutaredoxin (*GRX1*), phytaspase 4 (*PHYT4*).

2.8. 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 non-volatile L. maculans metabolites on tomato calli

Non-volatile metabolites from *L. maculans* applied on tomato calli did not produce a significant increase in dry weight of calli (Fig. 2b), although it significantly reduced browning (Fig. 2d). The addition of both abiotic stresses caused a significant reduction in dry weight of tomato calli (Fig. 2b) and a significant increase in browning (Fig. 2d). Under salinity and drought, applying non-volatile metabolites from *L. maculans* on tomato calli significantly increased their dry weight (Fig. 2b) and reduced their browning (Fig. 2d).

Regarding the vitality of tomato calli and the indirect quantification of ROS accumulation, non-volatile metabolites from *L. maculans* did not produce significant changes. Both salinity stress and drought stress significantly reduced the vitality of tomato calli and increased the indirect quantification of ROS accumulation. Under these abiotic stresses, the application of non-volatile metabolites from *L. maculans* caused a significant increase in tomato calli vitality (Fig. 3a) and a reduction in indirect quantification of ROS accumulation (Fig. 3b).

3.2. Effect of volatile L. maculans metabolites on tomato calli

Volatile metabolites from *L. maculans* caused a significant increase in dry weight of tomato calli (Fig. 4b) and a significant reduction in browning (Fig. 4d). The application of both abiotic stresses (salinity and drought) significantly reduced the dry weight of tomato calli (Fig. 4b) and increased browning (Fig. 4d). Under saline and drought stress, volatiles from *L. maculans* did not significantly affect the dry weight of tomato calli (Fig. 4b), but significantly reduced browning (Fig. 4d).

As far as vitality and indirect quantification of ROS accumulation in tomato calli are concerned, the application of volatile metabolites from *L. maculans* resulted in a significant increase and decrease, respectively. Salinity and drought significantly reduced the vitality of tomato calli and increased the indirect quantification of ROS accumulation. Whereas

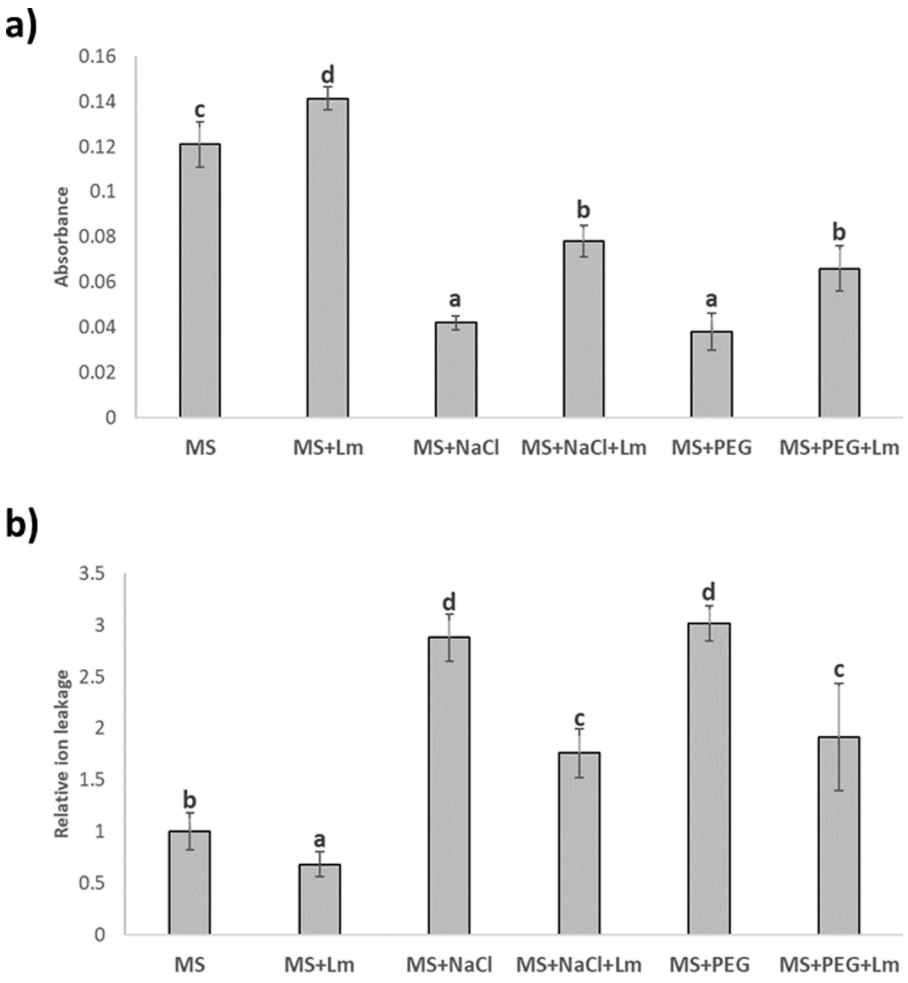

Fig. 5. Tomato calli vitality by TTC test (a) and indirect quantification of ROS (b). In tomato calli with volatile metabolites from *L. maculans* (+Lm), in MS basic medium (MS), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. Data are the mean of 9 calli for each condition with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

when volatiles from *L. maculans* were applied to tomato calli subjected to abiotic stresses, their vitality significantly increased and their indirect ROS accumulation quantification decreased (Fig. 5).

3.3. Expression of genes related to growth and stress

Without abiotic stresses, non-volatile metabolites from *L. maculans* did not modify the expression of any of the genes analyzed in tomato calli. Under salinity and drought, there was a significant decrease in the expression of genes related to plant growth (*ARF1* and *EXP*) and an increase in tolerance to abiotic stresses related genes expression (*DREB3* and *AREB1*), antioxidant activity (*GRX*) and cell death (*PHYT4*). The *L. maculans* non-volatile metabolites in salinity and drought conditions caused a significant increase in the expression of the plant growth genes (*ARF1* and *EXP*) and the gene for tolerance to abiotic stresses in response to ABA (*AREB1*), while it did not increase the expression of the ABA-independent tolerance gene (*DREB3*). In addition, the expression of genes related to antioxidant activity (*GRX*) and cell death (*PHYT4*) was reduced significantly (Fig. 6).

Regarding *L. maculans* volatile metabolites, their application in tomato calli significantly increased the expression of plant growth related genes (*ARF1* and *EXP*) and reduced that of the antioxidant activity related gene (*GRX*). Under salinity and drought, there was a significant decrease in the expression of growth related genes (*ARF1* and *EXP*), while the expression of tolerance to abiotic stresses related genes r (*DREB3* and *AREB1*), antioxidant activity (*GRX*) and cell death (*PHYT4*) increased significantly. The application of *L. maculans* volatiles in salinity and drought conditions produced a significant increase in the expression of the gene for tolerance to abiotic stresses in response to ABA (*AREB1*), while it did not increase the expression of the gene for tolerance independent of ABA (*DREB3*). In addition, the expression of antioxidant activity related genes (*GRX*) and cell death (*PHYT4*) was significantly reduced (Fig. 7).

4. Discussion

L. maculans produces a wide diversity of secondary metabolites, even allowing the chemo-taxonomical characterization of different isolates (Pedras et al., 2007). These secondary metabolites can have important biological capacities and industrial applications, such as fungicides and nematicides (Pedras and Sarma-Mamillapalle, 2012) or pigments (Pedras et al., 1995). The most studied secondary metabolites in *L. maculans* are different phytotoxins, such as sirodesmins (Gardiner et al., 2004; Elliott et al., 2011), maculansins (Pedras and Yu, 2008a) or leptomaculins (Pedras and Yu, 2008b). These phytotoxins appear to be specific against the plants of which it is pathogenic, without having an effect on other crops. The application of the phytotoxin sirodesmin in protoplasts, cells and plants of *B. napus* caused cellular and tissue damage, not observed in tobacco or tomato (Sjödin et al., 1988). Therefore, both for its productive capacity of bioactive metabolites and for its absence of toxicity in tomato, *L. maculans* represents a correct choice in the study proposed in this work.

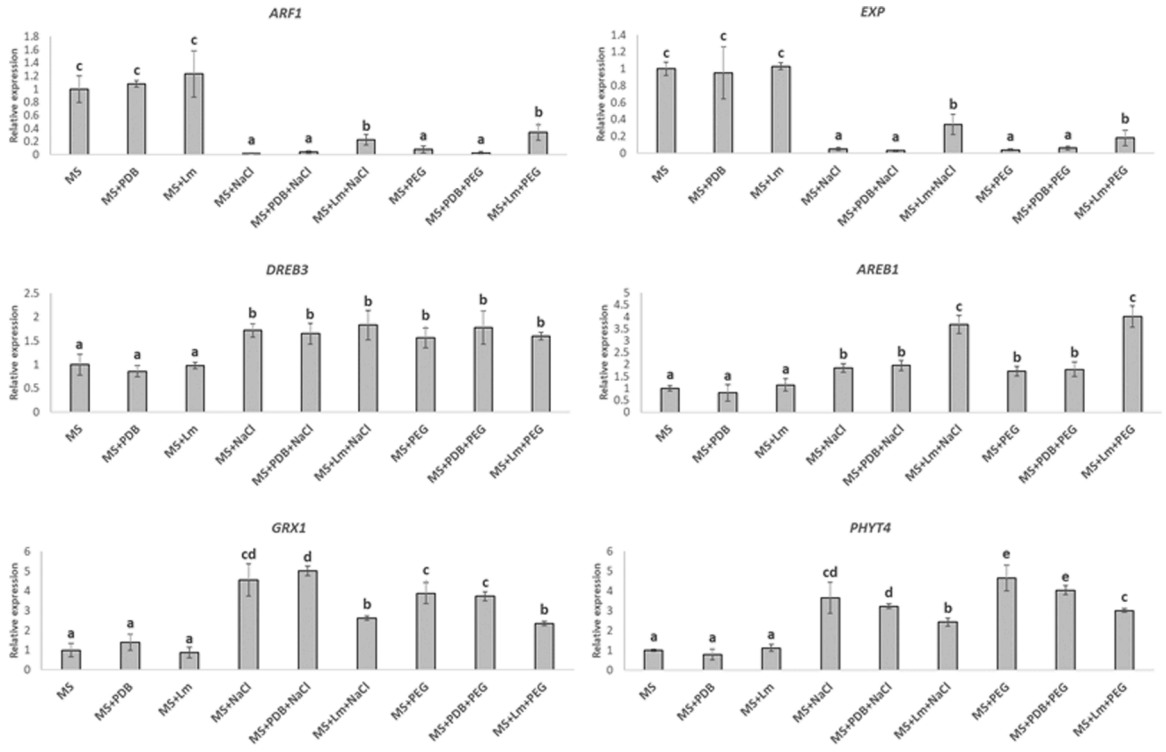

Fig. 6. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some growth and stress genes in tomato calli in MS basic medium (MS), with PDB (+PDB), or with non-volatile metabolites from *L. maculans* (+Lm), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. The expression of the genes of the auxin reponse factor 1 (*ARF1*), cytokinin-responsive expansin (*EXP*), dehydration responsive element-binding protein-3 (*DREB3*), abscisic acid (ABA)-response element binding factor 1 (*AREB1*), glutaredoxin (*GRX1*), phytaspase 4 (*PHYT4*). Values correspond to relative measurements against tomato calli in MS basic medium ($2^{-\Delta\Delta Ct} = 1$). The tomato *Act* 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, three tomato calli 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.

The use of calli has been reported as an important model system for the *in vitro* study of plant-fungi interactions (Wedge and Tainter, 1997; Peters et al., 1998; Cheng et al., 2019) and plant stress physiology (Gaspar et al., 2002). In this sense, the effect of abiotic stresses is more direct and rapid in plant callus than in whole plant, with a greater dehydration of calli under saline stress or drought, verified in tomato tissues (Rus et al., 1999). In this work we have been able to verify how, both the application of NaCl and PEG, caused in tomato calli a decrease in their growth and vitality, and an increase in browning and ROS production, in addition to a reduction in the expression of growth related genes and an increase in the expression of tolerance to abiotic stresses, antioxidant activity and cell death related genes. Likewise, it has been determined how the application of saline stress on *Solanum tuberosum* calli causes a decrease in growth and an increase in antioxidant activity, extrapolated to whole plants (Sajid and Aftab, 2009), like in tomato calli (Rus et al., 1999). Similarly, it has been reported in *Trigonella foenum* and *Taxus baccata* calli how PEG application decreased cell viability and growth, and induced oxidative stress (Sarmadi et al., 2019; Alzandi and Naguib, 2020). As far as *L. maculans* is concerned, Hura et al. in 2015, developed a work on the effect of secondary metabolites produced by the fungus on *B. napus* calli subjected to cold stress. In these calli, low temperatures led to an increase in the accumulation of phenolic compounds, leading to greater resistance to biotic stress (Hura et al., 2015a).

Under conditions without abiotic stresses, we have been able to verify how the application of non-volatile metabolites from *L. maculans* in tomato calli only reduces oxidation (browning), without reporting changes in gene expression; while the application of volatile metabolites produced an increase in biomass and vitality of tomato calli, together with a reduction in their oxidation and accumulation of ROS, reporting an increase in the expression of growth-related genes and a decrease in the gene related to antioxidant activity. This ability to promote the growth and to reduce the oxidation of plant calli by interaction with fungal metabolites had already been reported for *Myrotthecium* sp. in alfalfa calli by tichothecenes (verrucarin A, roridin A and 8-β-hydroxyroridin E) (Kobayashi et al., 1989), *Aspergillus flavus* in *Catharanthus roseus* calli (Tonk et al., 2016), and even in cell cultures of *Linum album* by *Piriformospora indica* and *Sebacina vermifera* interaction (Baldi et al., 2008). Regarding *L. maculans*, this work represents the first observation of plant growth promotion by its metabolites, mainly by volatile metabolites, group of metabolites whose possible biological activity had not been previously studied. In this sense, the ability of *L. maculans* to produce and release important growth phytohormones, such as cytokinins, mainly cis-zeatin (Trdá et al., 2017), and auxins, mainly indole-3-acetic acid (Leontovyčová et al., 2020), has been described.

Under salinity and drought, we have verified how the application of non-volatile and volatile metabolites from *L. maculans* reduces the detrimental effect of abiotic stresses on tomato calli. Non-volatile metabolites cause an increase in tomato calli biomass, not observed with volatile metabolites, although both types of metabolites increase vitality and reduce browning and ROS accumulation. These results agree with the gene expression data obtained, with an increase in the expression of genes related to plant growth (*ARF1* and *EXP*) only with the application of non-volatile metabolites. With the application of non-volatile and volatile metabolites, there is an increase in the expression of the ABA-dependent abiotic stress tolerance gene (*AREB1*) and the expression of the antioxidant activity (*GRX*) and cell death (*PHYT4*) genes is reduced.

Both under abiotic stress and without stress, secondary metabolites released by *L. maculans* are capable of reducing the oxidation of tomato calli. This could be due to the increased activity of antioxidant enzymes, such as catalases, superoxide dismutases, phenylalanine ammonia-lyase, guaiacol peroxidase, ascorbate peroxidase, or glutathione reductase

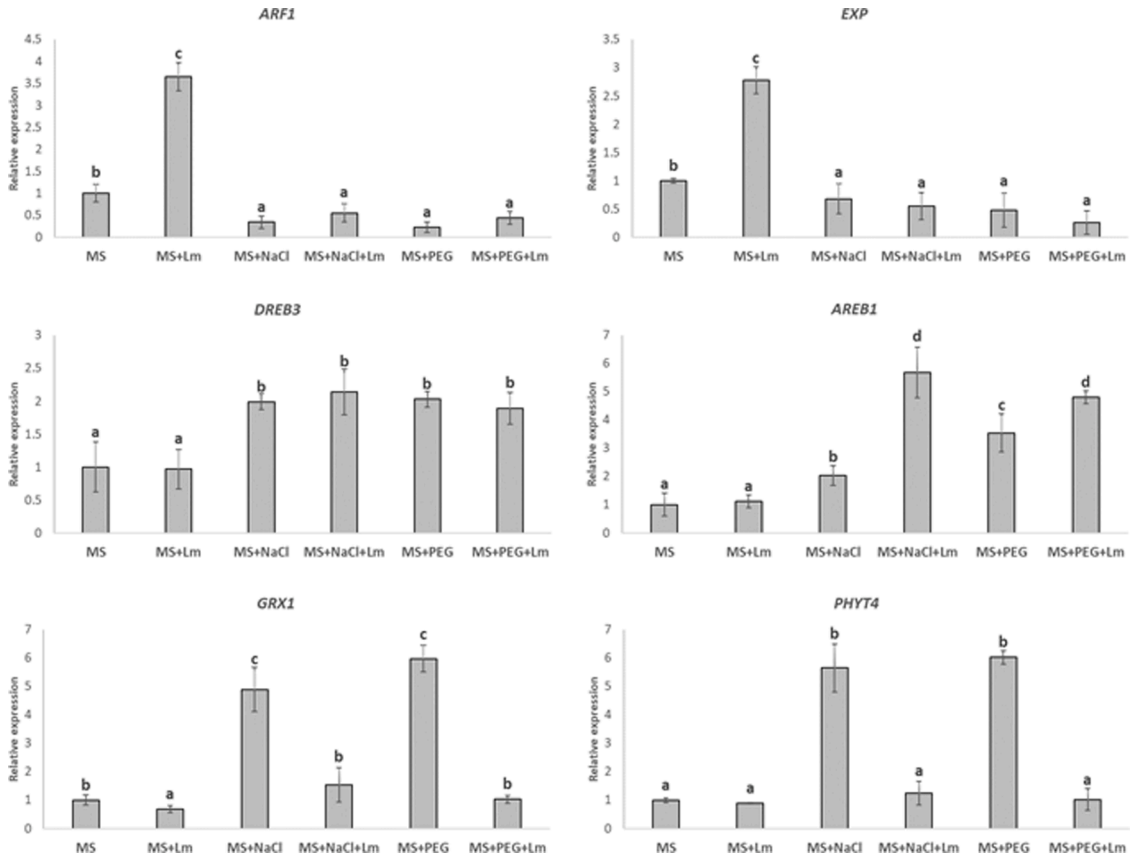

Fig. 7. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some growth and stress genes in tomato calli in MS basic medium (MS), with volatile metabolites from *L. maculans* (+Lm), and under salinity (200 mM) (+NaCl) or drought (−1.5 MPa) (+PEG) stress. The expression of the genes of the auxin reponse factor 1 (*ARF1*), cytokinin-responsive expansin (*EXP*), dehydration responsive element-binding protein-3 (*DREB3*), abscisic acid (ABA)-response element binding factor 1 (*AREB1*), glutaredoxin (*GRX1*), phytaspase 4 (*PHYT4*). Values correspond to relative measurements against tomato calli in MS basic medium ($2^{-\Delta\Delta Ct} = 1$). The tomato *Act* 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, three tomato calli 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.

(Jindřichová et al., 2011; Hura et al., 2014a), and the accumulation of phenolic compounds and low molecular antioxidants (Hura et al., 2014b, 2015b), how has been observed in *B. napus* tissues after pathogen recognition. In this sense, the ability of secondary metabolites released by *L. maculans* to increase catalase activity had already been reported previously in calli of *B. napus* (Hura et al., 2015a).

The ability of *L. maculans* secondary metabolites to increase tolerance of tomato calli could be related to the production of specific phytohormones against abiotic stresses. In this regard, the ability of *L. maculans* to produce abscisic acid has recently been described, with the corresponding gene cluster for synthesis identified (Darma et al., 2019).

As conclusions, the study of fungus-plant calli-abiotic stresses interactions represents a novel approach in understanding the complex interactions of plants with biotic and abiotic agents in the natural environment. In this sense, *L. maculans* has been described as a fungus with the ability to produce secondary metabolites with great biological potential, capable of increasing plant growth and preventing cellular stress processes in tomato calli. Furthermore, the production of these secondary metabolites is capable of increasing plant tolerance to salinity and drought, thanks to the increase antioxidant enzyme activity, accumulation of antioxidant compounds, or increase in the expression of plant-abiotic tolerance genes, such as *AREB1*. Furthermore, *L. maculans* can be a great resource for new metabolites of interest in agriculture, requiring the development of more studies, especially in its non-volatile metabolites, this being the first work with them.

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CRediT authorship contribution statement

Jorge Poveda: Investigation, Visualization, Methodology, Formal analysis, Writing – original draft, 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.

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