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Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation

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Abstract. 1. Fungal endophytes are ubiquitous associates of virtually all plant species. Although many studies have focused on the role of these microorganisms as mediators of plant–herbivore interactions, these studies have usually been conducted using short-term experiments.

2. Truly effective defences against herbivores may require normal functioning of the plant, as excised leaves may be less resistant as compared with those still attached to the plant. Yet, most studies investigating possible effects of endophytes in conferring host resistance to herbivores have been conducted with plant parts rather than intact plants.

3. Using the root endophytic fungus (*Acremonium strictum*)—broad bean (*Vicia faba*)—generalist herbivore (*Helicoverpa armigera*) model, we conducted experiments to examine whether endophyte effects on herbivory would depend on the experimental setting used in the investigation and whether they would translate into a subsequent generation of the herbivore.

4. *Acremonium strictum* negative effects on the fitness of *H. armigera* first generation were more evident when the larvae foraged freely on inoculated intact whole plants than when offered leaf discs of inoculated plants. Furthermore, these effects were carried over into *H. armigera* second generation reared on an artificial diet.

5. *Acremonium strictum* could not be re-isolated from *V. faba* leaves; hence direct contact between the endophyte and the insect could be excluded. Alternatively, loss of volatiles or inhibitory effects of compounds that were stronger *in situ* might have caused changes in larval feeding and performance on leaf discs as compared with intact plants, regardless of infection status.

6. We suggest that the reduction in fitness parameters of *H. armigera* across two generations is caused indirectly via an endophyte-triggered reduction in plant quality.

Key words. *Acremonium strictum*, experimental setting, *Helicoverpa armigera*, host–endophyte interactions, host plant quality, long-term effects, root endophytic fungi, successive generations, *Vicia faba*.

Introduction

Fungal endophytes (i.e. fungi that live internally within the tissues of their host plant without causing visible signs of infection) appear to be ubiquitous associates of all plants, as they have been found in virtually every organ from every plant species examined so far (Hartley & Gange, 2009). The most investigated and best understood group of these endophytes is the clavicipitaceous endophytic fungi that are vertically transmitted (via seeds) and systemically associated with the aboveground portions of grasses. They are thought to interact mutualistically with their host plants (but see Faeth, 2002; Faeth & Fagan, 2002); mainly by the production of secondary compounds, including alkaloids, which benefit

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plants by increasing their competitive ability and resistance to biotic and abiotic stresses (Kuldau & Bacon, 2008).

Host–endophyte symbioses are not restricted to this highly specialized group of endophytes in grasses. The vast majority of fungal endophytes form internal localized infections in foliage, roots, stems, and bark and are horizontally transmitted via spores (Faeth, 2002). However, the associations between those omnipresent unspecialised endophytes and their woody and herbaceous host plants remain less clearly understood, as relatively little is known about the interactions involved (Hartley & Gange, 2009). The mechanisms underpinning these interactions are mostly attributed thus far to the endophyte-mediated alteration of host plant nutritional quality (Bernays, 1993), growth, and competitive abilities (Marks *et al*., 1991; Faeth *et al*., 2004), or other cues, such as volatiles (Jallow *et al*., 2008) and secondary metabolites (Arnold, 2008) that may have major impacts on the organisms feeding on the endophyte-colonised host plant. In fact, both plant symbiotic endophytes and mycorrhizae have been shown to significantly affect the herbivores with which they are in relatively intimate contact. While work on endophytic fungi colonising foliage has been rare, even less attention has been paid to those colonising plant roots (Hartley & Gange, 2009). In contrast to both foliar and root endophytes colonising herbaceous and woody plants, the root-inhabiting mycorrhizae [especially the vesicular-arbuscular mycorrhiazae (VAM)] have been the subject of many studies and their beneficial effects (nutrient acquisition in addition to protection against environmental stresses and herbivore attack) are well established (see Brundrett, 2002 for a general review on myorrhizal fungi; Gange, 2007 for the most recent review of insect–mycorrhiza interactions).

Among the unspecialised root-colonising fungal endophytes, the genus *Acremonium* comprises a diverse group of soil-borne fungi that can be found in different host plants (Jallow *et al*., 2008; and references therein). Unlike the clavicipitaceous endophytic fungi of grasses, these endophytes are horizontally transmitted and commonly found in studies screening for endophyte diversity (Schulz *et al*., 1993; Gange *et al*., 2007). Previous work with a species of this genus (*Acremonium strictum* Gams) revealed an antagonism mediated by this endophyte towards herbivorous insects (Vidal, 1996; Jallow *et al*., 2004; Jaber & Vidal, 2009). However, these studies have been usually conducted over very short time periods (less than the time required for a single insect generation). In general, there have been very few studies on the long-term effects of endophytes as mediators of plant–herbivore interactions (e.g. Faeth & Hammon, 1997; Durham & Tannenbaum, 1998).

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a widespread agricultural pest (reviewed in Rajapakse & Walter, 2007) and one of the major polyphagous species in the subfamily Heliothinae (Fitt, 1989; Zalucki *et al*., 1986). Although *H. armigera* is known to feed on more than 200 host plant species (including both cultivated crops and wild plants) belonging to 47 families (reviewed in Zalucki *et al*., 1986), very few studies have ever associated it with broad bean, *Vicia faba* L. (e.g. Tripathi & Singh, 1989; Grundy *et al*., 2004). Johnson and Zalucki (2005) reported that larvae of generalist feeders do not behave in an equivalent manner on intact plants as compared with plant parts. This is most likely a result of volatiles emanating from intact plant surfaces and playing an important role in guiding larvae to their feeding sites (Singh & Mullick, 2002; and references therein). Such changes in larval foraging behaviour could have consequences for their growth and development (Johnson & Zalucki, 2005). These observations, coupled with the possibility that truly effective defences against herbivores may require normal functioning of host plants (as excised leaves may be less resistant as compared with those still attached to the plant; Klemola *et al*., 2007), suggests the importance of the experimental setting used in testing the influence of endophytes in conferring resistance to herbivores. Yet, most studies investigating the role of endophytes as mediators of plant–herbivore interactions have been conducted with plant parts rather than intact plants (e.g. Clay *et al*., 1993; Bultman & Conard, 1998; Raps & Vidal, 1998; McGee, 2002; Vicari *et al*., 2002).

In this study, we investigated (1) whether *A. strictum*-mediated effects on a range of *H. armigera* life history parameters would depend on the experimental settings, i.e. larvae foraging freely on inoculated intact whole *V. faba* plants versus leaf discs of inoculated plants and (2) whether these effects would translate into a subsequent generation of *H. armigera* reared on an artificial diet? We predicted that the negative influences of the root endophyte on plant–herbivore interactions would be enhanced on inoculated intact whole plants and would last across *H. armigera* successive generations.

Materials and methods

Study organisms

Vicia faba seedlings (cultivar, Hangdown Grünkernig, Gevo GmbH, NORTMOOR/OSTFR., Germany) were grown in a greenhouse chamber. Two-week-old plants were individually transplanted into plastic pots (15 cm diameter) with a mixture of non-sterile sand and soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany; 1:1 ratio). Plants were irrigated regularly and fertilised once a week with 15:10:15:2 NPKMg (COMPO GmbH, Münster, Germany).

A strain of *A. strictum* from DSMZ-GmbH, Braunschweig, Germany, was maintained in the laboratory on malt extract agar (MEA, 0.3%). Liquid MEA media (0.3%) was autoclaved at 120°C for 20 min. A spore suspension was prepared by adding a piece of malt extract agar containing fungus mycelia to the autoclaved medium. This suspension was then kept on a shaker (at 23°C and 100 rpm) for 12 days to ensure fungal growth and sporulation. After vacuum filtering, spore concentration in a drop of the suspension was measured under a microscope using a Thoma counting chamber (64×0.025 mm^2, chamber height 0.1 mm). Five days after transplanting, plants assigned to be inoculated (E+) were watered with 70 ml of spore suspension containing 10^6 *A. strictum* spores/ml, and control plants (E-) were watered with the same volume of culture filtrate. In previous studies, the inoculum density used here was found to be sufficient in colonising plant roots (Vidal, 1996; Jallow *et al*., 2004; Jaber & Vidal, 2009). Five days post-inoculation,

single plant replicates of E+ and E− plants near the five-leaf stage were used in experiment 1. In order to determine successful inoculation of the plants at the beginning of the experiment, root samples were taken from five inoculated and non-inoculated (non-treatment) plants 5 days post-inoculation. Sampled root segments were obtained and handled as described below.

Eggs of a laboratory strain of *H. armigera* were provided by Bayer Crop Science, Mohnheim, Germany, and kept in a climatic chamber at 25°C, 60% RH and LD 14:10 h photoperiod until hatching. Neonate first-instar larvae (hatching within 12 h) were later used as the first generation (F1) in experiment 1.

Establishment of Acremonium strictum *in roots and shoots of inoculated plants*

Six weeks after inoculating *V. faba* roots with *A. strictum* (at the end of experiment 1), growth of the fungus within the roots and leaves of E+ and E− plants was recorded by re-isolation from surface-sterilised root pieces and leaf discs. Surface sterilisation followed the method of Guo *et al.* (2000). Five leaves were randomly selected from 10 plants of each treatment. Roots of each plant, from which leaves were selected, were subsequently thoroughly washed, dried, and divided into five root zones. Samples were surface sterilised by consecutive immersion for 1 min in 70% ethanol, 2 min in 3.25% sodium hypochlorite (NaOCl), 2 min in sterile distilled water, and then vigorously rinsed with sterile distilled water. Less immersion time was used for sampled leaves than roots. Five leaf discs per leaf were cut with a sterile leaf punch and six equal 1-cm segments of root pieces were cut from each root zone using a sterile scalpel. Leaf discs and root segments were then evenly placed in 90-mm petri dishes containing potato dextrose agar (PDA) supplemented with 1 mg ml^{-1} streptomycin sulphate to suppress bacterial growth. Petri dishes were sealed and incubated at 24°C with a 12-h dark light cycle and examined periodically. When colonies developed, they were transferred to new Petri dishes with MEA. Fungi were then sub-cultured into low nutrient media and incubated under 12-h UV light and low temperature to induce sporulation. Subcultures of isolated fungi were identified when isolates sporulated by microscopic examination based on morphological characteristics.

Experiment 1. Responses of Helicoverpa armigera *first generation (F1) to* Acremonium strictum *infection in different experimental settings*

We conducted a greenhouse experiment, manipulating endophyte infection (I) and experimental setting (S) in a 2 × 2 factorial design. We used two endophyte infection levels: endophyte infected (E+) and endophyte free (*E*−), and two experimental settings: feeding on leaf discs of E+ or *E*− plants in petri dishes, and foraging freely on E+ or E− intact whole potted plants. Neonate larvae of uniform size (of the same full sib group; F1) were used in both experimental settings.

One hundred and twenty neonate larvae were randomly and individually placed in Petri dishes (90 mm diameter), lined with moistened filter paper. Half of the larvae were offered leaf discs (cut with a sterile leaf punch) from E+ plants, whereas the other half were offered leaf discs from E− plants. Leaf discs were replenished as necessary and filter papers were replaced with new ones every 48 h. Petri dishes of E+ and E− treatments were randomised inside an environmental-controlled climatic chamber (25°C, 60% RH and LD 14:10 h photoperiod). Another 150 neonate larvae (of the same sib group; F1) were used in the second experimental setting. The neonate larvae were randomly chosen and placed on the upper third of potted intact whole *V. faba* plants, being the major oviposition site for female moths (Jallow *et al.*, 2001), and allowed to forage freely. Half of the larvae were placed on E+ potted plants and the other half on E− potted plants (15 plants per treatment; five larvae per plant). Owing to technical reasons (i.e. potted *V. faba* plants did not fit inside the climatic chamber), treatments in the second experimental setting could not be kept with those of the first experimental setting. Instead, potted plants of E+ and E− treatments were randomized on a bench in a greenhouse chamber at controlled conditions similar to those of the first experimental setting (as described above). In order to prevent the introduced larvae from escaping or moving between plants from different treatments, we placed potted plants of either E+ or E− treatments on top of inverted pots immersed in a water-filled tray. During the course of the experiment, some larvae attempted to escape the plants on which they were released, and were thus found drowned in water. These larvae were therefore excluded from the calculation of per cent larval survival and the remaining analyses.

The larvae in both experimental settings were checked twice daily for moulting and survival until pupation. Larval weight was individually measured 9 and 11 days after the beginning of the experiment. The relative growth rate (RGR) was calculated according to Farrar *et al.* (1989) as follows: RGR = Biomass gained (mg fresh weight)/[(fresh weight at day 9 + fresh weight at 11 day)/2] × 2(days). Newly formed pupae were sexed and weighed individually 12 h after pupation, and then transferred into clean petri dishes lined with filter paper and kept at 22°C until adult emergence. Emergent adult moths from larvae reared on both treatments were kept separately in mating cages, supplied with 10% honey solution, and held for 3 days after eclosion to allow mating and egg maturation (Jallow & Zalucki, 1998). Twelve female moths per treatment were subsequently transferred to oviposition cages and fed 10% honey solution. Eggs were counted and recorded daily for 10 days. In order to determine the adult longevity, newly eclosed moths from larvae reared on each treatment were placed individually in transparent plastic cylinders and supplied with 10% honey solution. Twenty replicates were used per treatment and the survival time of each was recorded.

At the end of this experiment, percentage of larval survival, RGR, larval period (days from hatching to pupation), prepupal period, percentage of pupation, pupal weight, pupal period (days from pupation to adult emergence), percentage of adult emergence, female fecundity (average number of eggs per female), and adult longevity were determined. On E+ and E−

intact whole potted plants, the insect life history parameters were measured as the mean values of the surviving larvae per plant.

Experiment 2. Responses of Helicoverpa armigera *second generation (F2) to* Acremonium strictum *infection*

In order to determine whether there is an effect of endophytic infection on a subsequent generation of *H. armigera*, two egg groups laid within 12 h by F1 female adults (reared on E+ or E− intact whole plants in experiment 1; one female per treatment) were collected and incubated in a climatic chamber at 25°C, 60% RH and LD 14:10 h photoperiod until hatching. Sixty-four neonate larvae ($n = 64$) of the hatching sib group (full sib) from each treatment were reared on a standard bean flour-based artificial diet (Teakle, 1991) and served as the F2 generation. The life history parameters of the F2 generation were followed as described with the F1 generation in experiment 1.

Statistical analyses

Data (except *A. strictum* infection percentage) met assumptions of normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). *Acremonium strictum* infection percentage was calculated as the total number of plant-tissue segments infected by the fungus, divided by the total number of incubated segments. Differences in infection percentage of shoots and roots of E+ and E− plants were analysed using logistic regression. Differences in life history parameters of *H. armigera* reared on E+ and E− treatments in experiment 1 were tested with a two-way ANOVA (GLM procedure) with *endophyte infection* (I) and *experimental setting* (S) as the main factors, except the female fecundity data (number of eggs laid over 10 days) for which a two-way repeated-measures ANOVA was carried out. Bonferonni's correction for multiple testing (modified by Simes, 1986 for the test of an overall hypothesis which is a combination of n individual hypotheses) was carried out in order to control for the experiment-wide error. The Tukey–Kramer honestly significant difference (HSD) test (for unequal sample sizes) was then used to separate the treatment combinations only when the interaction between the two main factors was highly significant ($P < 0.001$), in order to deal with the restricted randomisation in this experiment (i.e. keeping E+ and E− treatments of each experimental setting in different locations). Differences in life history parameters of *H. armigera* between E+ and E− treatments within and across generations in experiment 2 were tested using one-way ANOVA with Bonferroni's correction for multiple testing. All analyses were performed using SYSTAT for Windows, version 12 (SYSTAT, 2008).

Results

Establishment of Acremonium strictum *in roots and shoots of inoculated plants*

The success of the endophyte inoculation procedure was confirmed at the beginning of experiment 1 by the outgrowth of the fungus of all incubated root segments sampled from inoculated (non-treatment) plants, whereas non-inoculated (non-treatment) plants did not show any *A. strictum* infection (data not shown). Six weeks post-inoculation, 77% of the root segments sampled from *A. strictum*-inoculated *V. faba* plants (E+) were found to be successfully infected by the endophyte, whereas root segments from non-inoculated plants (E−) showed no outgrowth of the fungus (z ratio = 237.82, df = 1, $P < 0.0001$; logistic regression) (Table 1). Out of the 273 fungal isolates recovered from E+ plants roots, 231 isolates were sporulating and identified as *A. strictum* isolates. The remaining 42 isolates (14%) did not sporulate (mycelia sterilia) and could not be identified. On the other hand, *A. strictum* was not established in the shoots of neither E+ nor E− *V. faba* plants, as none of the leaf discs sampled showed any outgrowth of the fungus. Interestingly however, some fungal pathogens were recorded in a small number (11%) of the leaf discs sampled from E− plants (Table 1).

Table 1. Re-isolation of *Acremonium strictum* from roots and shoots of inoculated (E+) and control (E−) *Vicia faba* plants.

	V. faba roots		*V. faba* shoots	
	E+ plants	E− plants	E+ plants	E− plants
Samples	300	300	250	250
Samples with isolates	273	None	None	27
A. strictum isolates recovered	231	None	None	None
% *A. strictum* infection	77 **a**	0 **b**	0	0

Values within rows followed by different letters are significantly different ($P < 0.0001$; logistic regression).

Experiment 1

Both main factors, *endophyte infection* and *experimental setting*, had strong significant effects on the life history parameters of *H. armigera* F1 generation, and there was a significant interaction between the two factors for all the sampled parameters except pupal weight (Table 2). *Acremonium strictum* negative effects on *H. armigera* fitness were dependent on the experimental setting used (Fig. 1). The F1 generation of *H. armigera* suffered significant reductions in larval survival rate (Fig. 1A), relative growth rate (Fig. 1B), female longevity (Fig. 1F), and fecundity (Fig. 1G) only when the larvae foraged freely on inoculated intact plants as compared with their non-inoculated counterparts ($P < 0.05$; Tukey–Kramer HSD test after two-way ANOVA with Bonferroni's correction). None of these parameters differed between the E+ and E− treatments when the larvae were offered leaf discs of inoculated or non-inoculated plants (Fig. 1). In addition, *A. strictum* infection significantly prolonged the larval (Fig. 1C), prepupal (Fig. 1D), and pupal (Fig. 1E) developmental periods in *H. armigera* larvae fed upon the E+ treatment on intact plants, but not on leaf discs. On the other hand, the pupal weight was not influenced by the endophyte infection, neither on leaf

discs, nor on intact plants (Table 2); although it was slightly larger on E+ treatment in both experimental settings (data not shown). Within each of the endophyte infection groups, significant differences were found in *H. armigera* fitness parameters sampled on intact plants as compared with leaf discs (Fig. 1).

Experiment 2

Larval survival rate ($F_{1,62} = 1.27$, $P = 0.26$; Fig. 2A; one-way ANOVA with Bonferroni correction), the relative growth rate ($F_{1,62} = 2.46$, $P = 0.12$; Fig. 2C), pupation rate ($F_{1,62} = 0.32$, $P = 0.57$; Fig. 2D), pupal weight ($F_{1,53} = 1.99$, $P = 0.16$; Fig. 2E), and pupal period ($F_{1,26} = 0.48$, $P = 0.49$; Fig. 2F) did not vary significantly across *H. armigera* generations reared on E+ treatment. On the other hand, adult emergence ($F_{1,53} = 4.13$, $P = 0.047$; Fig. 2G), longevity ($F_{1,28} = 4.43$, $P = 0.004$; Fig. 2H), and female fecundity ($F_{1,17} = 4.59$, $P = 0.047$; Fig. 2I) were significantly reduced further across *H. armigera* generations reared on E+ treatment as compared with those reared on E− treatment. Significantly shorter larval periods were observed in F2 generations of *H. armigera* reared on both treatments ($F_{1,62} = 85.65$, $P = 0.001$, E+ treatment; $F_{1,72} = 13.01$, $P = 0.001$, E− treatment; Fig. 2B). A significant increase in pupal weight across *H. armigera* generations was only found within the E− treatment ($F_{1,69} = 25.89$, $P = 0.001$; Fig. 2E).

Discussion

Although previous studies have already reported detrimental effects of endophytic fungi on *H. armigera* (McGee, 2002; Jallow *et al.*, 2004), results of the current study constitute the

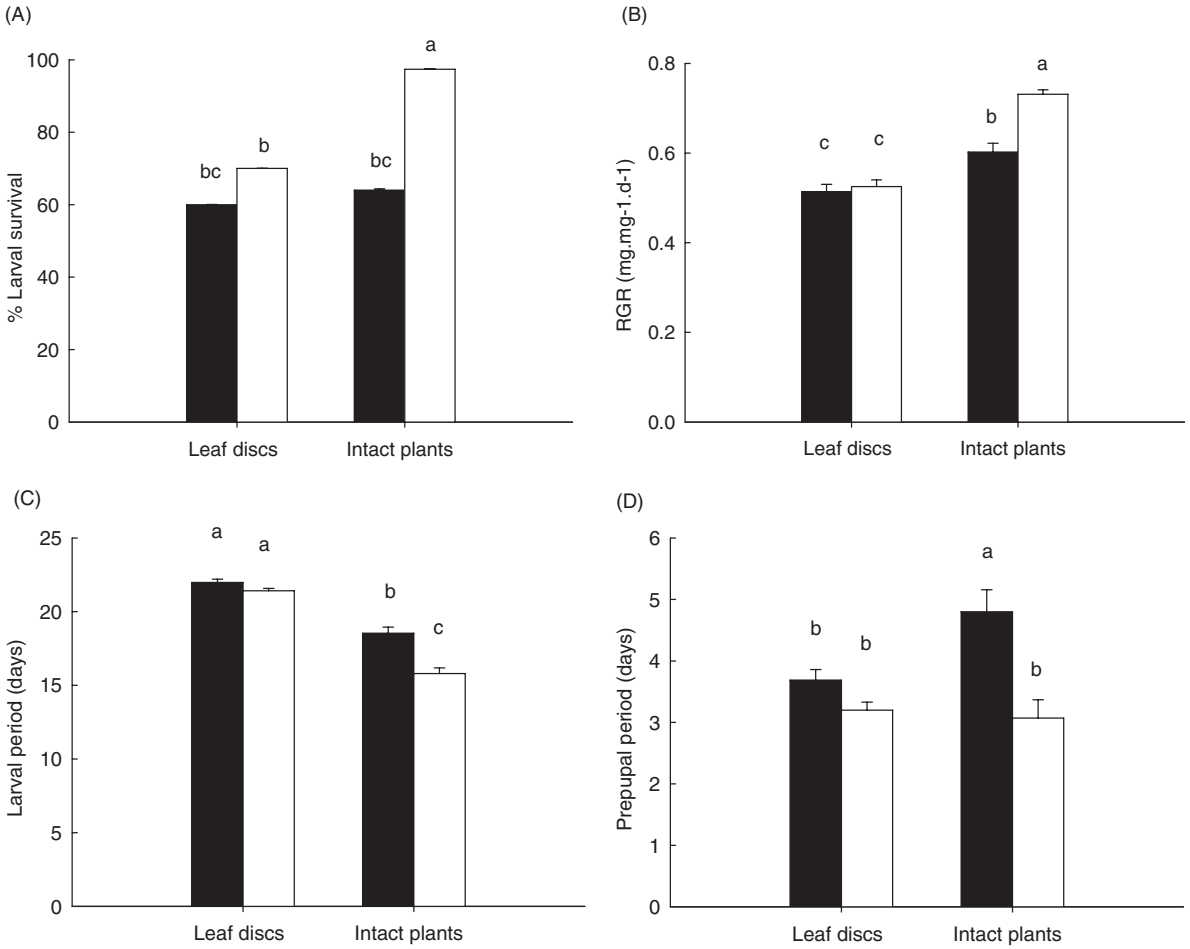

Fig. 1. Effect of endophyte infection and experimental setting on the life history parameters of *Helicoverpa armigera* first generation (F1; mean ± SE). (A) Per cent larval survival; (B) relative growth rate (RGR) (mg* mg^{-1}* d^{-1}); (C) larval period (days); (D) prepupal period (days); (E) pupal period (days); (F) female longevity (days); and (G) female fecundity (mean eggs/female). Insects were offered leaf discs of *Acremonium strictum*-inoculated plants (E+; black bars) or non-inoculated plants (E−; white bars), or foraged freely on *A. strictum*-inoculated (E+) or non-inoculated intact whole *Vicia faba* plants (E−). We used the Tukey–Kramer honestly significant difference (HSD) test to separate the treatment combinations (different letters denote means significantly different at $P < 0.05$) only when the interaction between endophyte infection and experimental setting was highly significant ($P < 0.001$; two-way ANOVA with Bonferroni's correction for multiple testing).

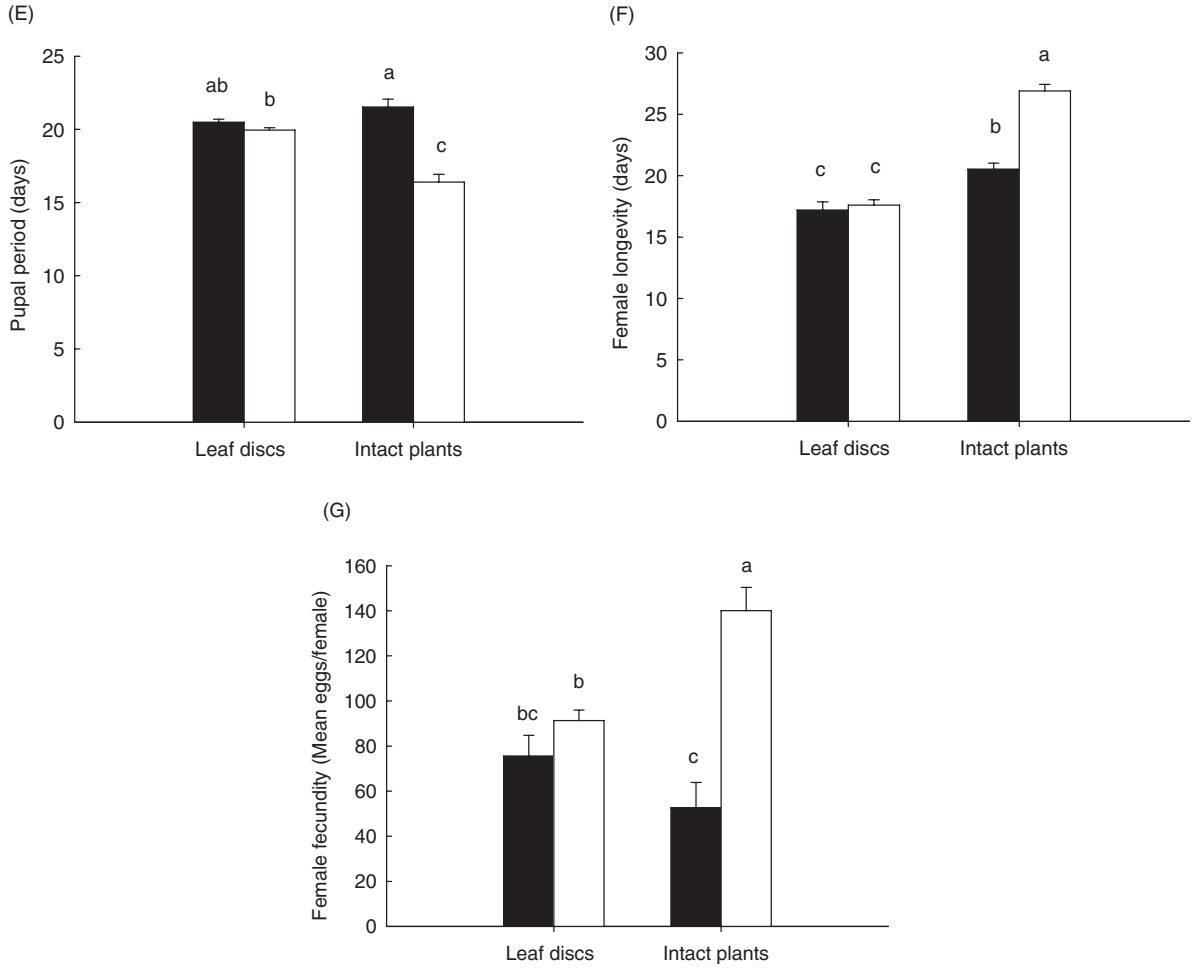

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Table 2. Two-way ANOVA (*P*-values) for the effects of endophyte infection (I) and experimental setting (S) on the life history parameters of *Helicoverpa armigera* first generation (F1).

H. armigera life history parameter (F1 generation)	Endophyte infection (I)	Experimental setting (S)	I × S
% larval survival	***	***	***
Relative growth rate (RGR) (mg* mg^{-1} * d^{-1})	**	***	***
Larval period (days)	***	***	***
Prepupal period (days)	***	*	***
% pupation	**	n.s.	*
Pupal weight (mg)	n.s.	***	n.s.
Pupal period (days)	***	***	***
% adult emergence	**	*	**
Adult longevity (days): total	***	***	**
♀	***	***	***
♂	**	***	*
Female fecundity (mean eggs/♀)	***	*	***

n.s., not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
P-values are adjusted by Bonferroni correction for multiple testing.

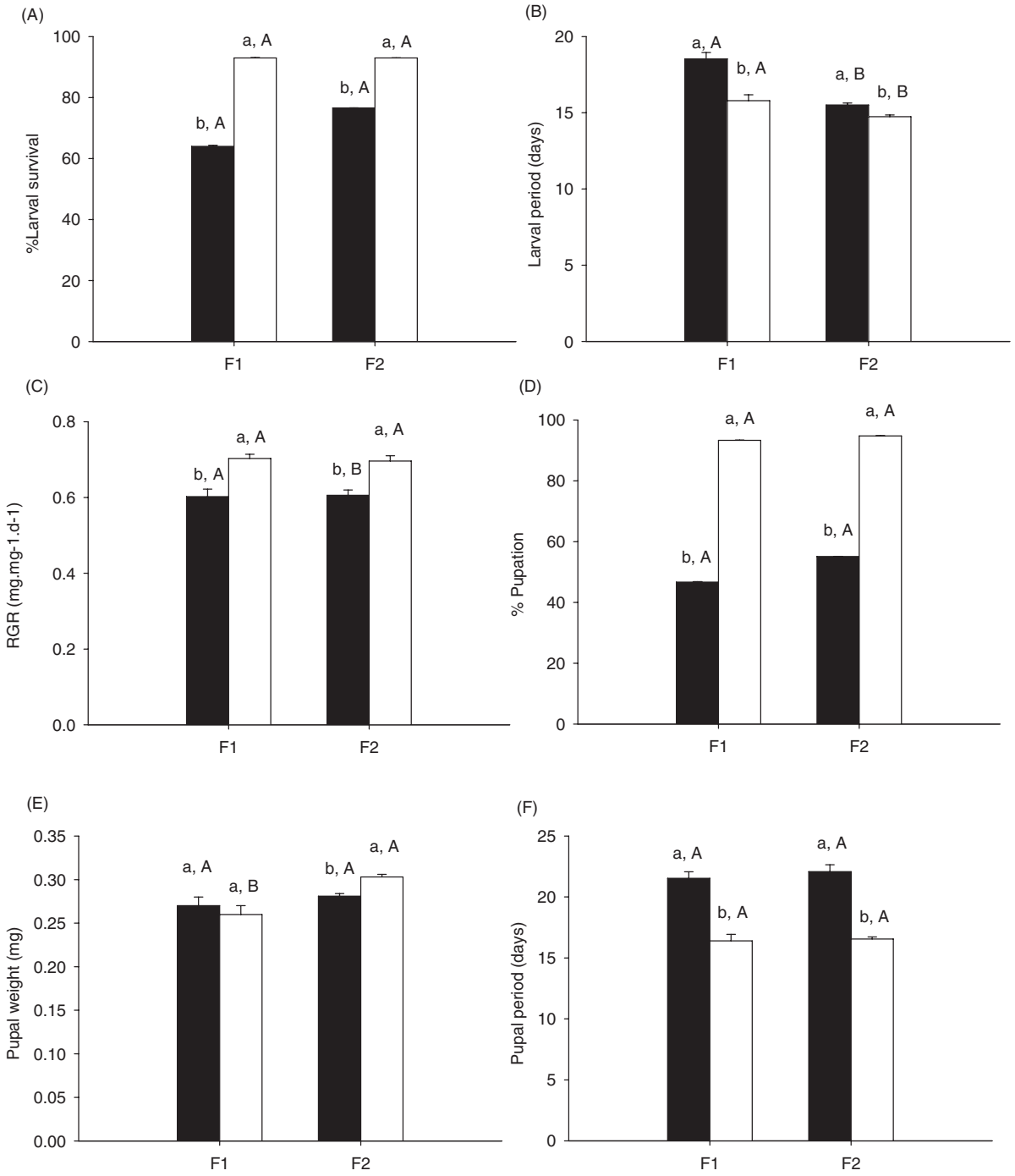

Fig. 2. Life history parameters of two successive generations of *Helicoverpa armigera* (mean ± SE). F1: first generation reared on *Acremonium strictum*-inoculated whole plants (E+; black bars) or non-inoculated plants (E−; white bars); F2: second generation reared on an artificial diet after hatching from eggs laid by females of the F1 generation (reared on E+ or E− plants; one female per treatment). (A) Per cent larval survival; (B) larval period (days); (C) relative growth rate (RGR) ($mg^{*}\, mg^{-1} {}^{*}\, d^{-1}$); (D) per cent pupation; (E) pupal weight (mg); (F) pupal period (days); (G) per cent adult emergence; (H) adult longevity (days); and (I) female fecundity (mean eggs/female). Different lowercases show significant difference between treatments within generations and different uppercases indicate significant difference within treatments across generations ($P < 0.05$; one-way ANOVA with Bonferroni's correction for multiple testing).

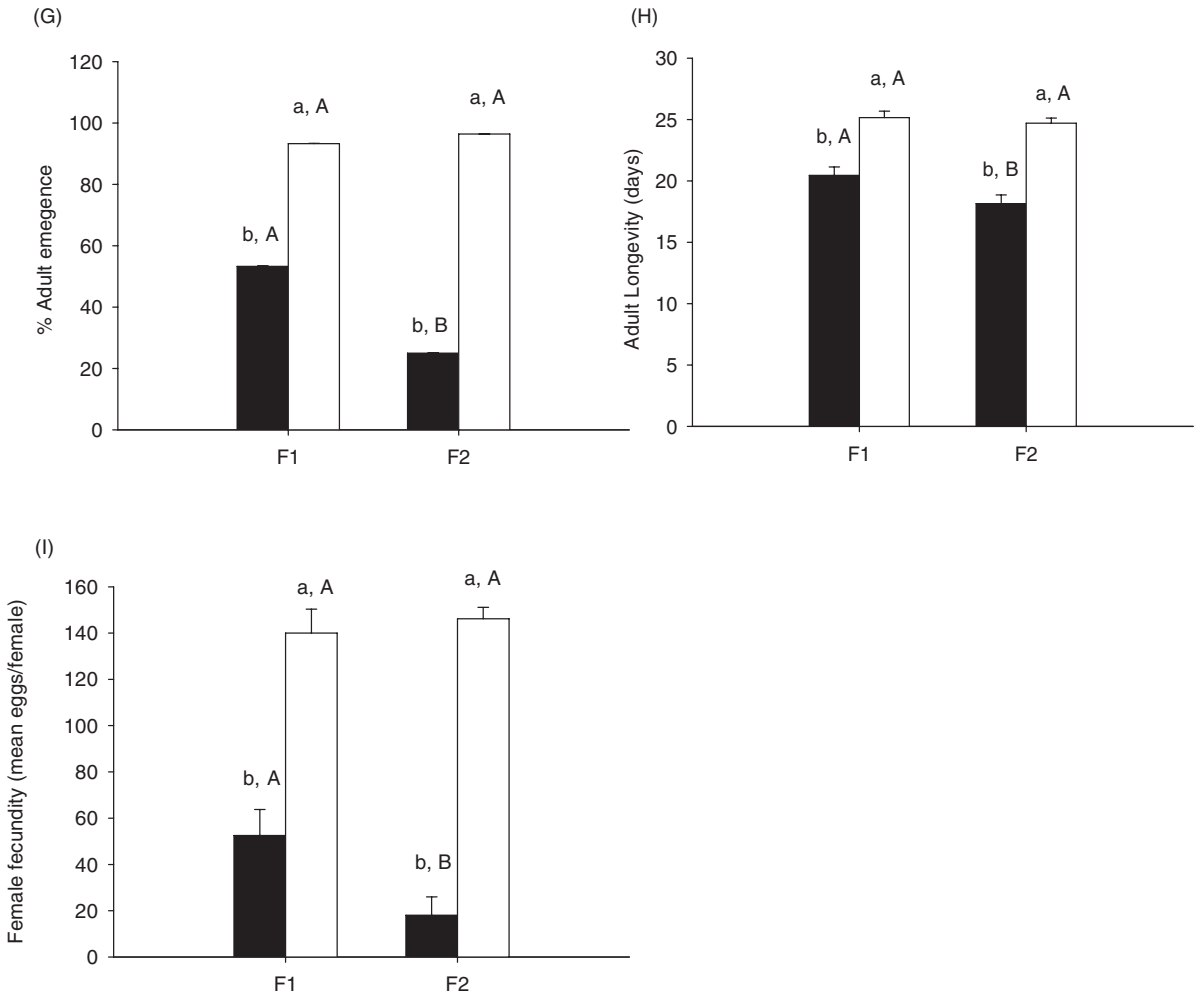

Fig. 2. Continued.

first documented evidence that endophyte-mediated negative effects on insect fitness depend on the experimental setting used in the investigation. Moreover, we demonstrate for the first time that these effects reach beyond insect individuals reared on the endophyte-infected plants, and may last across successive generations.

Acremonium strictum infection caused significant reductions in larval survival and growth rate, female longevity and fecundity, and a significant delay in moulting and eclosion of the *H. armigera* F1 generation. These endophyte-mediated negative effects were more evident when the larvae foraged freely on inoculated versus non-inoculated intact *V. faba* plants (i.e. the second experimental setting) in comparison to when offered leaf discs of inoculated or non-inoculated plants (i.e. the first experimental setting). Of interest, also, was the finding that significant differences in *H. armigera* fitness parameters between E+ and E− treatments found in the second experimental setting were not only a result of significant differences between larvae reared on E+ plants compared to leaf discs of E+ plants, but also to significant differences between those reared on E− plants as compared with leaf discs of E− plants. F2 generation larvae, reared on an artificial diet after hatching from eggs laid by females of the F1 generation reared on E+ plants, performed similarly to those of the F1 generation reared on intact plants. However, adult emergence, longevity, and female fecundity were further reduced in the F2 generation as compared with the F1 generation of *H. armigera* reared on E+ plants.

McGee (2002) reported that the presence of endophytes in cotton leaves was associated with reduced larval growth rate of *H. armigera*. In our study, however, *A. strictum* could not be re-isolated from *V. faba* leaves, even when the fungus was allowed time to grow within inoculated plants. Therefore, unlike a closely related species (i.e. *Acremonium alternatum*; Raps & Vidal, 1998), *A. strictum* colonisation is restricted to the *V. faba* root system and never spreads from below-ground

parts into the aerial plant parts. A direct contact between the endophyte (i.e. *A. strictum*) and the folivore (i.e. *H. armigera*) could thus be excluded. Alternatively, the possibility of translocation of *A. strictum*-derived products to the leaves that may have been interrupted by cutting out leaf discs, could account for the reduced insect fitness on inoculated plants as compared with leaf discs of inoculated plants. Production of inhibitors from the soil-borne *Acremonium spp*. has not been examined in any detail. Yet, two isolates of *A. strictum* were inhibiting the infection of leaves and leaf sheaths of rye grass (*Lolium perenne* L.) and an ornamental species of *Pennisetum* with pathogens (McGee *et al*., 1991). In that case, inhibition was related to compounds extracted by acetone from *A. strictum* cultures showing *in vitro* antibioses against three fungal pathogens. Interestingly, we found growth of some fungal pathogens in leaf discs sampled from E− plants, whereas none of the leaf discs sampled from E+ plants showed any outgrowth of fungal pathogens. We therefore speculate that the concentration of the inhibitor(s) may have been lower in detached leaf discs as compared with the concentration produced by the endophyte *in situ*, and thus translated into weaker effects against *H. armigera* larvae fed on leaf discs of inoculated plants in comparison to those fed on inoculated intact plants.

On the other hand, *H. armigera* is known to perform better on some plant parts than others, which is most likely due to factors such as shelter, nutrition, and attraction. In pigeon pea for example, larvae performed best (in terms of weight gain, developmental time, and survival) on pods, then flowers, and then leaves (Sison & Shanower, 1994), which were all available for the foraging larvae on intact whole plants. Moreover, there are reports regarding the attraction of *Helicoverpa* larvae to the volatiles emanating from plant surfaces and which play an important role in guiding them to their feeding sites. Interestingly, maceration (damage) was observed to affect the attraction of pigeon pea leaves for *H. armigera* neonate larvae; as whole leaves elicited significantly higher orientational responses of larvae than crushed leaves (Singh & Mullick, 2002). Therefore, cutting of leaf discs from E− intact plants might have caused the loss of such attractive volatiles (because of fast degradation), resulting in changes in larval feeding and performance, and consequently rendering the differences in fitness parameters of insects reared on leaf discs between the E+ and E− treatments hard to detect. We further suggest that such changes in larval feeding and performance on leaf discs of E− plants might also explain the large differences in all of the fitness parameters (except the prepupal period) of larvae reared on E− intact plants as compared with those reared on leaf discs cut from E− plants. Our assumption is in line with Haukioja (1980) who found that when leaves were mechanically damaged, their quality (as a food source for larvae) deteriorated within a few hours or days. He concluded that bioassays with detached plant materials may produce totally different results than tests with fresh growing intact plants.

As in some studies dealing with the unspecialized endophytes associated with woody and herbaceous plants, the exact mechanisms underlying the endophyte-based resistance to herbivory remain poorly understood, but are often attributed to indirect and complex factors (Faeth & Hammon, 1997; Jallow *et al*., 2004, 2008). *Acremonium strictum* negative effects on plant–herbivore interactions could also be due to an altered nutritional status of inoculated plants. Competition between an endophyte-induced sink in plant roots and the herbivore for resources essential for both organisms and supplied by their shared host plant (Raps & Vidal, 1998; L. Jaber, unpubl. data) could negatively affect the fitness of *H. armigera* larvae reared on E+ plants in comparison with E− plants. The negative effects of such nutritional competition are expected to be stronger in intact whole plants, on which both organisms (the fungus and insect) colonise different parts. In addition, changes in the overall content and composition of phytosterols (i.e. allelochemicals known to influence the feeding, growth, and development of insects) have been reported in *A. strictum*-inoculated tomato plants (Dugassa-Gobena *et al*., 1996) and may explain the reduced fitness parameters of *H. armigera* observed on E+ intact *V. faba* plants. Unlike some endophytes belonging to the same group (i.e. the highly-diversed horizontally-transmitted endophytes) that were reported to negatively impact plant growth (e.g. Schulz *et al*., 1998, 1999; Hashimoto & Hyakumachi, 2001), *A. strictum* did not alter several measures of *V. faba* fitness after inoculation (L.R.J., unpubl. data). If *A. strictum* had negative effects on plant fitness, then any endophyte-mediated detrimental effects on the herbivore might have been outweighed by this cost to the host plant. On the other hand, *A. strictum*-inoculated tomato plants were shown to release significantly less amounts of volatile compounds (but a similar volatile profile) and attract more *H. armigera* ovipositing females when compared to endophyte-free plants (Jallow *et al*., 2008). The increased oviposition preference of *H. armigera* moths on endophyte-infected plants might be an evolutionary adaptation to host plants with low amounts of volatile emissions, in order to escape egg predators or parasitoids using these volatiles as foraging cues for locating their prey (Dicke *et al*., 1990; Turlings *et al*., 1990; De Moraes *et al*., 1998). The hatching larvae feeding on endophyte-infected plants have yet to cope with the endophyte-triggered low nutritional quality of ingested food.

Albeit not quantitatively measured in the current study, food intake of F1 generation *H. armigera* larvae fed on E+ plants was apparently greater when compared to larvae fed on E− plants (L. Jaber, pers. observ.). Phytophagous insects feeding on plants with low nutritive quality show strong tendencies to compensate through increased consumption of plant tissues (Moran & Hamilton, 1980). Consequently, we suggest that larvae on E+ treatment had increased their intake, ostensibly to offset the inferior food quality and meet requirements for specific nutrients, and thus produced heavier pupae (although not significantly so) than those produced by larvae fed on E− treatment in both experimental settings. However, this marginal increase in F1 generation average pupal weight on E+ intact plants did not result in increased reproductive performance of the emergent adults in the F1 generation; neither did it result in improved performance of *H. armigera* individuals in the F2 generation. In contrast, larvae of the F1 generation fed on E− intact plants displayed a significant

further increase in average pupal weight and a maintained fitness in the F2 generation. Larval period was the only parameter showing a significant decrease across *H. armigera* generations within the E− treatment. This could be due to the standard artificial diet, on which insects develop faster (Teakle, 1991). Consistent with our findings, Jallow *et al*. (2004) found a significant increase in the relative consumption rate (RCR) of *H. armigera* larvae fed *A. strictum*-inocuated plants, and a significant decrease in the efficiency with which both ingested and digested food was converted to insect biomass. Therefore, we hypothesise that the reduction in fitness parameters of insects reared on E+ intact plants in the F1 generation may be caused indirectly via an endophyte-mediated reduction in plant tissue nutritional status, which had a significant long-term effect across *H. armigera* generations. Similar long-term detrimental effects of an endophyte-grass symbiosis were found on the food intake, growth rate, and especially the reproductive success of prairie voles. Ergot alkaloids (produced exclusively in endophyte-infected grass systems) were believed to be the primary agents responsible for these effects (Durham & Tannenbaum, 1998). Faeth and Hammon (1997), on the other hand, reported that the long-term survival and mass of lepidopteran leafmining larvae did not differ between larvae on control oak tree branches and those on branches with elevated infection levels of the horizontally-transmitted endophytic fungus *Asteromella sp*.

Several studies have shown a direct influence of larval food quality on the fitness components of herbivorous insects (e.g. Awmack & Leather, 2002; Moreau *et al*., 2006; Klemola *et al*., 2007). The possibility that such nutrition-based variations in herbivore fitness could be passed on to subsequent generations (as suggested by our results) has, however, never been demonstrated and merits further investigation. It is not clear how the endophyte-triggered low nutritional quality of ingested food by *H. armigera* F1 generation was carried over into the F2 generation in our study. Sequestration of several classes of plant secondary metabolites is known among many lepidopteran species (Nishida, 2002). Conceivably, there might have been a feedback interaction between a poorer quality of E+ plants and a larger consumption of possible allelochemicals or secondary plant metabolites, that if sequestered to the adult stage, could account for such cross-generational effects. Alternatively, the performance of *H. armigera* in the F2 generation might be due to a genotype rather than a treatment effect, as the hatchlings used in this experiment were obtained from only one female (F1 generation) per treatment. Choosing a few hatchlings from many females of each treatment would have certainly offered a more decisive effect. The advent of metabolomic techniques (i.e. techniques to investigate changes in the whole plant metabolome) should, on the other hand, allow researchers to assess the relative contributions of endophyte-mediated changes in nutrients and toxins on insect performance (Hartley & Gange, 2009) and hence offer new insights into the mechanisms underpinning the long-term endophyte–host interactions.

Our results have important conceptual and practical implications. First, studies conducted under very restricted one set conditions for a very short time, and thus failing to demonstrate an impact of endophytes on plant–herbivore interactions, should be revisited. Also, results emerging from studies using a highly controlled organism system (such as ours) might not extend to native species under natural conditions. Artificial greenhouse and growth chamber conditions used in most of these studies may not capture essential factors influencing endophyte–host interactions in the field (e.g. the variable colonisation of plants by different combinations of micorrhizal and endophytic fungi). Such factors might obscure the interactions in field populations, even when occurring at small spatial scales. However given our results prove general under field conditions, endophytes may not only have strong impacts on plant–herbivore interactions, but also on multitrophic assemblages. Finally, more work should be carried out to identify secondary metabolites (e.g. *A. strictum*-derived inhibitory compounds) potentially produced by fungal endophytes in pure cultures and inoculated plant tissues.

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