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The plant hormone salicylic acid interacts with the mechanism of anti-herbivory conferred by fungal endophytes in grasses

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Abstract

The plant hormone salicylic acid (SA) is recognized as an effective defence against biotrophic pathogens, but its role as regulator of beneficial plant symbionts has received little attention. We studied the relationship between the SA hormone and leaf fungal endophytes on herbivore defences in symbiotic grasses. We hypothesize that the SA exposure suppresses the endophyte reducing the fungal-produced alkaloids. Because of the role that alkaloids play in anti-herbivore defences, any reduction in their production should make host plants more susceptible to herbivores. *Lolium multiflorum* plants symbiotic and nonsymbiotic with the endophyte *Epichloë occultans* were exposed to SA followed by a challenge with the aphid *Rhopalosiphum padi*. We measured the level of plant resistance to aphids, and the defences conferred by endophytes and host plants. Symbiotic plants had lower concentrations of SA than did the nonsymbiotic counterparts. Consistent with our prediction, the hormonal treatment reduced the concentration of loline alkaloids (i.e., N-formyllolines and N-acetylnorlolines) and consequently decreased the endophyte-conferred resistance against aphids. Our study highlights the importance of the interaction between the plant immune system and endophytes for the stability of the defensive mutualism. Our results indicate that the SA plays a critical role in regulating the endophyte-conferred resistance against herbivores.

KEYWORDS

alkaloids, beneficial microorganisms, biotrophs, endophyte symbiosis, *Epichloë*, plant defences, sap-sucking insects

1 | INTRODUCTION

Plants are continuously challenged by herbivores. Due to the selective pressures exerted by the herbivores, plants have acquired mechanisms to respond to herbivory (Agrawal, Hastings, Johnson, Maron, & Salminen, 2012; Karban & Baldwin, 1997; Schoonhoven, van Loon, & Dicke, 2005). For example, the plant immune system can be a highly specific and effective defence against several groups of attackers (Bari & Jones, 2009; Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012; Thaler, Humphrey, & Whiteman, 2012). These responses are orchestrated by a group of interacting plant hormones that include salicylic acid (SA), jasmonic acid (JA), and ethylene (Ballaré, 2014; Dicke & Baldwin, 2010; Heil & Ton, 2008; Pieterse et al., 2012; Thaler et al., 2012). SA and JA govern the defensive responses of plants against pathogens and insect herbivores (Ballaré, 2014; Bari & Jones, 2009; Pieterse et al., 2012; Thaler et al., 2012; Vos, Pieterse, & van Wees, 2013). The current model posits that JA-dependent defences are effective against necrotrophic pathogens and chewing insect herbivores, whereas SA-dependent defences are effective against biotrophic pathogens and sap-sucking insects (Ballaré, 2014; Glazebrook, 2005; Kunkel & Brooks, 2002; Pineda, Dicke, Pieterse, & Pozo, 2013; Schwartzberg & Tumlinson, 2014; Schweiger, Heise, Persicke, & Muller, 2014; Thaler et al., 2012). Although this model of plant immunity has received considerable support, it is not clear how the model applies when host defences are assisted by other organisms. Beneficial symbiotic microorganisms may modulate the immune responses of plants and affect their interactions with natural enemies (Jung, Martinez-Medina, Lopez-Raez, & Pozo, 2012; Pieterse et al., 2014; Pozo & Azcón-Aguilar, 2007).

The association of plants with beneficial microorganisms generates changes in the plant immune responses (Cameron, Neal, van Wees, & Ton, 2013; Pieterse et al., 2014; Zamioudis & Pieterse, 2011). For

example, the presence of certain species of bacteria (e.g., *Rhizobium*) and mycorrhizal fungi activate systemic immune responses termed *induced systemic resistance* and *mycorrhiza-induced resistance*, respectively (Cameron et al., 2013; Jung et al., 2012; Pieterse et al., 2014; Pozo, López-Ráez, Azcón-Aguilar, & García-Garrido, 2015; Verhage, van Wees, & Pieterse, 2010). Induced systemic resistance and mycorrhiza-induced resistance differ from other types of induced resistance in that symbiotic plants show a "primed state" of defence, usually related to the JA pathway. In a primed state, plants exhibit an earlier, faster, stronger, and/or more sustained expression of defences against pathogen or insect attacks (Jung et al., 2012; Pieterse et al., 2014; Pineda et al., 2013; Van Wees, Van der Ent, & Pieterse, 2008). When the JA pathway is activated, symbiotic plants are generally more resistant to necrotrophic pathogens and chewing insects than nonsymbiotic ones (Jung et al., 2012; Pieterse et al., 2014; Pineda, Zheng, van Loon, Pieterse, & Dicke, 2010; Zamioudis & Pieterse, 2011). However, it has also been found that plants in symbiosis with mycorrhizal fungi are still susceptible to biotrophic pathogens and certain species of sap-sucking insects (Hartley & Gange, 2008; Jung et al., 2012; Pineda et al., 2013). This susceptibility seems to result from the symbionts' active suppression of the SA pathway (Cameron et al., 2013; Jung et al., 2012; Pozo et al., 2015; Zamioudis & Pieterse, 2011).

The modulation of the plant immune responses results from the continuous molecular crosstalk between plants and symbiotic microorganisms (Gutjahr, 2014; Ryu, Cho, Choi, & Hwang, 2012). This crosstalk has been primarily studied in symbiosis with mycorrhizal fungi and certain rhizobacteria, which have to colonize the roots to establish a stable symbiosis (Cameron et al., 2013; Gutjahr, 2014; Jung et al., 2012; Oldroyd & Downie, 2008; Pozo et al., 2015; Ryu et al., 2012). At the first step of the interaction (incipient symbiont colonization), plants recognize these microorganisms as biotrophic pathogens and activate the SA pathway. In response, the microorganisms produce specific enzymes that suppress the SA pathway (Martínez-Abarca et al., 1998; Siciliano et al., 2007), allowing themselves to colonize root tissues and establish the symbiotic interaction (Jung et al., 2012; Pozo & Azcón-Aguilar, 2007; Ryu et al., 2012; Stacey, McAlvin, Kim, Olivares, & Soto, 2006; Yasuda et al., 2016; Zamioudis & Pieterse, 2011). In addition to the SA modulation by symbionts, the JA pathway also responds to the communication between plants and microorganisms. Enhanced levels of JA and the up-regulation of JA precursors or JA-responsive genes have been observed in symbiotic plants (Jung et al., 2012; Pieterse et al., 2014; Pozo et al., 2015; Wasternack & Hause, 2013). However, the activation of the JA pathway seems to be related to a plant mechanism that regulates the functionality of the symbiosis (Jung et al., 2012; Nakagawa & Kawaguchi, 2006; Pieterse et al., 2014; Pozo & Azcón-Aguilar, 2007; Sun et al., 2006; Wasternack & Hause, 2013).

Some leaf fungal endophytes of the genus *Epichloë* (Clavicipitaceae and Ascomycota) establish persistent symbioses with cool-season grass species (Poöideae) and are strictly vertically transmitted from plant to progeny (Clay, 1988; Gundel, Rudgers, & Ghersa, 2011; Schardl, 2010). The endophyte prevalence in grass populations (i.e., the proportion of endophyte-symbiotic plants) depends on the host plant fitness (relative to endophyte-free plants), and the efficiency in which fungal endophytes are transmitted between generations (Gundel, Garibaldi, Martínez-Ghersa, & Ghersa, 2011). Among the benefits associated with endophyte symbiosis, alkaloid mediated herbivore defence has the most experimental support (Clay, 1988; Saikkonen, Gundel, & Helander, 2013; Schardl, 2010). There are four well-described alkaloid classes produced by *Epichloë* endophytes: ergot alkaloids (i.e., ergopeptine and ergovaline), indole-diterpenes (i.e., lolitrem B and terpendoles), pyrrolizidines (i.e., lolines), and peramine (Panaccione, Beaulieu, & Cook, 2014; Saikkonen et al., 2013; Schardl et al., 2013; Schardl, Young, Faulkner, Florea, & Pan, 2012; Young et al., 2015). The required enzymatic apparatus for synthesis of these alkaloids are encoded entirely in the fungal genome, and most of the biosynthesis routes have been already elucidated (Schardl et al., 2013; Schardl, Grossman, Nagabhyru, Faulkner, & Mallik, 2007; Young et al., 2015). The fungal species and strains determine the alkaloid profiles, whereas the production level can also depend on other factors such as the plant species, plant tissue/organ, environmental conditions, and the concentration of the fungus present in the plant (Ball, Prestidge, & Sprosen, 1995; Justus, Witte, & Hartmann, 1997; Rasmussen et al., 2007; Ryan, Rasmussen, Xue, Parsons, & Newman, 2014; Saikkonen et al., 2013).

The presence of *Epichloë* fungal endophytes leads to a modulation of the plant SA signalling pathway. A recent study showed a general pattern of down-regulation of genes related to biosynthesis and signalling of SA in *Lolium perenne* plants associated with its common endophyte *E. festucae* strain Fl1 (Dupont et al., 2015). Furthermore, several marker genes of the SA pathway can be down-regulated in endophyte-symbiotic plants (Dupont et al., 2015; Johnson, Johnson, Schardl, & Panaccione, 2003). The down-regulation of the SA pathway could be the result of an active control by the endophyte on the host immune system by specific fungal effectors (Ambrose & Belanger, 2012). Evidence of biotrophic pathogen susceptibility also supports the hypothesis that the presence of the endophyte decreases SA-dependent plant defences (Welty, Barker, & Azevedo, 1991, 1993; Wäli, Helander, Nissinen, & Saikkonen, 2006; Krauss, Härri, Bush, Power, & Muller, 2007; Dariusz, Małgorzata, & Mikołaj, 2011; Sabzalian, Mirlohi, & Sharifnabi, 2012).

Here, we studied the interaction between the SA hormone and leaf fungal endophytes on herbivore defences in symbiotic grasses. We subjected annual ryegrass plants (*Lolium multiflorum*), with and without the common endophyte *Epichloë occultans* (Moon, Scott, Schardl, & Christensen, 2000), to an exogenous application of SA followed by a challenge with the generalist aphid *Rhopalosiphum padi* (bird cherry-oat aphid). *Epichloë occultans* is known to produce the loline alkaloids N-formylloline (NFL) and its precursor N-acetylnorloline (NANL; Bastias et al., 2017; Moore, Pratley, Mace, & Weston, 2015; Sugawara, Inoue, Yamashita, & Ohkubo, 2006). It has previously been demonstrated that both the presence of *E. occultans* (Bastias et al., 2017; Gundel et al., 2012; Miranda, Marina, & Chaneton, 2011; Omacini, Chaneton, Ghersa, & Muller, 2001; Ueno et al., 2015) and particularly these fungal lolines (Eichenseer, Dahlman, & Bush, 1991; Johnson et al., 1985; Panaccione et al., 2014; Wilkinson et al., 2000) have negative effects on the performance of *R. padi*. We predicted that the exogenous application of SA would stimulate the immune system (specifically the SA pathway) in endophyte-free plants, increasing the overall resistance level and consequently reducing the population size of the aphids. However, because *Epichloë* fungal endophytes are biotrophic microorganisms, the exposure of symbiotic

plants to exogenous SA would impair the alkaloid-based defences decreasing the resistance level and consequently increasing the population size of aphids. Our study helps to elucidate the functional bases of the resistance responses of plants in symbiosis with leaf fungal endophytes.

2 | MATERIAL AND METHODS

2.1 | Plant stock and aphid colony

Plants of *Lolium multiflorum* Lam., symbiotic and nonsymbiotic with the fungal endophyte *E. occultans* (E+ and E−, respectively), were generated from one grass population collected from an old successional pampean grassland (Argentina; 36° 00′ S, 61° 5′ W). Nonsymbiotic plants were manipulatively generated by treating symbiotic seeds from F0 generation with a systemic fungicide (Triadimenol 150 g kg^{-1}; Baytan®). In order to multiply seeds, fungicide treated and untreated seeds were sown in contiguous plots (1 m^2, recall that the endophyte *E. occultans* is strictly vertically transmitted and cannot move from plot to plot except by seed dispersal, which was prevented through collection) at the experimental field of the Institute IFEVA—CONICET, College of Agronomy, Universidad de Buenos Aires, Argentina (34° 35′ S, 58° 28′ W). Plants from both plots were allowed to share pollen to mitigate any genetic differentiation (*L. multiflorum* is obligately outcrossing). Ripe seeds from each plot were harvested and stored in dry conditions until the experiments commenced. The F1 seed-symbiotic status in each seed lot was estimated by sampling 100 seeds from each lot, clearing, staining, and examining each individually under a microscope at 40X power (seed squash technique; Bacon & White, 1994; Card, Rolston, Park, Cox, & Hume, 2011). The frequency of F1 endophyte-symbiotic seeds was contrasting; 1% in fungicide-treated and 99% in nontreated seed lots. In addition, the symbiotic status of each experimental plant was reconfirmed by microscopic examination of the sheath base of the outermost leaf (Bacon & White, 1994). This protocol to obtain E+ and E− plants by removing the fungus has been widely used (Kauppinen, Saikkonen, Helander, Pirttilä, & Wäli, 2016), and some advantages are that (a) both plant groups share the same genetic background (natural grass populations can include E+ and E− plants with genetic differences; Gundel et al., 2013; Gundel, Omacini, Sadras, & Ghersa, 2010) and that (b) any eventual toxic effect of the fungicide on the F1 plants are likely to be negligible (similarly to other studies; Gundel et al., 2015; Ueno et al., 2015). In particular, we did not observe any evident effect of the fungicide treatment on the morphology, phenology, and reproduction of our experimental plants.

During the normal growing season (autumn-winter-spring), individual plants (E+: n = 50, and E−: n = 50) were grown in the field, in 1.5-L pots (soil, sand, and peat). The plants were watered periodically in order to avoid water stress. In early spring, individual bird cherry-oat aphids *Rhopalosiphum padi* (L.) were collected from the same experimental field to generate a colony for our experiments. The founder population of aphids was established from 200 apterous adults that were reared for 6 weeks on wheat plants (cultivar Cronox; Don Mario) under controlled conditions (21 °C [±1], photoperiod L16: D8 h, and radiation 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

2.2 | Design and set-up of the experiment

An experiment was conducted to test the defence responses of E+ and E− plants to *R. padi* aphids. In mid spring, 14 E+ and 14 E− 18 weeks old healthy plants were selected from the plant stock, and moved to a growth chamber with the same environmental conditions as the aphid colony. The plants had approximately 45 tillers each (range: 35–67) and were starting to flower. After a close examination to ensure that there were no insects on them, the plants were individually enclosed with a white voile fabric bag supported with a tubular plastic net. Plants were acclimatized to the chamber conditions for 1 week before applying the hormone treatments (see below).

The experiment was a 2 × 2 full factorial design, with endophyte (E+, E−) and SA (SA+, SA−) as main factors. Prior to the SA treatment, we recorded the number and phenological state (vegetative or reproductive) of all tillers. Half of the plants from each endophyte status (7 E+ and 7 E−) were sprayed with 10 ml of 0.5 mM of SA solution (Biopack; Buenos Aires, Argentina) and the other half with 10 ml of water. Three days after the SA application, each plant was challenged with five adult apterous aphids (from our colony) and immediately enclosed within the white voile fabric bags to avoid insect escape. The delay between the SA application and the aphid challenge was to allow the plants to develop a response prior to contact with aphids.

We followed aphid populations for 12 days. The number of aphids on each plant (aphid population size) was counted at Days 4, 7, and 12 of the aphid challenge, which corresponds with Days 7, 10, and 15 since the SA application. On the plants, three serial harvests of tissues were carried out in order to measure the physiological concentration of hormones and fungal alkaloids. At the first harvest, two leaves (from one tiller) per plant were removed just before aphid challenge (3 days after the SA application) to measure the concentrations of defensive hormones (SA and JA) by means of mass spectrometry (see below). At Days 7 and 15 after the SA application (or Days 4 and 12 of the aphid challenge), one tiller base (pseudostems formed by leaf sheaths) per E+ plant was harvested to measure the concentration of fungal alkaloids by means of gas chromatography (see below). The same procedure was performed on E− plants, although alkaloids were not quantified. We harvested tillers where aphids were feeding. Considering that tillers have some degree of independence (Yang & Hwa, 2008), all the samples were removed from distant tillers, thus reducing the effects of serial "clipping" of tissues on the physiological status of the whole plant. In addition, we avoided sampling senescing plant tillers. The total harvest of samples (for hormones and alkaloids) was around a 1% of the total aboveground biomass. At Day 15 after the SA application, we harvested, dried, and weighed the aboveground plant tissues with an analytical balance (±0.1 g Mettler Toledo).

2.3 | Quantification of SA and JA

2.3.1 | Extraction and derivatization

The analysis of trace amounts of SA and JA was achieved by GC-MS/MS after derivatization by N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). Leaf samples were freeze dried and ground using a 2010 Geno/grinder® (SPEX®SamplePrep); 50–100 mg of each sample was transferred to 2-ml screw-cap FastPrep

tubes (Qbiogene, Carlsbad, CA) containing two steel balls (4 mm; SPEX®SamplePrep). One millilitre of 100% acentronitrile (ACN) spiked with 10 µl of internal standards (d6-SA: 100 ng and d5-JA: 100 ng; CDN Isotopes; Pointe-Claire, QC, Canada) was added to the 2-ml tubes and shaken for 10 min at 1,000 strokes per minute with 2010 Geno/grinder. Samples were centrifugated at 13,200 rpm for 20 min at 4 °C, and supernatants were transferred to 2-ml glass vials (Phenomenex®). The extraction protocol was repeated adding 1 ml of 100% ACN without internal standards. After the centrifugation step, supernatants were combined. The volume of plant extracts were reduced to complete dryness in a concentrator (Savant SpeedVac, Thermo Fisher Scientific Inc.), and dry samples were derivatized with 100 µl of MSTFA at 60 °C for 2 hr. After silylation, samples were cooled down at room temperature prior to injection into the GC.

2.3.2 | GC-MS-MS method

A Scion TQ GC-MS/MS (Bruker Daltonics Inc.) was used with an Agilent DB-5MS column (30 m long with 10 m guard column, 0.25 mm inner diameter, and 0.25 µm film thickness) installed. The GC was operated in constant flow mode (1 ml min^{-1}) with helium as carrier gas. The inlet had temperature of 290 °C, and solution (1 µl) injection was performed in the split mode at 1:20 ratio. The GC oven was kept at 80 °C for 2 min before rising to 230 °C using a linear gradient of 10 °C/min. After keeping constant for 1 min at 230 °C, temperature was increased to 310 °C at 40 °C min^{-1} and held for 5 min resulting in a 25-min total run time. The MS transfer line temperature was set to 290 °C. The source temperature was set to 230 °C. The data were acquired in electron impact positive ionization mode at 70-eV energy and multiple reactions monitoring mode using 2-mTorr collision pressure and 30-eV energy for precursor ion fragmentation. For each compound, the precursor and product ion pair transition with their corresponding retention times were as follows: SA (267- > 73 m/z, 11.2 min), d6-SA (271- > 73 m/z, 11.1 min), JA (222- > 73 m/z, 13.7 min), d5-JA (287- > 73 m/z, 13.2 min).

2.3.3 | Isotope dilution analysis

The SA and JA were quantified by isotope dilution analysis by adding 10 µl of isotopically enriched salicylic and jasmonic acids to the plant extracts prior to *O*-TMS derivatization. We took advantage of chromatographic behaviour differences of natural abundance and deuterium-labelled compounds, the isotope effect in chromatographic separations results in retention time differences between compounds at natural abundance and isotopically labelled. The retention time shift for a given labelled compound depends on the number of deuterium atoms in the molecule. Both hormones and their isotopically labelled counterparts were found to have a less than 0.1-min difference in retention time. Initially, standards of SA, SA-d6, JA, and JA-d5 were run to determine optimal separation, retention times, and transitions to monitor. The small difference in retention time between of SA, SA-d6, JA, and JA-d5 was sufficient to reveal the presence and quantity of these phytohormones. The plant matrix interference was not observed. Two separate GC single reaction monitoring MS methods to explore the transition of SA (267- > 73 m/z) and d6-SA (271- > 73) as well as the transition of JA (222- > 73 m/z) and d5-JA (287- > 73 m/z) were conducted to ensure that detection of each compound was optimized for maximum sensitivity. In the final stage of method development, all compound separation differences were optimized in multiple reactions monitoring to achieve a 5-nmol detection limit with excellent chromatographic peak shape and signal to noise ratio.

2.4 | Quantification of lolines

Lolines were analysed using a modification of the method of Moore et al., (2015). Samples of lyophilized and ground grass tissue (50 mg) were additionally ground with a bead ruptor (FastPrep FP120, Savant Instruments Inc., Farmingdale, NY, USA) with 3 × 3-mm stainless steel beads in a 2-ml vial (10 s at 5 m s^{-1}) in order to ensure a fine powder to increase the efficiency of alkaloid extraction. Samples were extracted for 1 hr with 50 µl of 40% methanol/5% ammonia and 1 ml of the 1,2-dichloroethane (containing 54.8-ng ml^{-1} 4-phenylmorpholine as internal standard). After centrifuging (5 min at 8,000 G) the supernatant was transferred to glass GC vials via a 10-mm filter for analysis. The analysis was conducted on a gas chromatography-flame ionization detector (GC2010Plus, Shimadzu Corporation, Japan) equipped with a ZB-5 capillary column (30 m × 0.32 mm × 0.25 µm film; Phenomenex, Torrance, CA, USA). The detection limit using this technique is 25 µg g^{-1} DW (dry weight).

2.5 | Statistical analyses

The response variables of concentration of SA and JA hormones were analysed separately using linear effects models with the function gls from the package nlme in R software (Pinheiro et al., 2009). The models included the plant symbiotic status (E+, E−) and SA treatment (SA+, SA−) as categorical factors. To accommodate deviations in the variance homogeneity in both response variables (SA and JA), VarIdent variance structures were used on SA treatment and on the interaction between SA treatment and plant symbiotic status, respectively (Zuur, Ieno, Walker, Saveliev, & Smit, 2009). After that, all the analysis of variance (ANOVA) assumptions were met.

Each alkaloid concentration (total lolines, NFL, and NANL) was analysed separately using linear mixed-effects models with the same package as above (Pinheiro et al., 2009). An outlier in the SA− treatment was identified and removed from the dataset. This plant showed alkaloid concentrations with an order of magnitude higher than the treatment means (total lolines, NFL, and NANL: 1,930, 1,313, and 617 µg g^{-1} DW, respectively). Thus, for each alkaloid response variable, the dataset contained six SA− and seven SA+ replicates. The fixed effects of the model included SA treatment (SA+, SA−), experimental time (7 and 15 days since the SA application) as categorical factors and the aphid population size at Days 7 and 15 as continuous covariate; the random effect included the time nested in pot. Temporal autocorrelation across of the repeated measurements was not observed. Depending of the response variable, VarPower or VarExp variance structures were used on the aphid population size to accommodate deviations in the normality (Zuur et al., 2009). After that, all the ANOVA assumptions were met.

The relationship between the concentrations of NFL and NANL loline derivatives was analysed using mixed modelling and the nlme

package. The model considered the NFL concentration as response variable with the fixed effects including SA treatment (SA+, SA−), experimental time (7 and 15 days since the SA application) as categorical factors and the NANL concentration as continuous covariate; the random effect included the time nested in pot. We constructed the statistical model based on the chemical role that NFL and NANL derivatives have in the biosynthetic pathway, where NANL is the substrate molecule to produce NFL (Charlton et al., 2014). To accommodate deviations of normality and problems of autocorrelation between the repeated measures, we used VarExp variance structure on the NANL concentration variable and a correlation structure CorARMA (p = 1, q = 0), respectively (Pinheiro et al., 2009; Zuur et al., 2009). After that, all the ANOVA assumptions were met.

The aphid population size (number of individuals) was analysed with linear mixed-effects models using the package glmmADMB and negative binomial distribution in R (Fournier et al., 2012). The fixed effects in the model included plant symbiotic status (E+, E−), SA treatment (SA+, SA−), and experimental time (4, 7, and 12 days since the aphid challenge) as categorical factors, and the random effect included the time nested in pot. Temporal autocorrelation between the repeated measurements was not observed. All values are means ± *SE* of the mean.

3 | RESULTS

3.1 | Effects of SA and endophyte on plant physiological levels of hormones

The plant defence hormones (i.e., SA and JA) responded differently to the SA treatment and the endophyte presence. In line with our hypothesis, the presence of the endophyte reduced the plant physiological concentration of SA, endophyte status: $F(1, 24) = 14.08$, $p = .001$; Figure 1, but independently of the hormonal treatment, endophyte status × SA treatment: $F(1, 24) = 0.16$, $p = .693$; Figure 1. The endophyte effect was particularly evident on plants not exposed to SA, situation in which the E+ plants showed a reduction in SA concentration of about 41% (125 ng/g DW) compared to E− plants. In addition, both endophyte-symbiotic and endophyte-free plants exhibited a significant increase in SA concentration due to the hormone treatment. Following the exogenous hormone application, the physiological level of SA was around 12 times higher in plants sprayed with SA (SA+) than in nonsprayed plants and independent of the endophyte status of the plants, SA treatment: $F(1, 24) = 188.63$, $p < .001$; Figure 1. In the case of the JA, the physiological concentration of this hormone was not altered by either the SA treatment or the endophyte presence, SA treatment: $F(1, 23) = 0.53$, $p = .473$; endophyte status: $F(1, 23) = 0.38$, $p = .546$; endophyte status × SA treatment: $F(1, 23) = 3.05$, $p = .094$; Figure 1.

3.2 | Effects of SA on the concentration of fungal loline alkaloids

The defence conferred by fungal endophytes (i.e., alkaloids) was affected by the hormone treatment. The exposure of endophyte-symbiotic plants to exogenous SA (SA+) had a 28% lower concentration of total lolines than untreated plants, SA−; SA− and SA+, 643 ± 52 and 457 ± 65 μg g^{-1} DW, respectively; $F(1, 11) = 7.22$, $p = .021$; Figure 2. The same pattern of results was observed for each individual loline derivative; compared to SA− plants, the levels were a 26% and 36% lower in SA+ plants for NFL and NANL, respectively, NFL: $F(1, 11) = 9.97$, $p = .009$; NANL: $F(1, 11) = 4.86$, $p = .049$; Figure 2. There was no effect of time, total lolines: $F(1, 7) = 1.26$, $p = .297$; NFL: $F(1, 7) = 2.04$, $p = .196$; NANL: $F(1, 7) = 0.05$, $p = .822$, nor the interaction between the SA treatment and time, total lolines: $F(1, 7) = 0.31$, $p = .594$; NFL: $F(1, 7) = 0.39$, $p = .548$; NANL: $F(1, 7) = 0.44$, $p = .530$, on the concentrations of lolines.

3.3 | Effects of SA on the relationship between NFL and NANL alkaloids

The hormonal treatment also affected the relationship between the loline derivatives. Not surprisingly, there was a significant positive

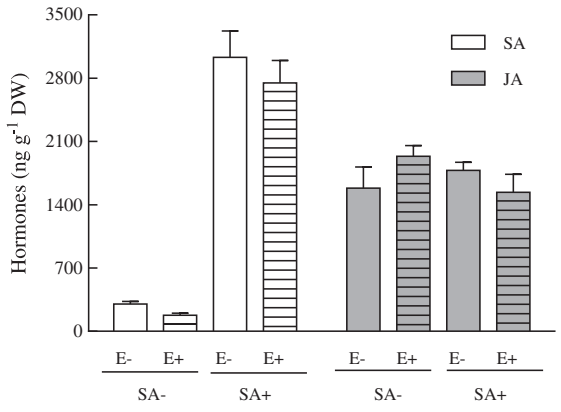

FIGURE 1 Effects of endophyte presence and salicylic acid treatments on the physiological concentration of salicylic acid (SA) and jasmonic acid (JA) hormones. White and grey bars show the plant endogenous SA and JA concentrations, respectively. Bars filled out with horizontal lines indicate plants symbiotic with the endophyte. Bars denote means ± *SE* of the mean

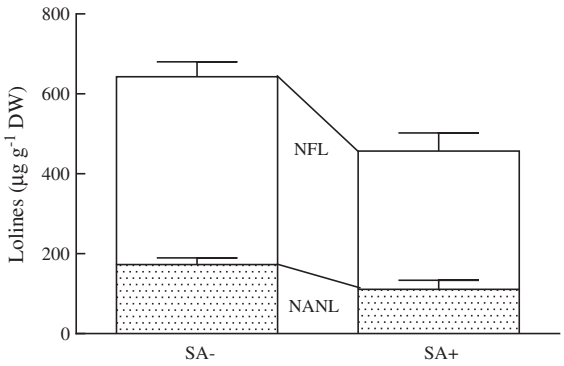

FIGURE 2 Effects of the salicylic acid treatment on the concentration of loline alkaloids in endophytic plants. Dotted and white bars indicate the N-acetylnorloline (NANL) and N-formylloline (NFL) concentrations, respectively. Note that NANL + NFL indicates the total loline concentration. Each bar contains all the loline concentrations measured along the experimental time (at Days 7 and 15 after the hormone treatment; $n_{SA+} = 14$, $n_{SA-} = 12$). Bars denote means ± SE of the mean

relationship between the concentrations of NFL and NANL alkaloids, mean slope = 1.42 ± 0.24, $F(1, 7) = 215.75$, $p < .001$; Figure 3a,b. However, this relationship varied with the time since the SA treatment application, SA treatment × experimental time: $F(1, 7) = 21.53$, $p = .002$; Figure 3a,b; the NFL concentration for a given level of NANL increased 40% and 36% from Days 7 to 15 for plants under SA− and SA+ conditions, respectively (SA− slopes at Days 7 and 15: 1.39 ± 0.58 and 2.32 ± 0.7 μg NFL μg^{-1} NANL, respectively; Figure 3a; SA+ slopes at Days 7 and 15: 1.41 ± 0.25 μg and 2.22 ± 0.29 μg NFL μg^{-1} NANL, respectively; Figure 3b). In addition, at Day 7, the NFL concentration in SA+ treated plants was 58% lower than in SA− treated plants, but this difference disappeared by Day 15 (intercepts in SA+ and SA− at Day 7: 165 ± 33 and 262 ± 102 μg NFL g^{-1} DW, respectively; intercepts in SA+ and SA− at Day 15: 131 ± 24 and 82 ± 124 μg NFL g^{-1} DW, respectively; Figure 3a,b).

3.4 | Effects of SA and the endophyte on aphid populations

The endophyte effect on the number of aphids per plant, a response variable that reflects the level of plant resistance, depended on the SA treatment, $F(1, 72) = 7.44$, $p = .006$; Figure 4a, and varied through time, $F(2, 72) = 10.26$, $p = .005$; Figure 4b. In agreement with our prediction, the endophyte-conferred resistance against aphids decreased with the plant exposition to SA. The number of aphids in E+ plants increased significantly threefold due to the application of SA (SA+ vs. SA−). Contrary to our expectations, the same treatment did not change the level of resistance to aphids in endophyte-free plants. The population size only tended to decrease in E− plants with the SA exposure (Figure 4a). In addition, the rate of aphid population increase was greater on the E− plants than that on the E+ plants (Figure 4b).

4 | DISCUSSION

We hypothesized that the exogenous application of the SA hormone, because of its role in plant defence against biotrophic pathogens, would also suppress symbiotic beneficial fungal endophytes such as *Epichloë* spp. Suppression of the symbiont would result in a concomitant reduction in endophyte-produced alkaloids. Because of the role that alkaloids play in anti-herbivore defences, any reduction in their production should make host plants more susceptible to herbivores. Our results support this hypothesis. Plants in symbiosis with *Epichloë* fungal endophytes had lower concentrations of the SA hormone than did the endophyte-free plants. Following the exogenous hormone

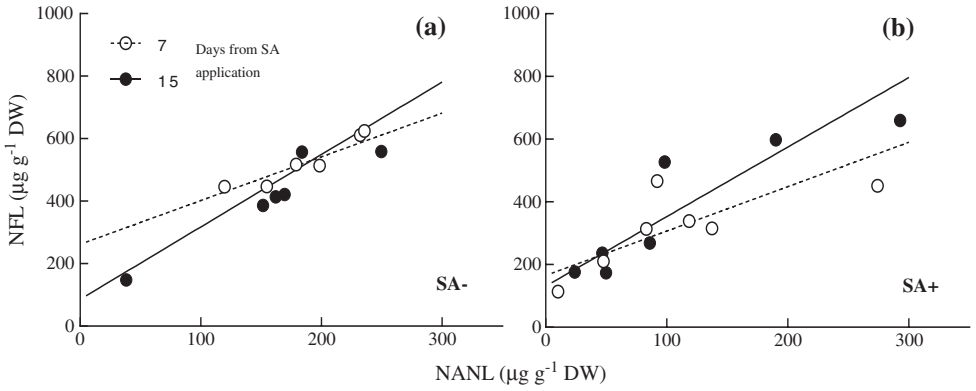

FIGURE 3 Relationship between the loline alkaloid derivatives N-formylloline (NFL) and N-acetylnorloline (NANL) in endophytic plants (a) not exposed and (b) exposed to salicylic acid hormone across the experimental time. Open circles and dashed lines indicate NFL–NANL relationship after 7 days from the SA application, and close circles and solid lines indicate NFL–NANL relationship after 15 days

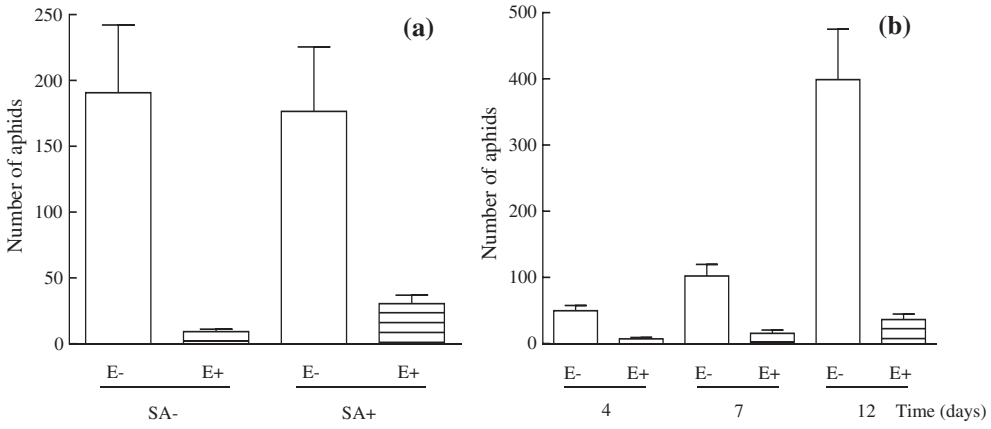

FIGURE 4 Effects of (a) endophyte presence and salicylic acid treatments and (b) endophyte and time on the aphid population size (number of aphids). Bars filled with horizontal lines indicate plants symbiotic with endophyte. Bars denote means ± *SE* of the mean

application, all plants achieved very high physiological concentrations of the SA hormone. In line with our prediction, the hormonal treatment reduced the concentration of loline alkaloids and consequently decreased the endophyte-conferred resistance against aphids. The endophyte-free plants did not show any significant change in the level of resistance to aphids.

As a consequence of the communication between plant and beneficial microorganisms, a well-established symbiosis normally results in a down-regulation of the SA pathway (Navarro-Meléndez & Heil, 2014; Stacey et al., 2006; Yasuda et al., 2016). Consistent with reports on other symbioses, we found that endophyte-symbiotic plants also had lower concentrations of SA than did endophyte-free plants. This is also consistent with the usually observed pattern of down-regulation of biosynthetic genes and markers of the SA signalling pathway in other grass–endophyte combinations (Dupont et al., 2015; Johnson et al., 2003) (but see Schmid et al., 2017). In the case of *Epichloë* endophytes, it is not known whether the down-regulation of SA production is strictly controlled by the plant, the fungus, or some interaction of mechanisms between them. The plants in our study were transitioning from vegetative reproduction to sexual reproduction. This is precisely the stage at which the endophyte is in an active growth phase, attempting to colonize the reproductive meristems, and hence success is likely dependent on the down-regulation of SA production (Gundel et al., 2011; Justus et al., 1997).

Our results contrast with the findings of Ambrose et al., (2015) who found that the presence of an *Epichloë* endophyte in red fescue plants (*Festuca rubra*) had a null or even positive effect on SA concentrations, depending on the plant tissue (leaf/sheath) and the endophyte strain. Apart from the obvious differences in terms of plant and endophyte species, plant phenology, and type of tissues harvested between the Ambrose et al. study and our study, there were other differences that could also explain the discrepancies. One difference is that Ambrose et al. used sexually reproducing fungi and the plants were infected with native and nonnative fungal strains, whereas in our study, plants were symbiotic with a native and asexual endophyte species. It seems possible that the host immune system might respond differently depending on the fungal life cycle and/or the endophyte–host compatibility. In fact, recent studies suggested that these factors could lead to a differential transcriptional reprograming of host plants (Dinkins, Nagabhyru, Graham, Boykin, & Schardl, 2017; Schmid et al., 2017). An enhanced concentration of SA could be a plant's mechanism to regulate the excessive symbiont proliferation (López-Ráez et al., 2010). This hypothesis has emerged from studies using mycorrhizal fungi where plants increase the SA concentration after that symbiont mycelium is above a certain threshold of biomass (Khaosaad, García-Garrido, Steinkellner, & Vierheilig, 2007; López-Ráez et al., 2010). Thus, the amount of SA may depend on the interaction between the endophyte life cycle and plant growth, the species-specific symbiotic association, and/or the plant tissues.

The SA-dependent resistance has been proposed to be an effective mechanism of control against biotrophic pathogens and sap-sucking insects (Ballaré, 2014; Glazebrook, 2005; Schwartzberg & Tumlinson, 2014; Schweiger et al., 2014; Thaler et al., 2012). We therefore expected that the exogenous SA application would increase the resistance level in endophyte-free plants. However, despite the fact that E− plants showed a significant increase in their physiological SA concentration due to the hormone treatment, their subsequent aphid population sizes were unaffected. Because the plant SA signalling pathway is usually activated by the exogenous application of SA the hormone (Feechan et al., 2005; Lawton et al., 1996; Loake & Grant, 2007; Uknes et al., 1992), our results suggest the possibility that another hormonal pathway may be involved in the defensive responses of endophytic plants to aphids. An increasing number of studies are showing that plants respond to aphid attacks by means of more complex signalling pathways that involve not only SA but also JA and ethylene (Boughton, Hoover, & Felton, 2006; Cooper & Goggin, 2005; Cooper, Jia, & Goggin, 2004; De Vos et al., 2005; Ellis, Karafyllidis, & Turner, 2002; Li et al., 2008; Li, Xie, Smith-Becker, Navarre, & Kaloshian, 2006; Moran, Cheng, Cassell, & Thompson, 2002; Moran & Thompson, 2001; Park, Huang, & Ayoubi, 2006; Zhu-Salzman, Salzman, Ahn, & Koiwa, 2004). In the case of endophyte-grass symbioses, a previous study explored the defensive role carried out by JA in the association between tall fescue plants (*S. arundinaceus*) and fungal endophytes (Simons, Bultman, & Sullivan, 2008). In particular, the application of methyl jasmonate to endophyte free-plants produced a significant increase in the plant's resistance to aphids, reducing the population size of *R. padi* by about 50% (Simons et al., 2008). In our study, the SA treatment did not affect the physiological concentration of JA. Thus, our results suggest that the SA hormone did not change the herbivory resistance level of endophyte-free grass plants either directly or indirectly through subsequent changes in JA production.

The exogenous application of SA has generally been found to disrupt the benefits provided by microbial symbionts to host plants (Hayat, Hayat, Irfan, & Ahmad, 2010). We observed that the benefit delivered by the endophyte was also negatively affected by the SA treatment, through a reduction in the concentration of fungal alkaloids. It is likely that the alkaloid production had been disrupted by the SA directly as a consequence of the hormone treatment; however, effects mediated by the SA signalling pathway could have also taken place (Herrera Medina et al., 2003; Khaosaad et al., 2007; López-Ráez et al., 2010; Stacey et al., 2006). In general, the amount of alkaloids can vary as result of two nonmutually exclusive mechanisms: variations (a) in fungal biomass concentration (see Rasmussen et al., 2007) and (b) in the rate of alkaloid production per unit of fungal biomass (see Ryan et al., 2014). Because SA has commonly been observed as a regulator of the symbiont proliferation in other symbioses (Fernández et al., 2014; Hayat et al., 2010; Zamioudis & Pieterse, 2011), it is possible that in our experiment, the variation in endophytic biomass was the mechanism behind the reduction in the concentration of alkaloids. Nevertheless, the possibility that the SA hormone directly or indirectly affects the biosynthetic routes of fungal alkaloids is an alternative that cannot be ruled out. For example, endophytic plants exposed to JA hormone (actually methyl jasmonate) showed a down-regulation of *LolC* gene that encodes for an enzyme of the biosynthesis of lolines (Simons et al., 2008).

The SA hormone treatment reduced the concentration of loline alkaloids, and consequently, it decreased the level of resistance of endophyte-symbiotic plants to aphids, resulting in higher insect population sizes. However, the loss of resistance in E+ plants was small

considering the fact that endophytic plants showed an initial 12-fold increase in their SA concentrations (due to the hormone treatment). E+ plants were still highly resistant to aphids, with population sizes fivefold smaller than those populations reared on E− plants. The reduced resistance seen in E+ plants is consistent with the fact that fungal lolines concentrations decreased 26–36% (depending of the type of loline) as a consequence of the SA exposure. Additionally, a subtle increase in the production of NFL from NANL loline derivatives was detected 15 days after the SA exposure. This higher NFL–NANL relationship could have partially compensated for the general reduction of lolines as a whole and thus help the plants to maintain a high level of resistance to aphids after the SA exposition. Moreover, it is worth noting that E+ plants not exposed to SA also showed an increment in NFL production after 12 days from the start of the aphid challenge (or 15 days after the SA exposition). This result suggests the possibility that changes in production of NFL (from NANL) could have been induced by the activity of aphid feeding on the plants.

In conclusion, this study highlights the importance of the interaction between the plant immune system and the presence of leaf fungal endophytes for the stability and persistence of the defensive mutualism. Our results indicate that the hormone SA plays an important role in regulating the endophyte-conferred resistance against sap-sucking insects. An important consequence of this interaction is that any ecological factor that significantly stimulates the SA hormone could impact negatively on the endophyte and the benefits that it provides. Consistent with this hypothesis is the evidence from the episodic exposure of *L. multiflorum* plants to ground level ozone gas, a contaminant of global change that elicits the plant SA (Kangasjarvi, Jaspers, & Kollist, 2005), resulted in a significant reduction of the effectiveness of the defences provided by the endophyte *E. occultans* (Ueno et al., 2015). Continued research on how changes in endogenous SA levels affect the defence provided by endophytes may give a more detailed idea about the plant regulation of the fungus. Transgenic or mutant plants with specific defects in SA signalling pathway would be useful for studying this issue. In the case of *Lolium spp*. plants, although generation of transgenic plants has been successful in certain cases (Bettany et al., 2003), better protocols are still needed to increase mainly the plant transformation frequency and the stability of transgenes in the *Lolium* genome (Lee et al., 2010). Finally, another aspect to consider are effects that the endophytes could have on the plant growth hormones (i.e., auxins, gibberellins, and others), which regulate the defensive hormones (SA and JA; Ballaré, 2014). Although it has been found that plant growth hormones are up-regulated in endophyte-symbiotic plants (Dupont et al., 2015; Schmid et al., 2017), the impacts that the activation of these pathways on the host defence responses are still unknown. To have a better understanding how different ecological factors affect the functionality of grass–endophyte symbiosis, more studies with a focus on the chemical crosstalk between partners are required.

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