Jasmonic acid regulation of the anti-herbivory mechanism conferred by fungal endophytes in grasses


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Summary

1. The most studied mechanism of protection against herbivores in grasses associated with *Epichloë* fungal endophytes has been the fungal production of alkaloids. However, the contribution of the plant immune response on the level of resistance to herbivores in symbiotic grasses has been poorly explored. We studied the relationship between the plant hormone, jasmonic acid (JA), and *Epichloë* fungal endophytes on herbivore defenses in symbiotic grasses. We hypothesized that an exogenous application of methyl jasmonate (MeJA), an activator of the plant JA defense response, would increase the level of resistance of endophyte-symbiotic and non-symbiotic plants to a chewing insect. As *Epichloë* endophytes produce alkaloids, an enhancement of the JA defense would complement the resistance given by these alkaloids.

2. *Lolium multiflorum* plants symbiotic and non-symbiotic with the endophyte *Epichloë occultans* were subjected to an exogenous application of MeJA followed by a challenge with the generalist chewing insect *Spodoptera frugiperda*. We measured the level of plant resistance to chewing insects, and the defenses conferred by host plants and fungal endophytes.

3. Symbiotic plants were more resistant to *S. frugiperda* than their non-symbiotic counterparts. However, despite the fact that the concentration of JA significantly increased in all plants exposure to MeJA, neither endophyte-symbiotic nor non-symbiotic plants showed an enhanced resistance to insects. Unexpectedly, the exposure of endophyte-symbiotic plants to MeJA led to a reduction in the concentration of loline alkaloids (i.e. N-formyllolines and N-acetylnorlolines), consequently decreasing the level of plant resistance to the herbivore.
4. *Synthesis*. Our results suggest that, rather than complementing the alkaloid-based
defense, the JA hormone weakens the anti-herbivore mechanism conferred by *Epichloë*
endophytes. The present study highlights that the interaction between the JA hormone
and the presence of leaf fungal endophytes can be of importance for the effectiveness of
the anti-herbivore defenses of symbiotic plants.

**Key words**: Jasmonates, *Epichloë*, endophyte symbiosis, alkaloids, beneficial microorganisms,
plant defenses, jasmonic acid.

**Second Summary in Spanish**

1. El mecanismo de anti-herboríoría más estudiado en los pastos asociados con los hongos
endofitos *Epichloë* es la producción de alcaloides fúngicos. Sin embargo, es
prácticamente desconocido el aporte que podría tener la respuesta inmune de los pastos
simbióticos en la resistencia frente a los insectos herbívoros. Aquí estudiamos la
interacción entre la hormona de defensa de la planta, el ácido jasmónico (JA), y los
hongos endofitos *Epichloë* sobre las defensas anti-herboríoría de los pastos. La hipótesis
es que la aplicación exógena de metil jasmonato (MeJA), un activador de la defensa
dependiente del JA, aumenta el nivel de resistencia de las plantas con y sin el endofito
frente a un insecto masticador. Dado que los endofitos *Epichloë* producen alcaloides, la
activación de la defensa del JA debería complementar la resistencia dada por los
alcaloides.
2. Plantas de *Lolium multiflorum* simbióticas y no simbióticas con el endofito *Epichloë occultans* fueron expuestas a una aplicación exógena de MeJA y posteriormente desafiadas con el insecto masticador generalista *Spodoptera frugiperda*. Se midió tanto el nivel de resistencia de la planta frente al insecto como las defensas conferidas por la planta hospedera y el hongo endofito.

3. Las plantas simbióticas fueron más resistentes frente al insecto *S. frugiperda* que las no simbióticas. Sin embargo, a pesar de que la concentración del JA aumentó significativamente en las plantas expuestas al MeJA, ni las plantas con el endofito ni las sin el hongo aumentaron la resistencia frente a los insectos. Inesperadamente, la exposición de las plantas simbióticas al MeJA redujo la concentración de los alcaloides (i.e. N-formilolinas y N-acetilnorlolinas), disminuyendo consecuentemente el nivel de resistencia de las plantas al herbívoro.

4. *Síntesis*. Nuestros resultados sugieren que, en lugar de complementar la defensa dada por los alcaloides, la hormona JA atenua el mecanismo anti-herbivoría conferido por los hongos endofitos *Epichloë*. Este estudio enfatiza que la interacción entre la hormona JA y la presencia de los hongos endofitos foliares puede ser de importancia para la efectividad de las defensas de las plantas simbióticas.

**Introduction**

During their entire life, plants are constantly threatened by herbivores. In order to defend themselves, plants have developed sophisticated mechanisms that can be either constitutively expressed or induced by the herbivore attack (Karban & Baldwin, 1997; Schoonhoven, van
Loon, & Dicke, 2005). Some of these herbivore-induced plant responses involve the production of toxins, anti-nutritional compounds, and plant volatile organic compounds (Karban & Baldwin, 1997; Schoonhoven et al., 2005). The induced responses are governed by a group of plant hormones of which jasmonic acid (JA) and salicylic acid (SA) play a central role (Ballaré, 2014; Dicke & Baldwin, 2010; Heil, 2008; Karban & Baldwin, 1997; Thaler, Humphrey, & Whiteman, 2012). Plants generally respond by activating JA-dependent defenses when attacked by chewing insects and necrotrophic pathogens, whereas SA-dependent responses are induced by attacks from sap-sucking herbivores and biotrophic pathogens (Ballaré, Mazza, Austin, & Pierik, 2012; Glazebrook, 2005; Halitschke & Baldwin, 2004; Kunkel & Brooks, 2002; Schwartzberg & Tumlinson, 2014; Schweiger, Heise, Persicke, & Muller, 2014; Thaler et al., 2012). In addition, plants usually interact and establish symbiotic relationships with beneficial microorganisms (Pineda, Dicke, Pieterse, & Pozo, 2013). These mutualistic symbioses between plants and microorganisms involve a complex molecular dialogue that affects several plant functions including the expression of plant defenses (Gutjahr, 2014; Pozo, Lópezh-Ráez, Azcón-Aguilar, & García-Garrido, 2015). While certain symbionts increase plant resistance by modulating the host hormonal pathways involved in defenses (Pieterse et al., 2014; Pineda et al., 2013; Pozo et al., 2015), other microorganisms (e.g. Epichloë fungal endophytes) produce bioactive compounds, which protect their host plants against attackers (Panaccione, Beaulieu, & Cook, 2014; Schardl et al., 2013a). Although the role of the defense compounds produced by endophyte-symbionts in protecting plants have been well described (Potter, Tyler Stokes, Redmond, Schardl, & Panaccione, 2008; Wilkinson et al., 2000), the possibility that this endophyte-conferred mechanism of resistance is complemented by the plant’s own defenses, has not been explored.
The establishment of symbiotic relationships between plants and beneficial microorganisms entails changes in the plant’s immune response (Jung, Martínez-Medina, Lopez-Raez, & Pozo, 2012; Pieterse et al., 2014; Pozo et al., 2015; Zamioudis & Pieterse, 2011). The effects of beneficial symbionts on the host immunity have been mainly studied in plant symbioses with rhizobacteria and mycorrhizal fungi, where the colonization of roots by these microorganisms is essential for the establishment of a stable interaction (Cameron, Neal, van Wees, & Ton, 2013; Gutjahr, 2014; Jung et al., 2012; Oldroyd & Downie, 2008; Pozo et al., 2015; Ryu, Cho, Choi, & Hwang, 2012). From the plant’s point of view, a consequence, in terms of defense, of establishing a symbiotic interaction with these microorganisms is that symbiotic plants are generally more 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 an active suppression of the plant’s SA pathway by symbionts (Cameron et al., 2013; Jung et al., 2012; Pozo et al., 2015; Zamioudis & Pieterse, 2011). As biotrophic microorganisms, beneficial symbionts would control the SA pathway, by means of specific effectors, in order to avoid the plant immune response (Jung et al., 2012; Martínez-Abarca et al., 1998; Paszkowski, 2006; Pozo et al., 2015; Siciliano et al., 2007; Zamioudis & Pieterse, 2011). Another change usually observed is that symbiotic plants with these microorganisms are more resistant to necrotrophic pathogens and chewing herbivores (Jung et al., 2012; Pieterse et al., 2014; Pineda, Zheng, van Loon, Pieterse, & Dicke, 2010; Zamioudis & Pieterse, 2011). This resistance seems to be related to the activation of the JA signaling pathway, rendering the symbiotic plants in a ‘primed state’ of defense (Martinez-Medina et al., 2016; Pieterse et al., 2014; Pineda et al., 2013; Van Wees, Van der Ent, & Pieterse, 2008). A primed state of defense differs from other induced immune responses in that plants show an earlier, faster, stronger, and/or more sustained
defensive response against attackers (Jung et al., 2012; Pieterse et al., 2014; Pineda et al., 2013; Van Wees et al., 2008).

Some asexual leaf fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) form mutualistic symbioses with specific grass species of the subfamily Poöideae. These *Epichloë* species are strictly vertically transmitted from one plant generation to the next. The symbiotic interaction persists across generations by the clonally-multiplied mycelium colonizing developing seeds (Clay, 1988; Gundel, Rudgers, & Ghersa, 2011; Schardl, 2010; Zhang et al., 2017). One benefit that these endophytes provide is that they are the source of bioactive alkaloids, nitrogen-based compounds, that protect host plants against herbivores (Clay, 1988; Popay & Bonos, 2005; Saikkonen, Gundel, & Helander, 2013; White & Torres, 2009). Fungal alkaloids produced by *Epichloë* endophytes belong to four main classes, ergot alkaloids (i.e. ergopeptine and ergovaline), indole-diterpenes (i.e. lolitrem B and terpendoles), pyrrolizidines (i.e. lolines), and peramine (Panaccione et al., 2014; Saikkonen et al., 2013; Scharld et al., 2013a; Schardl, Young, Faulkner, Florea, & Pan, 2012; Scharld et al., 2013b; Young et al., 2015). The genes for the biosynthesis of alkaloids are entirely encoded within the fungal genome, and the biosynthetic routes are almost entirely elucidated (Scharld et al., 2013a; Scharld, Grossman, Nagabhryru, Faulkner, & Mallik, 2007; Young et al., 2015). The particular *Epichloë* species and strain determine the alkaloid profiles, whereas the level of alkaloid production depends on factors such as the plant species and genotype, plant growth stage, plant tissue/organ, and growing conditions (Ball, Prestidge, & Sprosen, 1995; Justus, Witte, & Hartmann, 1997; Ryan, Rasmussen, Xue, Parsons, & Newman, 2014; Saikkonen et al., 2013). Furthermore, the effectiveness of a particular fungal alkaloid will depend on the particular herbivore species attacking the plant (Saikkonen et al., 2013; Saikkonen, Lehtonen, Helander, Koricheva, & Faeth, 2015).
2006; Saikkonen, Saari, & Helander, 2010; Schardl et al., 2013a) and the environmental conditions (Ueno et al., 2015).

The role of fungal alkaloids in protecting the host grasses has been extensively documented (Potter et al., 2008; Wilkinson et al., 2000), while the effects of *Epichloë* endophytes on the host immune system have received less attention. Recent studies, however, revealed that the presence of fungal endophytes can induce changes in the molecular components of the host plant immune system (Dinkins, Nagabhyru, Graham, Boykin, & Schardl, 2017; Dupont et al., 2015; Schmid et al., 2017). For example, various WRKY genes were up-regulated in plants of *Schedonorus arudinaceum* (syn. of *Lolium arudinaceum* and *Festuca arundinacea*) symbiotic with the endophyte *E. coenophiala* (formerly *Neotyphodium coenophialum*), which suggests that the endophyte-symbiotic plants could have an enhanced level of resistance to attackers (Dinkins et al., 2017). Plants of *Lolium perenne* symbiotic with the endophyte *E. festucae* strain Fl1 have shown a general down-regulation of genes related to the biosynthesis and signaling of the SA pathway (Dupont et al., 2015). Similar to other systems involving symbioses between plants and biotrophic symbionts, the downregulation of the SA pathway could be the result of an active suppression exerted by *Epichloë* endophytes on the host defense system (Ambrose & Belanger, 2012; Martínez-Abarca et al., 1998; Siciliano et al., 2007). Other studies suggest that the presence of the endophyte could also affect the JA-dependent plant defense. For instance, endophyte-symbiotic plants of *S. arundinaceus* showed an enhanced expression of the plant gene *TFF41*. The protein encoded by the gene *TFF41* is highly similar to the potato and parsley enzyme ω-3 FAD, which increases the concentration of JA precursor molecules (Johnson, Johnson, Schardl, & Panaccione, 2003). Additionally, endophyte presence has also been associated with higher concentrations of plant metabolites involved in defenses, such as...
phenylpropanoid and phenolic compounds, and the up-regulation of genes related to the biosynthesis of these compounds (Dupont et al., 2015; Malinowski, Alloush, & Belesky, 1998; Pańka, Piesik, Jeske, & Baturo-Cieśniewska, 2013; Rasmussen, Parsons, & Newman, 2009; Rasmussen, Parsons, Popay, Xue, & Newman, 2008).

Study System

Here, we studied the interaction between the JA hormonal pathway and Epichloë endophytes on plant defenses against herbivores. We subjected symbiotic and non-symbiotic grass plants to an exogenous application of methyl jasmonate (MeJA) followed by a challenge with a chewing insect. MeJA was used to elicit a JA-dependent defense response (Koo, Yoon, Seo, Kim, & Choi, 2013; Wu, Wang, & Baldwin, 2008). For our model system we used larvae of the generalist folivorous herbivore Spodoptera frugiperda (fall armyworm), Lolium multiflorum host plants (Italian ryegrass), and the fungal endophyte Epichloë occultans. This endophyte species is known to produce loline alkaloids (Bastías et al., 2017; Moon, Scott, Schardl, & Christensen, 2000; Moore, Pratley, Mace, & Weston, 2015; Sugawara, Inoue, Yamashita, & Ohkubo, 2006), which have been shown to provide the plant with protection from insect herbivores (Charlton et al., 2014; Schardl et al., 2007). Loline alkaloids are not present in endophyte-free grasses (Clay, 1988). Despite the wide range of plant species that can be consumed by S. frugiperda, grasses are among the most preferred at the larval stage (Luginbill, 1928). Previous studies have demonstrated a negative effect of Epichloë endophytes on the performance of S. frugiperda (Ahmad, Govindarajan, Johnson-Cicalese, & Funk, 1987; Ball et al., 2006; Crawford, Land, & Rudgers, 2010).
We used a group of response variables related to insect performance as indicators of plant resistance, and both the physiological concentrations of loline alkaloids and hormones (SA and JA) to characterize the defensive state of plants. Since chewing insects and biotrophic symbionts are assumed to be negatively affected and unaffected by the JA pathway, respectively (Halitschke & Baldwin, 2004; Thaler et al., 2012), we predicted that the exogenous application of MeJA will enhance the resistance level in endophyte-symbiotic and non-symbiotic plants, and thus reducing the performance of fall armyworms. However, the increased resistance will be higher in endophyte-symbiotic than in non-symbiotic plants. Whereas the resistance will be only related to the JA-dependent defense in non-symbiotic plants, the same hormonal mechanism will be complemented by the alkaloid-based defense in endophyte-symbiotic plants. This study helps to understand how the interaction between the JA pathway and *Epichloë* fungal endophytes can modulate plant herbivore defenses.

**Materials and Methods**

**PLANT STOCK AND LARVAE COLONY**

Plants of *Lolium multiflorum* (Lam.) symbiotic and non-symbiotic with the endophyte *Epichloë occultans* (E+ and E-, respectively) were generated from one population harvested from a successional grassland in the Argentinean Pampas (36° 00’ S, 61° 5’ W). E- plants were generated by subjecting endophyte-infected seeds to a systemic fungicide (Triadimenol 150 g kg⁻¹; Baytan®). In order to increase the number of seeds, fungicide treated and untreated seeds were sown in contiguous 1 m² plots at the experimental field of the Institute IFEVA - CONICET, Universidad de Buenos Aries, Argentina (34° 35’ S, 58° 28’ W). Cross-pollination between plants was allowed to mitigate any genetic differentiation between symbiotic and nonsymbiotic
plants (Gundel et al., 2012). Mature seeds from each plot were harvested and the proportion of endophyte-infected seeds from each seed lot was determined by microscopy. For each seed lot, 100 seeds were sampled, cleared, stained, and examined individually under a microscope at 40× magnification (Bacon & White Jr, 1994; Latch & Vaughan, 1995). The efficacy of the fungicide treatment to remove the endophyte was very high as only 3% of the seeds were endophyte-infected in the seed lot produced by plants grown from fungicide-treated seeds. On the other hand, 99% of the seeds produced by plants grown from untreated seeds were endophyte-infected. We did not observe any phytotoxic effects of the fungicide on the experimental plants.

The experimental plants (E+: n = 50, and E-: n = 50) were grown individually in 1.5 L pots (containing equal parts of soil, sand, and peat) during the normal growing season for the species (autumn-winter-spring). Plants were placed outdoors in the Institute’s experimental field site and were periodically watered to avoid stressful situations. The symbiotic status of each experimental plant was re-confirmed by looking for the endophyte in the sheath base of the outermost leaf using microscopy (Bacon & White Jr, 1994). Larvae of fall armyworms (Spodoptera frugiperda, Smith) were obtained from a colony maintained permanently in our lab (>80 generations), reared on a synthetic diet (composed of pinto beans, wheat germ, soybean protein, brewer yeast, ascorbic acid, tetracycline, methyl paraben, sorbic acid, and agar) under controlled conditions [21 °C (±1), photoperiod L16:D8 h, and radiation 150 μmol m⁻² s⁻¹].

DESIGN AND SETUP OF THE EXPERIMENT

We conducted an experiment to test the anti-herbivory responses of endophyte-symbiotic and non-symbiotic plants against S. frugiperda larvae. In early-spring, 36 healthy plants (18 each of E+ and E-), each 16 weeks old, were chosen from the plant stock and moved to a growth chamber with identical conditions to those experienced by the S. frugiperda colony. After
examining each plant to ensure that there were no insects on them, they were individually enclosed in a white voile bag supported by a tubular plastic frame. These plants were subjected to chamber conditions for one week prior to the application of the jasmonate treatment (see below). The plants had approximately 32 tillers each (range: 24-46) and were starting to flower. In addition, the number of tillers was positively affected by the endophyte presence (Symbiosis: $F_{1.34} = 14.70, P < 0.001$); E+ plants had 17% more tillers than E- plants (E+: 35.33 ± 1.36 and E-: 29.17 ± 0.89). The positive effect of the endophyte on plant growth has been commonly observed in other studies (Clay & Schardl, 2002; Saikkonen et al., 2006).

The experiment was a 2 x 2 full factorial design with 9 replicates of each treatment combination. Endophyte status (E+, E-) and methyl jasmonate application (MeJA+, MeJA-) were the treatments. The MeJA treatment was carried out by means of exogenous application. Half of the plants from each endophyte status (9 E+ and 9 E-) were sprayed with 10 mL of 1 mmol L$^{-1}$ of MeJA (Sigma-Aldrich®) and the other half with 10 mL of water. Three days after the MeJA application, a single fourth instar larva was placed on each plant, and the plant was immediately enclosed with the white voile bag to avoid insect escape. All the larvae used in the experiment came from a single hatching clutch and insects were followed for 70 days. Prior to placement on the plants, larvae were weighed [12.40 ± 0.57 mg (mean ± SE)] and starved for 4 hours. The 3-day period between the MeJA application and the larval placement allowed the plants to reach a high physiological level of JA prior to contact with the herbivores. We did not observe signals of senescence in aboveground tissues after MeJA exposure.

We used insect body mass, development time, and survival to characterize the individual performance of $S. frugiperda$. Every 48 hours, larvae from each plant were gently removed to measure individual body mass, determine their developmental instar/stage, and quantify their...
survival. When the larvae reached the pupal stage, pupae were removed from plants and placed individually in Petri dishes until adult emergence (keeping the identity of each experimental unit; a plant = a Petri dish). For insect body mass, we used the larval live mass at day 15 of the insect challenge (or day 18 post-MeJA exposure), pupal live mass, and adult live mass. At day 15, most of the larvae were at instar 6 and close to their maximum weight. With respect to insect development, we calculated the days to pupation, days as pupa, and days to adult eclosion as response variables. When larvae reached the pupal stage, the aboveground biomass of plants was removed, dried (in an oven for 48 h at 60 °C), and weighed.

The physiological concentrations of hormones and fungal alkaloids were quantified from plant tissues sampled in three serial harvests. At the first harvest, two leaves (including both sheath and blade) from one tiller were removed just before the insect challenge (day 3 after the MeJA application), to measure the concentrations of defense hormones (SA and JA) by means of GC-mass spectrometry (MS, see below). At day 5 post-MeJA application, one tiller base (pseudostems formed by leaf sheaths) per E+ plants was harvested to measure the concentration of fungal alkaloids by means of gas chromatography (see below). While the same procedure was performed on E- plants, loline alkaloids were not quantified since E- plants do not produce these compounds (Clay, 1988). The third harvest was performed at day 21 post MeJA application to quantify both fungal alkaloids and hormones, respectively, following the same protocols previously mentioned. Considering that tillers have some degree of independence (Yang & Hwa, 2008), all the samples were removed from distant tillers, thus minimizing the effects of serial “clipping” of tissues on the physiological status of the whole plant. In addition, we avoided sampling senescing plant tillers, and particularly for alkaloid measurements, we harvested samples from tissues with signs of larval herbivory. The sampling biomass for hormones and
alkaloids represented 4-5% of the total plant aboveground biomass [~0.3 g dry weigh (DW) of harvested tissues from 6.40 ± 0.25 g DW of aboveground biomass].

QUANTIFICATION OF SA AND JA

Extraction and derivatization

Freeze dried leaf samples of 50-100 mg were extracted using 1 mL of 100% acetonitrile (ACN), spiked with 10 μL of internal standards (d6-SA: 100 ng and d5-JA: 100 ng) (CDN Isotopes Inc.) for GC-MS/MS. Two 4 mm steel balls were included in each 2 mL vial (FastPrep tubes, Qbiogene Inc.), and samples were shaken for 10 min at 1000 stokes min⁻¹ with a Geno/Grinder (model 2010, SPEX®SamplePrep). Plant extracts were centrifuged at 13,200 rpm for 20 mins at 4 °C, and supernatants were transferred to 2 ml glass vials (Phenomenex Inc.). The extraction step was repeated adding 1 mL of 100% ACN without internal standards, and supernatants were combined. The supernatants were evaporated to complete dryness using a SpeedVac™ (Thermo Fisher Scientific Inc.), and dry samples were further derivatized using 100 μL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 60 °C for two hours. After silylation, samples were cooled down at room temperature and then injected into the GC.

GC-MS-MS method

One microlitre of derivatized sample was injected in a split mode at 1:20 ratio into an Agilent DB-5MS column (30 m long with 10 m guard column, 0.25 mm inner diameter, 0.25 μm film thickness). The inlet had a temperature of 290 °C. The gas flow rate was 1 ml min⁻¹ with helium as a carrier gas, and the column temperature was held at 80 °C for 2 min, followed by an increase to 230 °C using a linear gradient of 10 °C min⁻¹. After keeping constant for 1 min at 230 °C, the temperature was risen to 310 °C at 40 °C min⁻¹ and held for 5 min resulting in a total run time of
25 min. The column effluent was added into the ion source of a Scion TQ GC-MS/MS (Bruker Daltonics Inc.). The MS transfer line temperature was set to 290 °C and the source temperature was at 230 °C. The data were acquired in electron impact (EI) positive ionization mode at 70eV energy and multiple reactions monitoring (MRM) mode using 2 mTorr collision pressure, and 30eV energy for precursor ion fragmentation. For salicylic acid and jasmonic acid, the ion pair transition (precursor to product) and the retention time for each transition were as follow: 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).

Isotope dilution analysis

SA and JA were quantified using isotope dilution analysis by adding 10 μL of isotopically-labeled SA and JA molecules to the plant extracts. We took advantage of the chromatographic differences between the hormones naturally present in the samples and the deuterium-labeled hormones. The isotope effect in chromatographic separations results in different retention times between both types of compounds. The retention time shift for a given labeled compound (in our case, SA or JA) depends on the number of deuterium atoms in the molecule. SA and JA hormones and their isotopically-labeled counterparts had a less than 0.1 min difference in retention time. Initially, hormone standards (SA and JA) and isotopically-labeled compounds (SA-d6 and JA-d5) were run to determine optimal separation, retention times, and transitions to monitor. The small differences in retention times between SA, SA-d6, JA, and JA-d5 were sufficient to reveal the presence and quantity of these hormones. The plant matrix interference was not observed. Two separate GC single reaction monitoring (SRM) MS methods meant to explore the transition of SA (267->73 m/z) and d6-SA (271->73 m/z) as well as the transition of JA (222->73 m/z) and d5-JA (287->73 m/z), were conducted to ensure that the detection of each
compound was optimized for maximum sensitivity. Lastly, the separation differences between all compounds were optimized in MRM, achieving a detection limit of 5 nmol with excellent chromatographic peak shape and signal to noise ratio. Similar protocols have been used to quantify plant hormones, and these studies report hormone concentrations comparable to those reported here (Bastías et al., 2018; Davis, Bosque-Pérez, Popova, & Eigenbrode, 2015; Eberl, Hammerbacher, Gershenzon, & Unsicker, 2017; Ohnmeiss & Baldwin, 2000; Paulmann et al., 2018).

QUANTIFICATION OF LOLINES

Lolines were analyzed using a modification of the method of Moore et al. (2015). Plant samples were freeze-dried and ground using a Geno/Grinder® (model 2010, SPEX®SamplePrep) with 6 mm steel balls, in order to ensure a fine powder to increase the alkaloid extraction efficiency. 50 mg of ground samples were extracted using 50 µL of 40% methanol/5% ammonia and 1 mL of 1,2-dichloroethane (containing 54.8 ng mL⁻¹ of 4-phenyl morpholine as an internal standard) for 1 hour. Plant extracts were centrifuged at 8000 G for 5 min, and the supernatants were transferred to glass GC vials via a 10 mm filter. The analysis was conducted using a gas chromatography-flame ionization detector (GC2010Plus, Shimadzu Corporation), equipped with a ZB-5 capillary column (30 m x 0.32 mm x 0.25 µm film; Phenomenex Inc.). The detection limit observed using this technique was 25 µg g⁻¹ DW (dry weight).

STATISTICAL ANALYSES

The response variables of SA and JA concentrations were analyzed separately with linear mixed-effects models, using the nlme package in R software, and assuming independent, identically distributed normal random errors (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2009). The

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fixed part of each model included the categorical variables of symbiotic status (E+, E-), MeJA treatment (MeJA+, MeJA-), and the experimental time (days 3 and 21 since the MeJA application), and the random part included the time nested in the pot. *VarIdent* variance structure was used in the interaction between symbiotic status and MeJA treatments to accommodate heteroscedasticity of residuals (Zuur, Ieno, Walker, Saveliev, & Smit, 2009). After that, the model assumptions were met (residuals independence, normality, and variance homegeneity).

The aboveground plant biomass was analyzed with a linear effect model, using the function *gls* from the package *nlme* in R software, and assuming independent, identically distributed normal random errors (Pinheiro et al., 2009). The model included the plant symbiotic status (E+, E-) and MeJA treatment (MeJA+, MeJA-) as categorical variables. A *VarIdent* variance structure on MeJA treatment was used to minimize the heteroscedasticity of residuals. After that, the model assumptions were met (residuals independence, normality, and variance homegeneity).

The concentrations of alkaloids (total lolines, NFL, and NANL) were separately analyzed with linear mixed-effects models, using the *nlme* package in R software, and assuming independent, identically distributed normal random errors. This time the fixed part of each model included the categorical variables of the MeJA treatment (MeJA+, MeJA-) and the experimental time (5d and 21d post-MeJA exposure), and the random part included the time nested in the pot. For each response variable, a *VarIdent* variance structure on MeJA treatment was used to accommodate heteroscedasticity of residuals, and a *CorARMA* \( (p = 1, q = 0) \) was adjusted to fix autocorrelation problems across repeated measurements. After that, all the model assumptions were met (residuals independence, normality, and variance homegeneity).
The response variables related with insect body weight (larval mass at 15d, pupal mass, and adult mass) were analyzed separately with linear effect models, using the function gls from the package nlme in R software, and assuming independent, identically distributed normal random errors. The initial insect body mass (at day 1) was used as a covariate for larval weight and pupal weight, and pupal mass as covariate for adult weight. The models included the categorical variables of symbiotic status (E+, E-) and MeJA treatment (MeJA+, MeJA-) and their respective covariate as a continuous factor. In all analyses, the model assumptions were met (residuals independence, normality, and variance homogeneity). For the variables related to insect development (time to pupation, time at pupa, and time to adult eclosion), each one was analyzed separately with generalized linear models, using the R software function glm from the package lme4, and assuming independent, identically distributed Poisson random errors (Bates, Maechler, Bolker, & Walker, 2015). The models included the categorical variables of symbiotic status (E+, E-) and MeJA treatment (MeJA+, MeJA-). Data overdispersion was not observed. All the analyses met the model assumption (residuals independence). Insect survival curves were estimated using the Kaplan-Meier model and the treatment effect was tested with the non-parametric Mantel-Cox test using the package Survival in R (Therneau & Grambsch, 2000). Since the Mantel-Cox test cannot evaluate the effect of interactions, we separately analyzed the effect of the symbiotic status on the insect survival for MeJA- and MeJA+ treatments. Pupae that did not emerge into adults after 70 days were considered dead. When significant interactions were detected, we used the lsmeans function from the lsmeans package (in R software) to test for differences between groups (Lenth, 2016). For all variables we report the mean ± SEM.
Results

EFFECTS OF MEJA AND FUNGAL ENDOPHYTES ON THE PHYSIOLOGICAL LEVELS OF HORMONES AND PLANT GROWTH

The endophyte reduced the physiological concentration of SA irrespective of the MeJA treatment (i.e. Symbiosis x MeJA) and the experimental time (i.e. Symbiosis x time) (Table 1). On average, the SA concentration in endophyte-symbiotic plants was ~ 38% lower than in non-symbiotic plants (E+: 41.67 ± 2.64 ng SA g\(^{-1}\) DW, and E-: 66.94 ± 3.62 ng SA g\(^{-1}\) DW) (Fig. 1a).

In addition, the concentration of SA increased with time (Table 1). The concentration of SA at day 21 was 35% higher compared to the concentration on day 3 following the application of MeJA ([SA] at days 3 and 21: 43.11 ± 2.99 and 65.50 ± 3.62 ng SA g\(^{-1}\) DW, respectively; Fig. 1a).

The effect of the MeJA treatment on the physiological concentration of JA was independent of the symbiotic status of plants (i.e. Symbiosis x MeJA), and varied with time (i.e. MeJA x time) (Table 1; Fig. 1b). Whereas the concentration of JA at day 3 was 2.8-fold higher in MeJA-treated plants than in the untreated counterparts (MeJA- and MeJA+: 1672 ± 85.81 and 4685 ± 186.50 ng JA g\(^{-1}\) DW, respectively), it went back to the initial level by 18 days later (MeJA- and MeJA+: 1410 ± 73.50 and 1407 ± 131.80 ng JA g\(^{-1}\) DW, respectively; Fig. 1b).

The plant aboveground biomass at the end of the larval stage of insects (23-28 days post MeJA exposure) was 16% higher in endophyte-symbiotic than non-symbiotic plants (E+: 6.92 ± 0.34 g DW and E-: 5.88 ± 0.33 g DW), and it was not affected by the exogenously added jasmonate (Table 1).

EFFECTS OF MEJA ON THE CONCENTRATION OF FUNGAL LOLINE ALKALOIDS

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Neither the total lolines as a whole nor the constituents by themselves (i.e. NFL and NANL) were affected by the interaction between MeJA treatment and time (Table 1). Irrespective of the time since application, the jasmonate treatment significantly reduced the concentration of total lolines (Table 1). The concentration of total lolines was 57% lower in MeJA-treated plants than in the untreated plants (Fig. 2). The same pattern of results were observed for each individual loline (Table 1). The MeJA treatment reduced the concentrations of NFL and NANL to 58% and a 54% respectively (Fig. 2). The concentrations of these two loline derivatives did not change with the experimental time (Table 1).

EFFECTS OF MEJA AND THE FUNGAL ENDOPHYTES ON FALL ARMYWORM PERFORMANCE

The body mass of *S. frugiperda* larvae (at day 15 post MeJA exposure) was affected by the endophyte fungus interacting with the MeJA treatment (Table 2). In plants not treated with MeJA, the endophyte reduced the larval body mass by 21%; but in MeJA-treated plants, larvae grown on endophyte-symbiotic plants weighed 31% more than those grown on non-symbiotic plants (Table 2). However, neither the body weights of pupae nor the adults were affected by the treatments (Table 2). Apart from the indicated effects, the final weight reached by each individual at the larval and adult stages was influenced by the initial body mass (Table 2).

The fungal endophyte interacted with the MeJA treatment in affecting the elapsed time of insects as larva and the time to adult eclosion (Table 2). For the plants not exposed to MeJA, endophyte presence delayed insect pupation and adult eclosion for about 4 days; but in plants that were exposed to MeJA, the insects pupated and the adults emerged 3 days earlier on endophyte-
symbiotic plants than on non-symbiotic ones (Table 2). The time taken for insects to emerge as pupa was not affected by any of the treatments or the interaction (Table 2).

The overall survival of *S. frugiperda* individuals evaluated 70 days from the start of the experiment was 58.5% and it was independent of the endophyte presence on either MeJA-exposed or MeJA-non-exposed plants (Table 2). Of the adult insects grown on endophyte-symbiotic plants, 50% showed wing deformations (Table 2).

**Discussion**

The JA hormone plays a central role in plant defenses controlling necrotrophic pathogens and chewing insect herbivores (Halitschke & Baldwin, 2004; Thaler et al., 2012). We hypothesized that an exogenous application of MeJA, an activator of the JA signaling pathway, would increase the level of resistance of endophyte-symbiotic and non-symbiotic plants to the chewing insect *S. frugiperda*. In addition, as biotrophic *Epichloë* fungal endophytes produce alkaloids, which are chemical compounds with anti-herbivory properties, we predicted that an enhancement of the JA defense would complement the resistance provided by the alkaloids. We found that *L. multiflorum* plants, symbiotic with the endophyte *E. occultans* were more resistant to *S. frugiperda* than their non-symbiotic counterparts. However, despite the fact that the JA concentration significantly increased with exposure of plants to MeJA, neither endophyte-symbiotic nor non-symbiotic plants showed enhanced resistance to the insects. Unexpectedly, the exposure of endophyte-symbiotic plants to MeJA led to a reduction in the concentration of loline alkaloids, consequently increasing the herbivore performance. Irrespective of the JA hormone concentration, the fungal endophyte was associated with a lower plant concentration of the SA hormone.

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The presence of beneficial microorganisms within plants is generally associated with a repression of the plant SA pathway (Navarro-Meléndez & Heil, 2014; Stacey, McAlvin, Kim, Olivares, & Soto, 2006; Yasuda et al., 2016). Like other symbiotic associations, we found that plants associated with fungal endophytes showed a lower concentration of SA hormone compared to non-symbiotic plants (see also Bastías et al., 2018). Consistent with our result, other grass-endophyte symbioses have shown a general downregulation of genes related to the SA pathway (Dupont et al., 2015; Johnson et al., 2003) (but see Schmid et al., 2017), as well as a susceptibility to certain species of biotrophic pathogens (Welty et al., 1991, 1993; Wäli et al., 2006; Krauss et al., 2007; Pańka Dariusz et al., 2011; Sabzalian et al., 2012). The endophyte production of specific enzymes controlling the SA pathway may be a plausible mechanism that explains the suppression of the SA pathway (Ambrose & Belanger, 2012). In addition to the endophyte effect on the SA, we found the hormone in increased concentrations when the plants were challenged with S. frugiperda larvae. A similar response was found on poplar trees (Populus nigra) in response to the biotrophic pathogen Melampsora larici-populina (a rust fungus) alone or in combination with larvae of the herbivore Lymantria dispar (Eberl et al., 2017). However, the high SA concentration found in the Eberl et al. study was mainly attributed to the pathogen infection rather than to the chewing herbivore. Other studies on Nicotiana attenuata plants have reported increases in SA concentrations in response to the single attacks of certain generalist Lepidopteran herbivores such as S. exigua, S. littoralis and Trichoplusia ni (Diezel, von Dahl, Gaquerel, & Baldwin, 2009; Heidel & Baldwin, 2004). It has been hypothesized that specific effector molecules or biotrophic-symbiotic microorganisms inhabitants of the insects oral secretions could be the potential elicitors of the plant SA-immune responses (Chung et al., 2013; Diezel et al., 2009). Thus, while our results suggest that the plant
concentration of SA would be mainly controlled by the fungal endophyte presence (see also Bastías et al., 2018), it could also respond to the activity of herbivores such as *S. frugiperda* larvae.

The presence of fungal endophytes in different grass species generally impairs the performance of *S. frugiperda* individuals (Afkhami & Rudgers, 2009; Ball et al., 2006; Boning & Bultman, 1996; Brem & Leuchtmann, 2001; Bultman & Bell, 2003; Bultman & Conard, 1998; Bultman & Ganey, 1995; Crawford et al., 2010; Davidson & Potter, 1995; Hardy, Clay, & Hammond, 1986; Mark & Lincoln, 1996; Salminen, Richmond, Grewal, & Grewal, 2005). Similarly, we found that the fungal endophyte *E. occultans* reduced the larval weight and extended the development time of *S. frugiperda* individuals. Moreover, it is interesting to note that even though insect survival and performance of pupae (i.e. body mass and development time) were not affected by the endophyte, about 50% of the adults that emerged from individuals reared on endophyte-symbiotic plants displayed deformed wings. This suggests that the effect of the endophyte on individual performance might continue beyond the larval stage, increasing the chances for insects to show further developmental failures (Thakur, Kaur, Kaur, & Singh, 2013).

Considering that the larvae developed faster when reared on non-symbiotic plants than on endophyte-symbiotic plants, one would expect that, after a fixed interval, the larval body masses would be higher in the former than in the latter type of plants (Boning & Bultman, 1996). In addition, the lack of endophyte effects on the pupae can be explained by the fact that larvae of *S. frugiperda* generally loose sensitivity to fungal alkaloids as they progress in growth and development (Bultman & Conard, 1998; Hardy et al., 1986).

The enhancement of plant resistance against chewing insects by means of exogenous application of MeJA has been shown in several plant species (Heijari, Nerg, Kainulainen, Vuorinen, &
In the case of grass species of the subfamily Poöideae, there has only been one study, to the best of our knowledge, showing that the exogenous application of MeJA increased the resistance level of tall fescue plants (*S. arundinaceus*) to a species of sap-sucking insect herbivore, namely the aphid *Rhopalosiphum padi* (Simons, Bultman, & Sullivan, 2008). In our study, despite the fact that the exogenous application of MeJA increased the plant concentration of JA hormone approximately 3-fold, the treatment did not enhance the level of resistance of non-symbiotic plants against the chewing herbivore *S. frugiperda*, nor did the MeJA treatment affect the plant aboveground biomass. Although counter to our expectations, the lack of effects of the MeJA exposure on the plant resistance to *S. frugiperda* could be explained by two non-exclusive mechanisms: (i) the plant tolerance to insect herbivory, and/or (ii) the insect tolerance to plant defenses. The level of plant tolerance to herbivores can increase in response to MeJA exposure. This enhanced level of tolerance has been termed “herbivory-induced resource sequestration (HIRS)” (Babst et al., 2005; Ferrieri, Agtuca, Appel, Ferrieri, & Schultz, 2013; Frost & Hunter, 2008; Gómez, Ferrieri, Schueller, & Orians, 2010; Meuriot et al., 2004), and has been identified in several plant species (Babst et al., 2005; Ferrieri et al., 2013; Frost & Hunter, 2008; Gómez et al., 2010; Meuriot et al., 2004) including grasses (Bazot, Mikola, Nguyen, & Robin, 2005; Fahnestock & Detling, 1999; Green & Detling, 2000; Hokka, Mikola, Vestberg, & Setälä, 2004; Wilsey, Coleman, & McNaughton, 1997). A HIRS response is explained by the transport of chemical resources, such as carbon and nitrogen-based compounds, toward roots in order to protect them from aboveground-herbivores (Orians, Thorn, & Gómez, 2011; Schwachtje

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& Baldwin, 2008). Thus, if the application of MeJA contributed to a rapid reallocation of resources rather than to an activation of defenses in plants of *L. multiflorum*, it could explain in part the lack of resistance observed in MeJA-exposed plants to *S. frugiperda*. Alternatively, *S. frugiperda* larvae could have been naturally tolerant to the defense metabolites produced by *L. multiflorum* plants. Among these metabolites, the most important are the polyphenol oxidases and proteinase inhibitors (Faville et al., 2004; Humphries, 1980; Lee, Olmos Colmenero, Winters, Scollan, & Minchin, 2006), for which *S. frugiperda* larvae have shown certain biochemical and physiological mechanisms to metabolize them (Brioschi et al., 2007; Giraudo et al., 2015; Paulillo et al., 2000).

We expected that symbiotic plants treated with MeJA would activate the host plant defenses, complementing the resistance effects due to the fungal endophyte and its alkaloids. However, despite the fact that MeJA exposure increased the JA concentration significantly, the jasmonate treatment reduced the concentration of loline alkaloids and consequently, it decreased the level of resistance of endophyte-symbiotic plants to *S. frugiperda*. A similar result was found in tall fescue plants where the endophyte production of alkaloids as well as the endophyte-conferred resistance to a sap-sucking insect herbivore were impaired by the exogenous application of MeJA (Simons et al., 2008). In studies with mycorrhizal symbionts, the JA can positively or negatively regulate fungal growth, depending on the degree of fungal colonization in plant roots (Fernández et al., 2014; Hause, Mrosk, Isayenkov, & Strack, 2007). This conditional effect of JA on fungal growth would be related to the dual role that the hormone plays on the plant metabolism, the re-allocation of resources or the activation of defenses (Hause et al., 2007).

Since mycorrhizal fungi associate with plant roots, the JA-mediated re-allocation of resources toward belowground tissues could increase fungal growth; but an opposite effect could be
triggered by the hormone activation of plant defenses (Fernández et al., 2014; Gutjahr, Siegler, Haga, Iino, & Paszkowski, 2015; Isayenkov, Mrosk, Stenzel, Strack, & Hause, 2005; Landgraf, Schaarschmidt, & Hause, 2012). In the case of leaf fungal endophytes, the JA stimulation of either of the two processes (i.e. re-allocation of resources or activation of defenses) could negatively affect the fungus. Since leaf fungal endophytes, such as *E. occultans*, live in the apoplast of the aboveground plant tissues, the reallocation of plant nutrients toward roots would reduce the pool of compounds available for fungal metabolism. In fact, the endophytes growth and the production of alkaloids are processes highly dependent on the amount of carbohydrates and nitrogen available in the aboveground grass tissues (Krauss et al., 2007; Rasmussen et al., 2007; Ryan et al., 2014).

Although, the possibility that the JA-dependent defenses can negatively affect biotrophic fungal endophytes seems counterintuitive, there is evidence of this in mycorrhizal fungi, but the underlying mechanisms are not completely understood (Gutjahr et al., 2015; Herrera-Medina, Tamayo, Vierheilig, Ocampo, & García-Garrido, 2008). Alternatively, the MeJA exposure could have directly affected the biosynthetic routes of fungal alkaloids. For instance, the gene *LolC* encoding for an enzyme of biosynthesis of lolines has been found to be downregulated in endophyte-symbiotic plants of *S. arundinaceus* exposed to MeJA (Simons et al., 2008). Thus, the unexpected MeJA effect on the herbivory resistance level of endophyte-symbiotic plants may be explained by indirect mechanisms, including the reallocation of plant resources or the activation of plant defenses, and/or by a direct mechanism influencing the biosynthesis of alkaloids.

In conclusion, the present study highlights that the interaction between the plant JA hormone and the presence of *Epichloë* endophytes can affect the anti-herbivore defenses of symbiotic plants. However, our results suggest that, rather than complementing the alkaloid-based defense, JA

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interferes with the anti-herbivore mechanism conferred by endophytes. An important ecological consequence of our results is that any biotic or abiotic factor activating the JA pathway may reduce the effectiveness of the defense provided by *Epichloë* fungi. An example of a JA pathway elicitor is UV-B radiation (Ballaré, 2014). Consistent with this hypothesis, the level of resistance against the locust *Shistocerca gregaria* was significantly reduced when plants of *Festuca pratensis* symbiotic with the endophyte *E. uncinatum* were exposed to UV-B radiation (McLeod, Rey, Newsham, Lewis, & Wolferstan, 2001). It is worth noting that our predictions and results might have been different if endophyte-symbiotic plants were challenged by specialist insect herbivores. Since specialist herbivores are likely to evolve specific detoxification mechanisms, they would likely be less affected by the fungal alkaloids than generalist herbivores (Ali & Agrawal, 2012; Faeth & Saari, 2012). For example, *Sipha maydis* aphids are specialist herbivores of grasses that naturally feed on *Lolium multiflorum* plants, and do not seem to be affected by the loline alkaloids produced by *E. occultans* endophytes (Miranda, Omacini, & Chaneton, 2008; Omacini, Chaneton, Ghersa, & Muller, 2001). Including the plant hormones as endogenous regulators of the endophyte, performance would be helpful to understand the mechanisms that explain the impacts of ecological factors on the defenses of endophyte-symbiotic plants against herbivores.

**Author contribution**

D.A.B., M.A.M.G., and P.E.G. planned and designed the research; D.A.B., S.D.C, W.J.M, and J.A.N. collected the data; D.A.B. analyzed the data; D.A.B., P.E.G., and J.A.N. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.
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Data accessibility

Data associated with this article are available on DRYAD:

https://doi.org/10.5061/dryad.k87m52h (Bastías et al., 2018).

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Boning, R. A., & Bultman, T. L. (1996). A test for constitutive and induced resistance by tall Fescue (*Festuca arundinacea*) to an insect herbivore: impact of the fungal endophyte,

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**Table legends**

**Table 1.** ANOVA table showing the effects of plant symbiosis status, methyl jasmonate application, and experimental time on the concentration of plant hormones salicylic and jasmonic acids, endophytic loline alkaloids [(total, N-formylloline (NFL), and N-acetylnorloline (NANL)] and weight of aboveground plant tissues of *Lolium multiflorum* plants symbiotic with the endophyte *Epichloë occultans*. Statistically significant effects are highlighted in bold. Values of means, SEM, and post hoc statistical differences are shown at Figures 1 and 2 and on the text.

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Table 2. Performance of *Spodoptera frugiperda* individuals at the different life stages (larva, pupa, and adult) grown on *Lolium multiflorum* plants with (E+) and without (E-) the fungal endophyte *Epichloë occultans* and treated (MeJA+) and untreated (MeJA+) with methyl jasmonate. Statistically significant effects are highlighted in bold. Different letters on performance values indicate significant differences at $P < 0.05$ (post hoc test). Replicate numbers are indicated in parenthesis. For the analysis of larval weight and pupal weight, the insect body mass at day 1 was used as a covariate (larval weight: $F_{1,30} = 11.08$, $P = 0.002$; pupal weight: $F_{1,29} = 0.11$, $P = 0.742$), and pupal mass ($F_{1,15} = 4.74$, $P = 0.045$) as covariate for the adult body weight. # indicates the $\chi^2$ value calculated from the non-parametric Mantel-Cox analysis. Values are mean ± SEM.

Figure legends

Figure 1. Physiological concentrations of salicylic acid [SA, top panel (a)] and jasmonic acid [JA, bottom panel (b)] at day 3 and 21 since the methyl jasmonate application [treated: MeJA+ (shaded bars), and untreated: MeJA- (unshaded bars)] in *Lolium multiflorum* plants with (E+, striped bars) and without (E-, plain bars) the endophyte fungus *Epichloë occultans*. Asterisks and letters indicate significant differences at $P < 0.05$ (post hoc test). Bars represent mean values ± SE ($n = 9$).

Figure 2. Concentrations of loline alkaloids [total (black bars) and derivatives: N-formylloline (NFL, grey bars) and N-acetylnorloline (NANL, white bars)] produced by the fungal endophyte *Epichloë occultans* in *Lolium multiflorum* plants treated (MeJA+) and untreated (MeJA-) with methyl jasmonate. Total lolines are NFL+NANL derivatives. Non-symbiotic plants do not
produce loline alkaloids. Each loline was analyzed separately (see Material and Method section). Asterisks indicate significant differences at $P < 0.05$. The bars represent mean values ± SEM, averaging across time (at days 5 and 21 post MeJA application; $n = 18$).

**Tables**

**Table 1.**

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<th>$df_2$</th>
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<th>$P$-value</th>
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**Figures**

![Graph a)](image1.png)

**Figure 1.**
Figure 2.