**Jasmonic acid pathway is required in the resistance induced by *Acremonium sclerotigenum* in tomato against *Pseudomonas syringae*.**

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**Abstract**

The use of fungal endophytes is considered as a new tool to confer resistance in plants against stresses. However, the mechanisms involved in colonization as well as in the induction of resistance by the endophytes are usually unclear. In this work, we tested whether a fungal endophyte isolated from an ancestor of wheat could induce resistance in plants of a different class from the ones that were isolated from the beginning. Seeds of *Solanum lycopersicum* were inoculated with *Acremonium sclerotigenum* and after four weeks, seedlings were inoculated with the bacterium *Pseudomonas syringae pv* tomato. Plants inoculated with endophytes showed significantly lower symptoms of infection as well as lower levels of colony forming units compared with control plants. Moreover, the presence of the endophytes induced an enhancement of Jasmonic acid (JA) upon inoculation with *P. syringae* compared with endophyte free plants. To ascertain the implication of JA in the resistance induced by *A. sclerotigenum*, two mutants defective in JA were tested. Results showed that the endophyte is

not able to induce resistance in the mutant *spr2*, which is truncated in the first step of JA biosynthesis. On the contrary, *acx1* mutant plants, which are unable to synthesize JA from OPC8, show a phenotype similar to wild type plants. Moreover, experiments with GFP-tagged endophytes showed no differences in the colonization in both mutants. In conclusion, the jasmonic acid pathway is required for the resistance mediated by the endophyte *A. sclerotigenum* in tomato against the biotrophic bacterium *P. syringae* but is not necessary for the colonization.

**Keywords:** Fungal endophytes; plant protection; Jasmonic acid; plant-microbe interaction, *Acremonium sclerotigenum*

## 1. Introduction

Control of pathogens attack is one of the biggest adversities that agriculture should face to avoid losses in the production and, currently, this control is mainly based on the application of chemical pesticides. Despite this, the society is demanding a new agriculture with a low impact on the environment. However, the control of pathogens based on natural compounds is not widely used in conventional agriculture.

Plants benefit from symbiosis with microorganisms that, in exchange for the protective environment that the plant offers, they have developed mechanisms to increase the resistance and survival of the host. These microorganisms could be present on the surface of the plant as epiphytes or colonize inner tissues as endophytes. This relation between plants and microorganisms could have influenced plant evolution, in which the presence of a certain microbiome provides flexibility of responses and improve plant adaptation to adverse conditions [1–3].

In the last years, the importance of the plant microbiome as a modulator of plant resistance is emerging as a potential tool to achieve an environmentally friendly agriculture [4]. The presence

of certain microorganisms could induce a reprogramming of plant metabolic pathways involved in defense, which may improve the ability of the plant to survive under adverse conditions [5]. Fungal endophytes such as *Penicillium*, *Neothypodium* or *Piriformospora* species have demonstrated to improve resistance in plants against drought or salinity [6–9]. Other microorganisms have shown protective effects against biotic stress via hyperparasitism, competition with other pathogenic microorganisms, counteracting virulence factors, or inducing innate plant resistance [2,10]. In this way, it has been previously described that *Trichoderma* species are able to protect grapevine against *Agrobacterium vitis* [10], or *Piriformospora indica* which is able to reduce the effects of *Verticillium dahliae* and Pepino Mosaic Virus in tomato [11].

Among beneficial microorganisms, those with an endophytic lifestyle need to be particularly well adapted to the host plant, since they have to develop most of their life cycle internally colonizing living plant tissue, without causing any negative effects [2]. According to Rodriguez et al. [12], these endophytes could be divided into different classes depending on their taxonomy and localization in the plant. The most common are those belonging to class 2, which is composed of non-clavicipitaceous endophytes that can grow in plant tissues both above and below ground. Moreover, a big number of these endophytes belonging to class 2 are related to necrotrophic pathogens, which, under certain conditions, can switch to a pathogenic lifestyle [13]. In this way, after the colonization, these microorganisms initiate a complex interaction with plant receptors, to be recognized as helpful for the host. Otherwise, the plant could trigger a set of defensive responses to remove the possible threat.

The interaction with microorganisms is highly dependent on the level of certain plant hormones. The plant response against biotrophic pathogens is usually controlled by the activation of SA-dependent responses [14]. However, it was also observed that SA pathway is also involved in the interaction with beneficial symbionts. Previous researchers showed that the plant induction of salicylic acid (SA) can inhibit the development and establishment of symbiosis with rhizobacteria and mycorrhiza [15,16]. Moreover, activation of SA pathway can also affect the

performance of beneficial symbionts. In grasses and legumes, the induction of SA pathway was related to a lower alkaloid production and nitrogen fixation by beneficial symbionts [17–19]. Similarly, the presence of beneficial microorganisms can modulate the SA pathway. For example, the presence of the fungal endophyte *Epichloe festucae* provoke a downregulation of SA biosynthesis genes [20] which may be related to a suppression of SA-dependent responses to facilitate the growth of the microorganism into plant tissues [18,21]

Jasmonic acid (JA) is one of the most important phytohormones involved in plant defense. This hormone is part of a family of signaling molecules derived from oxylipins that regulate different processes related to plant development, symbiotic interactions, and plant responses against insects and necrotrophic pathogens [22–24]. Together with ethylene (ET), JA is the hormone that triggers the phenomenon known as Induced Systemic Resistance (ISR) that is activated by the association between plants and certain beneficial microorganisms. This ISR is characterized by an accumulation of the JA derivative Jasmonoyl-isoleucine (JA-Ile), which is perceived by the protein complex Coronatine insensitive1 (COI1) and Jasmonate ZIM-domain (JAZ) protein. Upon the perception of JA-Ile the JAZ repressors are degraded releasing MYC2, allowing the expression of defensive genes. As we mentioned above, the big number of class 2 endophytes are related to necrotrophic pathogens, for this reason, it may be possible that the modulation of JA signaling could be involved in the symbiosis, keeping fungal endophytes in an asymptomatic stage [25].

It is generally accepted that there is an antagonism between Ja and SA pathways [26]. This antagonism is exploited by some hemibiotrophic pathogens such as *Pseudomonas syringae* pv. tomato (*Pst*). In this way, during the infection, *Pst* releases the phytotoxin Coronatine (COR) which is a structural homologous of jasmonic-isoleucine. Due to its structural affinity, COR binds to the JA-receptor COI1, triggering the jasmonic mediated responses such as the expression of SAMT1 and SAMT2 encoding enzymes that provoke the methylation of SA and, therefore, its deactivation. Thereby, this mechanism promotes an opening of the stomata by the

inactivation of the SA. However, some works hint to a synergistic interaction between the jasmonic and the salicylic pathways [27,28],

The genus *Acremonium* contains about 100 species most of which are saprobic species or plant pathogens [29–31]. In the recent years, several isolates of different species have been isolated as endophytes in healthy plants. Moreover, it was also observed that some of these endophytic species of *Acremonium* can improve the performance of the plant or protect the hosts against biotic or abiotic stress [32,33]. In recent works, we demonstrated that a new strain of *Acremonium, Acremonium sclerotigenum* isolate 13237 [34] isolated from *Aegilops sharonensis* was able to induce resistance in wheat against drought stress. The management of the plant microbiome has been proposed as a new platform for a revolution in the plant protection; however, the mechanisms underlying the relationships with the plant and with beneficial microbes are still understudied. For this reason, the aim of this work is to test whether this strain of *A. sclerotigenum* is able to colonize tomato plants and induce resistance against *Pseudomonas syringae* pv tomato as well as to study the resistance mechanisms that could be induced by the endophyte.

## 2. Materials and methods

### 2.1 Tomato seed inoculation and plant growth

*Acremonium sclerotigenum* strain 13237 [34] was cultured in erlenmeyer flasks containing 150 mL of Potato Dextrose Broth medium, which were incubated at 27º C under agitation at 180 revs $min^{-1}$ for 7 days. Conidia were collected from 7-day-old PDB cultures by filtration through two layers of Miracloth (Calbiochem) and adjusted to a concentration of $10^6$ conidia/ml with water. Then, tomato seeds of *Solanum lycopersicum* L. cv. Ailsa Craig, *Solanum lycopersicum* L. cv. Castlemart (wildtype) and JA pathway mutants, *acx1* and *spr2*, in the background Castlemart were soaked in conidia suspension (inoculated) or in sterile water (control).

After two hours, seeds were sowed in 100ml pots containing wet vermiculite. During the first week, seedlings were irrigated with water. Then plants were irrigated with Hoagland solution

for 3 weeks approx. until third and fourth true leaves were fully developed.48 hours before inoculation with *Pst*, plants of the same developmental stage were introduced in plastic boxes with near 100% of relative humidity and divided into four groups. Control: mock plants; inoculated: plants treated with *A. sclerotigenum*; infected: plants infected with *Pst*; inoculated and infected: plants treated with *A. sclerotigenum* and infected with *Pst*.

**2.2 Inoculation procedures**

For inoculation*, Pseudomonas syringae* pv. tomato strain DC3000 was grown on a shaker in agitation in KB supplemented with Rifampicin (50 mg $mL^{-1}$) at 28ºC for 24 h. Bacterial suspensions were adjusted to $5x10^5$ colony-forming units (CFU)/mL in sterile $MgSO_4$ (10 mM) containing 0.01% of the surfactant Silwet L-77 (Osi Specialties, Danbury, CT, USA).

Pathogen inoculation was performed by dipping the third and fourth leaves into the bacterial suspension. The disease rate was scored at 72 hpi by determining the percentage of dark-brown spots on the leaf surface and by counting the colony-forming units in KB medium. At least 20 samples for colony counting and 20 samples for disease rate scoring were taken for each treatment.

For chemical treatments, plants were grown and inoculated as described section 2.1. Treatment was performed as described by Scalschi et al.[35] In brief, two days before inoculation, plants were spray-treated with 50 µm JA (Santa Cruz Technologies) whereas mock plants were spray-treated with water. Plants were harvested and assessed for disease symptoms 72 h after inoculation.

**2.3 Evaluation of hormones related to plant defense by chromatographic analysis**

Fresh material (10 leaves per treatment and experiment) was frozen in liquid nitrogen, ground, and freeze-dried. 0.05 g of freeze-dried material was homogenized in 1ml of ultrapure water, and a mixture of internal standard {deuterated abscisic acid ($[^2H_6]$ ABA), deuterated salicylic acid ($[^2H_4]$ SA), and dihydro jasmonic acid (dhJA)} was added at 100 ng $ml^{-1}$ prior to extraction

in order to quantify the level of hormones JA, 12-oxo-phyto dienoic acid [OPDA], SA and ABA. After extraction, a 20 µl aliquot was injected directly into an Acquity ultra-performance liquid chromatography system (UPLC) with an ACQUITY UPLC BEH C18 column (1.7 µm 2.1 × 50 mm) (Waters, Mildford, MA, United States), which was interfaced with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, United Kingdom). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples.

**2.4 Gene expression**

Leaves were grounded in liquid nitrogen, and RNA was extracted using E.Z.N.A Plant RNA kit OMEGA biotek (http://www.omegabiotek.com), according to the manufacturer's instructions. 1 µg of total RNA after DNase treatment (Promega, http://www.promega.com) was reverse transcribed into cDNA using an oligodT primer and primescript RT enzyme mix 1 (Primescript RT reagent kit, TaKaRa). Quantitative real-time PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher) in a StepOneTM Real-Time PCR System (Thermo Fisher). For the analysis of plant genes, primers described by Scalschi et al[28] were used for *LoxD, AOS, AOC, OPR3, Jaz, PR1, PR5, ASR1,* and Actin as a control to normalize gene expression in each sample.

Quantification of fungal biomass in plants was performed by measuring eGFP. DNA was extracted using CTAB method [36]. The presence of the GFP gene was quantified using the following primers For: cgaccactaccagcagaaca and rev: gcatggacgagctgtacaag. Values were normalized to *Solanum lycopersicum* Actin gene.

**2.5 PEG-mediated protoplast transformation**

Mycelia of *Acremonium sclerotigenum* were obtained from one-week-old PDA cultures and used to inoculate flasks containing 150 mL of Potato Dextrose Broth (PDB) medium. The cultures were incubated for four days in a growth chamber at 28°C and agitation at 180 rpm. One day before the transformation the agitation was stopped in order to improve the amount of

mycelium. On the day of the transformation, the medium was filtered through miracloth and 0.5 gr of mycelium (approx.) were mixed in a petri dish with 10ml of protoplasting solution containing 40mg of lysing enzymes (SIGMA), 30 mg of Driselase (FUCA), 3mg of Lyticase (SIGMA) 2mg BSA and 2mg Yatalase (TAKARA). After3 hours of incubation at 25° C and 85 rpm, protoplasts were resuspended in STC buffer (1.2 M sorbitol, 50 mM $CaCl_2 \cdot 2H_2O$, 10 mM Tris–HCl pH 7.5) to obtain a final concentration of $10^8$ protoplasts/mL. 2 µg of Vector plasmid gGFP (containing GFP; hyg resistance and GPD promotor) was added to 100 µL of thawed protoplasts in a 50 mL centrifuge tube and then supplemented with STC buffer up to 300 µL. The protoplasts–DNA suspension was blended gently and cooled on ice for 20 min, then 2 mL PTC (60 % PEG3350, 10 mM Tris–HCl, 50 mM $CaCl_2$) solution was added dropwise and cooled for 20 min on ice. Next, 30 mL STC buffer was gently mixed into the suspension. The protoplasts were recovered by centrifugation at 2,000 g at 4ºC for 15 min. The supernatant was discarded, and the protoplasts were resuspended in 3 mL liquid regeneration medium (LR; 0.1 % yeast extract, 0.1 % casein enzymatic hydrolysate, 1 M sucrose) at 28ºC for 12 h. The culture was poured into a Petri dish with about 12 mL solid regeneration medium (SR; LR plus 0.7 % agar) and mixed. After the medium solidified, 1 % water agar with hyg B (50 mg/mL) was poured onto the plate.

**2.6 Root colonization analysis.**

Wild type, *spr2*, and *acx1* tomato seeds were surface sterilized and germinated in a petri dish with agar-water. Four days later, seeds with similar germination stage were transferred to a petri dish with Hoagland solution with agar for one week. Once the root was developed, a mycelium plug (0.6cm) of GFP tagged *Acremonium sclerotigenum* was deposited at 1cm from the root. One week later, roots were examined by confocal microscopy to check for the successful colonization of the fungi on the roots. In order to visualize root tissue, plant material was stained with propidium iodide (Merck). The GFP signal was collected on an Inverted Confocal Microscope Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) using 488 nm ray line of the argon laser for their excitation. GFP fluorescence was collected between 500 and 540 nm by

a HyD detector, while the fluorescence emitted by the propidium iodide was collected between 600 and 640 nm by a PMT detector. The same gain and offset settings were used for the different treatments. The images were processed using the LAS X (Leica Microsystems). Five roots were observed for each treatment and for each time point.

### 2.7 Statistical analysis

All experiments were conducted at least three times. Data from different repetitions were analyzed together since the analysis of variance (ANOVA) did not show significant differences ($P > 0.05$) between repetitions in each experiment. All data of this study were tested for normality, homogeneity of variances, and residual patterns. Mean values were compared using the Fisher's protected least significant difference (LSD) test at $p = 0.05$. Statistical analyses were performed by using the software Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA, USA).

## 3. Results

### 3.1 *Acremonium sclerotigenum* can induce resistance in tomato against Pst

In order to ascertain whether the inoculation with, *Acremonium sclerotigenum* was able to protect tomato plants against the bacterial pathogen *Pseudomonas syringae* pv tomato, the disease rate was scored by quantification of lesions and quantification of the bacterial population. Tomato plants treated with the endophyte *Acremonium sclerotigenum* showed significantly lower symptoms after inoculation with the bacteria *Pseudomonas syringae* in comparison with control plants. Visually, in figure 1a it can be observed that the area of necrotic specs, characteristically produced by this bacterium at 72h post-infection, was reduced in treated plants compared with the controls (fig 1a and b). The quantification of the symptoms showed a reduction in the percentage of diseased area of leaves from 67% in control leaves to 50% in *A. sclerotigenum* treated plants. In the same way, the size of the bacterial population was measured by determining the colony forming units (CFU) per gram of leaf. Results obtained showed that,

72 h post-infection, the viable bacterial population in leaves was 4 times higher in control plants than in plants treated with the endophyte (fig 1c).

### 3.2 Inoculation with acremonium induces the accumulation of jasmonic acid.

The inoculation of *Acremonium sclerotigenum* in tomato plants did not change the levels of abscisic acid, salicylic acid or OPDA *per se* in comparison with control plants. In detail, the level of abscisic acid was not affected neither by the presence of the endophyte nor by the inoculation with *Pseudomonas syringae* (fig. 2a). Regarding to the salicylic acid, results obtained showed that this hormone is enhanced by the infection with *Pst*, regardless of the inoculation with the endophyte (fig. 2b). Similar results were observed in the accumulation of OPDA. This compound, related to the JA pathway, was strongly enhanced in the plants infected with *Pst*, showing similar values in the plants inoculated and non-inoculated with *A. sclerotigenum* (fig. 2c). Interestingly, results obtained for JA showed that the presence of the endophyte enhances the accumulation of this hormone, and moreover, this enhancement is much stronger after the inoculation with *Pseudomonas syringae* (fig. 2d).

### *3.3 A. sclerotigenum* inoculation modifies the expression of defense related genes

Since the accumulation of JA was altered by the inoculation with the endophyte, we analyzed the expression of several marker genes for different steps of the jasmonic biosynthetic pathway, as well as, for other genes related to plant resistance.

The results of the expression of genes belonging to the initial steps of jasmonic biosynthesis showed that the inoculation with the endophyte does not enhance the expression of *LoxD* or *AOS* genes. Both genes showed a slight enhancement of the expression after the inoculation with *Pst*, regardless of the presence of endophyte (figure 3 b and c). However, when we observe the expression of the genes downstream in this pathway, results showed that, in the absence of *Pst*, the presence of the endophyte *per se* can induce the expression of *AOC* and *OPR3* showing significantly higher levels in comparison with control plants. Interestingly, in the presence of

the pathogen, the inoculation with *A. sclerotigenum* significantly enhances the expression of *OPR3* gene.

According to the results observed for the hormones, the expression of *ASR*, the marker gene for Abscisic acid, showed no significant differences between the different plant groups (Fig 4a). In the same way, the genes *PR1* and *PR5,* which are marker genes for salicylic acid, also confirm the results observed for the accumulation of that hormone. In both cases, we can observe an enhancement of the gene expression in the plants infected with *Pst* and treated with the endophyte. Interestingly, it could be observed that the plants treated with *Acremonium* and inoculated with *Pst* showed higher levels of *PR* genes compared with those inoculated with *Pst*. This result could be due to the higher presence of bacteria in plants inoculated with *Pst* in comparison with those treated with *Acremonium*. One of the mechanisms used by the *Pst* to infect the plants is the release of Coronatine that suppress the SA-derived responses including the expression of several PR genes [37]. However, the high variability on the results observed for endophytes treated plants provokes that despite the mean values are higher, there are no significant differences between plant inoculated with the endophyte and plants non-inoculated (Figure 4b and c). Interestingly, when we measure the expression of the *JAZ* gene, which is a jasmonic responsive gene, results showed a significant enhancement in plants infected with *P. syringae*. However, there are no significant differences between plants treated with the endophyte and plants non-treated for the measurement of the hormone. (fig. 4d).

### 3.4 JA mutants showed different defensive phenotypes after inoculation with *A. sclerotigenum*

In order to ascertain the role of the JA pathway in the resistance induced by *Acremonium sclerotigenum*, the mutants of JA *spr2* and *acx1* were used. The mutant *spr2*, which are defective in the initial steps of the JA biosynthesis, showed a complete lack of resistance. When we infected this mutant with *Pst*, no significant differences were observed between the plants that were previously inoculated with *A. sclerotigenum* and the non-inoculated plants. In this

case, both the percentage of the leaf with symptoms and the number of UFC per gram of leaf were similar in inoculated and non-inoculated plants (fig. 5 a and b). On the other hand, results showed by the mutant *acx1* were completely different. The mutant *acx1* showed that, after inoculation with the endophyte and the challenge infection with *Pst*, the phenotype is similar to that observed in the wild-type plants. In this mutant, which has impaired the β-Oxidation of OPDA to JA, the presence of the endophyte significantly reduced both the percentage of the leaf symptoms as well as the number of UFC per gram of leaf (fig. 5 c and d).

According with the results, we analyzed the hormonal levels of wild type, *spr2* and *acx1*. As expected, both mutants showed lower levels of JA in comparison to wild type plants. Interestingly, wild type plants and *acx1* mutant showed similar levels of OPDA in plants inoculated with Pst regardless the treatment with *A. sclerotigenum*, whereas *spr2* showed reduced levels of OPDA (Fig 6).

**3.5 Deficiencies of JA do not alter the colonization by *Acremonium sclerotigenum***

In order to study whether the deficiencies in the JA pathway affect the colonization of tomato plants by the endophyte, a study of the presence of *Acremonium sclerotigenum* in the different mutants used was performed. With the purpose of visualizing and quantifying the endophyte inside the plant, the isolate of *A. sclerotigenum* was tagged with GFP.

The success of the transformation was evaluated by microscopic examinations of selected transformants. In the selected transformants it was observed an intense fluorescence in all the structures of the fungi, including conidia, mycelia, and germinating conidia (fig 7a). In the same way, when the endophyte was inoculated in tomato plants, the fluorescence was easily visualized within the plant tissue by confocal microscopy (fig 7b).

When we compare the presence of the endophyte in the roots of the wild-type plants, with those observed in the mutants *acx1* and *spr2*, there are no visible differences. In all the samples, it was possible to visualize the fungi on the surface as well as within the tissue of the plant (fig. 7c). Moreover, to quantify the presence of the endophyte in each mutant as well as in the wild type,

the amount of the GFP gene was determined by qPCR. Results obtained showed that there are no significant differences in the amount of GFP gene in the root tissue of different mutants as well as in the wild-type tomato plant (fig. 7d).

### 3.6 Mutants resistance is not restored by JA treatment

In order to ascertain if the observed phenotype is due to the presence or absence of JA, mutants inoculated with *A. sclerotigenum* were treated with exogenous JA. Results showed that treatment with JA induce susceptibility in *acx1* mutants, increasing the percentage of infected leaf and CFU in both plants treated with *A. sclerotigenum* and control plants (fig. 8a and b). On the other hand, the treatment with JA had no changes in the susceptibility of *spr2* mutants, showing the same levels of infection and CFU regardless of the treatment with JA or the inoculation with the endophyte (fig 8 c and d).

## 4. Discussion and conclusions

Plant evolution has been highly influenced by the interaction with microorganisms in the environment, leading to symbiotic associations [38]. Despite that the most known symbiotic interactions are those formed with arbuscular mycorrhizal and rhizobia, growing evidence indicates that endophytic associations can benefit the plant either by the direct production of secondary metabolites or by the modulation of plant defense [39]. In this way, recent studies have demonstrated that different endophytes are able to induce plant resistance against biotic or abiotic stresses [34,40,41]. However, it is well known that the plant microbiome is highly influenced by the host. During the colonization, the endophytes should be recognized by the plant and the perception of the signal molecules, and correct recognition of the microbe as a beneficial partner would become a successful symbiosis [39,42]. In the same way, the plant genotype, which influences the ulterior morphological characteristics such as root architecture, growth, or exudates will have an impact on the microbiome assembly [43]. All these factors provoke that different plant species, even collected in the same field, show different microbiome [44]. For this reason, different species of wild plants could be considered as a source of new

endophytes for taxonomically related species, which could be expected to be more compatible than species isolated from unrelated plants [45]. However, several studies showed that some endophytes can induce resistance in crops independently from the species that were isolated from the beginning [46]. In our case, the genus *Acremonium* was previously observed as an endophyte in several plants [29,47,48]. However, our isolate was previously reported only in *Triticum*-related plants. Despite that, the results showed that the inoculation of the endophyte successfully reduce the symptoms of *Pseudomonas syringae* as well as the number of CFU per leaf, suggesting that this endophyte is able to induce resistance regardless of the plant species.

The analysis of different hormonal pathways suggests that the effect of the endophyte in the plant is due to an enhancement of the plant defenses. It is well known that the presence of endophytes can mediate resistance in plants by the alteration of endogenous hormones. Some authors suggest that a symbiosis with an endophytic fungus is usually related to a repression of the salicylic acid pathway and an enhancement of JA [18,25]. In this way, the resistance induced in plant-associated *Trichoderma asperellum*, *Penicillium* sp., and *Serendipita indica* have been related to an increase of JA [49–51]. However, the symbiosis with other endophytes displayed a different hormonal pattern. It was also observed that *S. indica* is able to induce resistance independently of the JA/ET pathway [52], as well as *T. asperellum* induced resistance is mediated by SA [53]. Moreover, Martinez-Medina et al [54] observed a shift between salicylic and jasmonic acid induced by *Trichoderma* which adapts its response according to the life stage of the pathogen *Meloidogyne incognita.* Our results showed that in plants control and inoculated with *A. sclerotigenum,* the infection with *Pst* induces a similar enhancement of the salicylic acid, suggesting that the *A. sclerotigenum* does not provoke repression of this molecule. However, the fact that non-inoculated plants showed no significant difference in the levels of SA, suggests that the levels of this hormone observed are not related to the presence of the *A. sclerotigenum* but due to the defense against *Pst*.

On the other hand, we observed that the presence of the *A. sclerotigenum per se* increase the levels of JA and, after inoculation with *Pst,* the endophyte provokes a significant increase in the

level of this hormone. This accumulation was also confirmed by the analysis of different genes related to the biosynthesis of JA, which revealed that, in some cases, the presence of the endophyte was also enough to induce the gene expression. It has been previously suggested that JA-dependent signaling could be involved in the establishment of the symbiosis, keeping fungal endophytes in a non-pathogenic lifestyle. Thus, the colonization by certain endophytes itself activates the jasmonic pathway increasing the resistance of the plant to other pathogens [25]. According to that, the increase of JA level observed in control plants inoculated with the endophyte, could suggest a plant response against the colonization by a foreign endophyte.

Despite that, the mechanisms by which the enhancement of JA induced by *A. sclerotigenum* is involved in resistance against Pst are not evident. Usually, the induction of JA is associated with the resistance against necrotrophic pathogens and insects[14], whereas the enhancement and the manipulation of the JA responses is one of the mechanisms used by *Pseudomonas syringae* to colonize the plant [55,56]. Our results showed an increase of OPDA, a precursor of JA in response to the infection with *Pst* in both control plants and plants treated with *A. sclerotigenum*. However, we can observe that the levels of JA are significantly higher in plants treated with the endophyte and infected with *Pst*. This suggests a possible implication of the JA pathway. To clarify this point, we used different tomato mutants to ascertain the implication of this pathway in the resistance induced by *A. sclerotigenum*. Previous studies suggested that several metabolites from JA biosynthetic pathway, such as OPDA, JA, and JA-Ile may possess a biological role [35,57]. For this reason, we used the *spr2* mutant which is affected upstream the biosynthetic pathway of jasmonic acid, in the generation of α-linolenic acid and consequently has lower levels of α-linolenic acid, OPDA, and JA. The use of this mutant showed that the endophytes was not able to induce the protective effect against *Pst* suggesting that some molecules derived from the JA biosynthetic pathway could be the responsible for the induction of resistance against *Pst*, by itself or acting as a signal molecule. On the other hand, the tomato mutant *acx1* is affected in steps of the β-oxidation of the oxo-pentenyl-cyclopentane-(OPC)-8 and, consequently, has lower levels of JA, but does accumulate OPDA [58]. Interestingly,

mutant *acx1* inoculated with the endophyte and infected with *Pst* showed the same levels of resistance than observed in wild type plants. Since, that mutant is impaired in the levels of JA and JA-Ile, it seems plausible that these molecules are not necessary for the induction of resistance mediated by *A. sclerotigenum.* These results suggest that some metabolites related to the initial steps of JA are required for the resistance induced by *A. sclerotigenum*. In order to confirm this hypothesis, an exogenous treatment with JA was performed in mutant plants treated with *A. sclerotigenum.* The results showed that the treatment enhanced the susceptibility of *acx1* mutant to *Pst* both in inoculated and non-inoculated plants with the endophyte. This result is not surprising since it has been previously described that *Pst* is able to overcome the natural defenses of the plant by releasing a structural homolog of JA-ile (Coronatine) that impairs the Salicylic acid mediated responses [59]. In this way, the exogenous application of JA would benefit the bacteria. On the other hand, the exogenous treatment with JA does not change the susceptibility of *spr2* mutants either inoculated or non-inoculated with the endophyte. These results were also expected since previous experiments using *spr2* mutants observed that the phenotype of resistance against aphids was not affected by exogenous application of JA. In this way, the authors suggested that the mutation of *spr2* may influence JA defenses through independent effects on JA synthesis [60].These results confirm that JA is not required for *A. sclerotigenum* induced resistance against *Pst*

The relationship between endophytic colonization, endophyte-induced resistance, and JA was previously described for several plant species. Ren and Dai (2012) described that the inoculation of *Atractylodes lancea* plants with the endophyte *Gilmaniella sp.* enhanced plant endogenous JA levels [61]. Navarro-Melendez and Heil observed that the inoculation of Lima Beans with *Fusarium sp*. or *C. lunatus* induced the emission of Volatile Organic Compounds, indicating an up-regulation of, at least, parts of the classical JA-dependent defenses [25]. The colonization by several beneficial microorganisms was studied using *AOC* and *spr2* mutants. In this case, the decrease in JA levels due to silencing of AOC and *spr2* gene was related to a delay in the mycorrhiza colonization as well as in the number of arbuscule. However, the exogenous application of JA in *spr2* mutants restored the normal colonization of plants [62,63]. Similarly,

Han et al.[33] observed that JA could be important for the *Acremonium* colonization of rice. These authors observed lower colonization of *Acremonium* sp. D212 in the rice mutant Coi1-18, defective in JA, compared with wild type plants. According to this, it would be plausible that the loss of resistance phenotype that we observed in the *spr2* mutants could be related to lower colonization by the *A. sclerotigenum.* To clarify this point, the presence of the endophyte in the roots was monitored by direct visualization of the GFP-tagged endophyte and by PCR. Our results showed that no differences in colonization or biomass between control plants and in *spr2* or *acx1* mutants, suggesting that the JA pathway is not related to the colonization of *Acremonium sclerotigenum* in tomato. In this way, similar results were obtained in nicotiana plants silenced for COI or AOS, in which the colonization of *Glomus intradices* reached the same levels as in wild-type plants [64].

In conclusion, the inoculation of *Acremonium sclerotigenum* in tomato plants is able to induce resistance against *Pseudomonas syringae*. Hormonal levels, as well as the use of jasmonic pathway mutants, suggest that the JA pathway is not necessary for the colonization by the endophyte. However, the lack of the complete pathway provokes a loss of the induced resistance, indicating a possible need for, at least, some components of the classical JA biosynthetic pathway. Although it has been described that the defense of the plant against *Pst* is determined by the SA pathway, and there is an antagonistic relationship between the SA and JA pathways, recent studies have shown that the JA pathway can act as a core signal in the phytohormone signaling network in induced resistance [35]. These authors suggest that some of the intermediary molecules of this pathway, especially OPDA could play this role in the crosstalk between different phytohormones [35,56].

These results agree with our observations, since the behavior of the JA pathway mutants suggests that nor the JA, JA-Ile it, or MeJA are the key molecules in the induction resistance in mediated by *A. sclerotigenum* against *Pst*,. Nevertheless, more experiments will be needed to ascertain the role of the jasmonic pathway in the resistance induced by *A. sclerotigenum*. The fact that an endophyte isolated from wild wheat species is able to induce resistance in tomato

hints that wild plants could be a source for new endophytes with a broad host range, which could be applicable to crops of agronomic interest.

**Acknowledgements**

This work was supported by FEDER/ Ministry of Science Innovation and Universities-Spanish State Research Agency (Project AGL2017-85987-C3-1-R), Programa I+D+I Generalitat Valenciana/ Subvenciones a grupos de investigación emergentes (GV/2019/028), and by the Pla de Promoció de la Investigació of Universitat Jaume I (UJI-A2019-19). The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Figure legends

**Fig 1: Phenotype analysis 72 hours after infection with *Pseudomonas syringae* of four-week-old tomato plants control or treated with *Acremonium sclerotigenum*.** A) detail of the control plants (left) and plants treated with *Acremonoium* (right) after inoculation with *Pst*. B) Infection rate scored measuring the percentage of the leaf covered by bacterial specks and C) Count of bacterial populations after plating in agar–King's B medium leaf extracts of control, or *Acremonium* treated plants. Different letters indicate statistically significant differences between treatments at the same time point ($p < 0.05$; least-significant difference test).

**Fig. 2. Hormone levels in control and A. sclerotigenum inoculated plants upon Pst infection.** Leaves were collected at 72 hpi and ABA(A), SA (B), cis-(+)-12-oxo-

phytodienoic acid (OPDA) (C), and JA(D) levels were determined by ultra-performance liquid chromatography (UPLC)-mass spectrometry. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Different letters indicate statistically significant differences between treatments at the same time point ($p < 0.05$; least-significant difference test).

**Fig 3. Scheme of Jasmonic acid biosynthetic pathway and gene expression profile of jasmonic biosynthetic pathway in tomato plants inoculated with *Acremonium sclerotigenum* and after *P. syringae* infection**. Right: scheme of JA pathway highlighting the step impaired in the different mutants used. Right: the expression of the LoxD(A), AOS (B), AOC (C), and OPR3 (D), genes were analyzed in cDNA from *control and A. sclerotigenum inoculated plants upon Pst infection* at 72 h post-inoculation. The results were normalized to the EF1α gene expression measured in the same samples. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Statistical analysis was carried out between samples collected at the same time point. Different letters indicate statistically significant differences between treatments ($p < 0.05$; least-significant difference test).

**Figure 4. Gene expression profile of plant defense pathways in tomato plants inoculated with *Acremonium sclerotigenum* and after *P. syringae* infection.** Expression levels of marker genes of ABA (ASR1) (A), SA (PR1 and PR5) (B, C), and JA (JAZ) (D) signaling pathways were analyzed. The results were normalized to the EF1α gene expression measured in the same samples. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Statistical analysis was carried out between samples collected at the same time point. Different letters indicate statistically significant differences between treatments ($p < 0.05$; least-significant difference test).

**Fig 5: Phenotype of Four-week-old tomato plants control or *A. sclerotigenum* treated 72 hours after infection with *Pseudomonas syringae*.** Infection rate scored measuring the percentage leaf covered by bacterial specks for A) *spr2* mutants and B) *acx1* mutant. Count of bacterial populations by plating in agar–King's B medium by plating leaf extracts of control, or *A. sclerotigenum* treated plants for C) *spr2* mutant and D) *acx1* mutant.

**Figure 6. Hormone levels wild type (Castelmart), *spr2* and *acx1* tomato plants treated with *A. sclerotigenum* and inoculated Pst infection**. Leaves were collected at 72 hpi and cis-(+)-12-oxo-phytodienoic acid (OPDA), and JA levels were determined by ultra-performance liquid chromatography (UPLC)-mass spectrometry. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Different letters indicate statistically significant differences between treatments at the same time point ($p < 0.05$; least-significant difference test).

**Fig 7: Presence of *A. sclerotigenum* in different backgrounds tested.** Fungal biomass in Castelmart, *acx1* and *spr2* mutants (A) measured by presence of GFP gene in roots. Visualization of GFP-tagged A. sclerotigenum in tomato roots of Castlemart (B), *acx1* (C), and *spr2* (D) by confocal microscopy.

**Fig 8: Effect of JA exogenous application on phenotype of Four-week-old tomato plants control or *A. sclerotigenum* treated 72 hours after infection with *Pseudomonas syringae*.** Infection rate scored measuring the percentage leaf covered by bacterial specks and count of bacterial populations by plating in agar–King's B medium by plating leaf extracts of control for A) and B) *acx1* mutants, C) and D) *spr2* mutants.