

**The effects of the root endophytic fungus *Acremonium strictum* on plant-herbivore interactions**

**Dissertation**

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*In the name of God, Most Gracious, Most Merciful*

*To Dad*

*to his spirit that resides in mine and inspires me to aim high all the time*

*&*

*Mom*

*my best friend who made it all possible*

*Hope I make you proud every single day..*

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## Summary

The widespread occurrence of endophytic fungi in virtually all plant species has prompted an increasing number of investigations into the ecological significance of these cryptic microorganisms as mediators of plant-herbivore interactions. In my studies, I investigated the role of the fungal endophyte *Acremonium strictum* Gams, restricted to the roots of the extrafloral (EF) nectary-bearing broad bean plant *Vicia faba* L., in induction of EF-mediated defences and reduction of herbivory. In the first experiment, I manipulated the presence/absence of *A. strictum* in plant roots and inflicted *Aphis fabae* damage at a specific time and location in order to examine whether the endophyte colonization would induce the EF-mediated indirect defences in response to herbivory. Separately, the endophyte colonization and the herbivore infestation induced the production of two EF traits (EF nectar volume and EF nectary number). On the other hand, both EF traits were significantly reduced in plants simultaneously colonized with the endophyte and infested with the herbivore; which was predicted (from a cost/benefit perspective) as a trade-off between EF- and endophyte-mediated defences.

In a subsequent experiment, these interactions were examined under variable levels of nutrient availability. Following herbivory, the level of variation in EF nectar and nectary in the absence of endophyte infection was only slightly affected by nutrient addition; whereas these EF rewards responded to nutrient addition in a more complex way in endophyte-infected plants depending on herbivore damage. Also, increasing nutrient supply increased the extent of root colonization with *A. strictum* and alleviated the negative effects of herbivory on plant fitness in both endophyte-infected and endophyte-free plants. Several measured parameters of the insect fitness were improved by nutrient addition on endophyte-free plants, but were less responsive on endophyte-infected plants. Results from this part suggest that plants regulate multiple mutualisms (i.e. EF- and endophyte-mediated mutualisms) in response to variation in resource availability so as to attain a favourable cost/benefit ratio.

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Finally, experiments were conducted to examine whether endophyte effects on herbivory would depend on the experimental setting used in investigation and whether they would translate into a subsequent generation of the herbivore. *A. strictum* negative effects on the fitness of *Helicoverpa armigera* first generation were more evident when the larvae foraged freely on inoculated intact whole plants than when offered leaf discs of inoculated plants, and these endophyte-mediated negative effects were carried over into the herbivore second generation. A 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 to intact plants, regardless of infection status. Furthermore, the reduction in fitness parameters of the herbivore across two generations might have been due to the endophyte-triggered reduction in plant quality.

Results from these studies should have far-reaching conceptual and practical implications for future endophyte research and should also set the stage for a better understanding of the context under which organisms interact, adapt, and evolve.

## Introduction

Most plant species associate with microbial symbionts (such as mycorrhizal fungi, nitrogen-fixing bacteria, and fungal endophytes; Smith & Read, 1997; Bacon & White, 2000) which are increasingly recognized for their potential to influence how their host plants respond to environmental stresses, including herbivory (Rudgers *et al.*, 2009; and references therein). The ability or not of fungal endophytes to protect their host plants from herbivory has become a focus for debate among plant-herbivore ecologists (Clay, 1997; Saikkonen *et al.*, 1998). Endophytic fungi (*sensu* Wilson, 1995) have been isolated from all plants studied to date (Hyde & Soyong, 2008). They are generally categorized as clavicipitaceous (C-endophytes) and nonclavicipitaceous endophytes (NC-endophytes; see Rodriguez *et al.*, 2009). Clavicipitaceous endophytes are vertically-transmitted and systemically colonizing the aboveground parts of grasses, and are best known for their ability to produce alkaloidal mycotoxins that deter or sicken herbivores (Clay, 1992; Breen, 1994). These grass endophytes may also benefit their host plants by increasing germination success and plant competitive abilities (Clay, 1992), in addition to ameliorating the negative effects of drought stress (Kannadan & Rudgers, 2008). Whereas the clavicipitaceous endophytes in grasses and their functions are generally thoroughly investigated and well understood, much less work has been done on the roles of the more ubiquitous nonclavicipitaceous endophytes inhabiting non-grass host plants (Hyde & Soyong, 2008).

The great abundance and diversity of the unspecialized horizontally-transmitted nonclavicipitaceous fungal endophytes in woody and herbaceous plants (Petrini, 1986; Petrini *et al.*, 1992) provide the potential for a wide variety of direct (via mycotoxins; e.g. Findlay *et al.*, 2003) and indirect (by altering the host plant; e.g. Gaylord *et al.*, 1996; Preszler *et al.*, 1996; Faeth & Hammon, 1997; Raps & Vidal, 1998) interactions between plants and herbivores. In addition to their role in increasing resistance to herbivores, nonclavicipitaceous endophytes have also been implicated in increased disease resistance (e.g. Arnold *et al.*,

2003), increased abiotic stress tolerance (e.g. Rodriguez *et al.*, 2008), and enhancement of plant growth (e.g. Ernst *et al.*, 2003). However, the generality of mutualism between this group of fungal endophytes and their non-grass host plants has been questioned because of inconsistent results from some studies (e.g. Gange, 1996; Faeth & Hammon, 1997; Sieber, 2007). As compared to the clavicipitaceous endophytes in grasses which are generally considered as plant mutualists (Cheplick & Clay, 1988; Clay, 1992; Clay *et al.*, 1993), there are three main hypothesis regarding the roles of the nonclavicipitaceous endophytes: (1) that they are neutral inhabitants, (2) parasites, or (3) mutualists of their hosts (Arnold, 2008). Given their tremendous phylogenetic diversity (Rodriguez *et al.*, 2009), the capacity of this group of endophytes to play each of these roles or to change roles overtime and under certain circumstances comes as a little surprise.

In response to attack by many different species of herbivore during their lifetimes, plants have evolved an enormous variety of *direct* (operating directly on herbivores) and *indirect* (operating via attracting natural enemies of herbivores) defence strategies (Price *et al.*, 1980). The costs of these anti-herbivore defences, which are central to *the optimal defence theory* for plant-herbivore interactions (see Mckey, 1974, 1979; Rhoades, 1979), also provide the basis for other ecological and evolutionary theories concerning plant allocation of limited resources that when used for defence would not be available for growth and reproduction (e.g. Feeny, 1976; Rhoades, 1979; Coley *et al.*, 1985; Simms & Fritz, 1990; Herms & Mattson, 1992). From an evolutionary perspective, any organism should respond to the resulting trade-off in a way that maximizes fitness (i.e. reducing costs and increasing benefits). One example for such an evolutionary optimization response is the evolution of herbivore-induced plant defences, which is generally regarded as a cost saving strategy by expressing defences only when they are needed (see Karban & Baldwin, 1997; Cipollini *et al.*, 2003; Dicke & Hilker, 2003). Particularly common in nature is a form of inducible indirect defence that entails extrafloral (EF) nectary resources and comprises mutualistic interactions with natural enemies (mainly

ants) that defend plants against herbivores (Davidson & Mckey, 1993; Heil *et al.*, 2001; Holland *et al.*, 2009). EF nectaries are secretory glands occurring on shoots, petioles, stipules, and leaves of plants belonging to at least 330 genera among 93 families (Koptur, 1992). Despite an ever-increasing number of studies demonstrating the important role EF nectaries serve in reducing herbivory rates in nature (reviewed in Heil, 2008); we are only beginning to understand the investment costs in EF-mediated defences, including how common induction of EF nectar and nectaries is among plants (Holland *et al.*, 2009). Besides, even though there is an enormous potential for interactions between endophytes (as frequent inhabitants of plants) and the widespread EF rewards, there has been no experimental manipulation of endophytic colonization in EF nectary-bearing plants to examine these interactions.

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## Objectives

I conducted a series of greenhouse experiments in order to explore the role of the fungal endophyte *Acremonium strictum* Gams, restricted to the roots of the EF nectary-bearing broad bean plant *Vicia faba* L., in the induction of EF-mediated defences and reduction of herbivory. In this context, the objectives of this dissertation are three-fold:

1. to synthesize a *first-time* knowledge regarding the interactions between endophytes, herbivores, and extrafloral nectary-mediated defences (**Chapter I**)
2. to examine how these interactions are expressed under variable levels of nutrient availability (**Chapter II**)
3. to highlight two important findings for future endophyte research; i.e. the effects of experimental design and setting on endophyte-plant-herbivore interactions as well as the little-known long-term endophyte-mediated effects on plant-herbivore interactions (**Chapter III**)

**Interactions between an endophytic fungus, aphids, and extrafloral nectaries:  
do endophytes induce extrafloral-mediated defences in *Vicia faba*?**

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## Summary

1. There is increasing evidence that extrafloral nectaries, described in approximately 1000 plant species from more than 90 families, have a defensive function. Endophytic fungi are an important group of microorganisms asymptotically colonizing host plants, and promoting their defences against natural enemies. We aimed at investigating the role of these microorganisms in inducing extrafloral nectary defences in plants against herbivory.
2. We conducted a full factorial experiment to study the effects of a soil-borne endophytic fungus, *Acremonium strictum*, alone or in combination with the aphid, *Aphis fabae*, on the production of extrafloral (EF) nectar and nectaries in broad beans. By manipulating the presence/absence of the fungus in the roots of the host plants and by inflicting herbivore damage at a specific time and location, we tested the hypothesis that endophyte inoculation induces EF-mediated indirect defences. The quantity of EF nectar production and the number of EF nectaries produced were assessed by repeated samplings at fixed intervals.
3. Endophytic inoculation of bean plants induced a significant short-term increase in total EF nectar production and a significant prompt increase in number of EF nectaries per expanded leaf. On the other hand, aphid infestation resulted in a prolonged increase in total EF nectar production and a delayed induction of EF nectaries. Conversely, when plants were simultaneously inoculated with the endophyte and infested with aphids, both EF traits were significantly reduced.
4. The effect of endophyte inoculation was further examined by recording the life history traits of *A. fabae*. Aphid performance was generally lower on inoculated plants; however, relative fecundity was the only fitness parameter significantly reduced on endophyte inoculated plants.
5. The organism model in the present study serves as a model for investigating how endophytic colonization alters the response of EF nectary traits to herbivory. From a cost/benefit perspective, variable responses in EF-mediated indirect defences

as influenced by endophytes could be explained as trade-offs in defence. In addition, other possibilities that may have contributed to the EF response patterns reported in this study are discussed.

*Key-words:* *Aphis fabae*, cost/benefit framework, extrafloral-mediated defences, fungal endophytes, mutualism, trade-offs in defence.

## **Introduction**

Plants have evolved a suite of morphological and chemical adaptations to protect themselves against herbivory. Such adaptations are manifested in either a direct or an indirect form. Direct defences, by definition, have a direct negative impact on herbivores and include trichomes, spines, and a high diversity of secondary plant metabolites (Karban & Baldwin 1997). On the other hand, indirect defences are those plant attributes that have a positive impact on the natural enemies of herbivores (Price *et al.* 1980) and encompass herbivory-induced plant volatiles (Agrawal 1998), domatia (Walter 1996), and nutritional supplements (food bodies and extrafloral nectaries, henceforth referred to as EF nectaries) (Koptur 1989), among others. EF nectaries have been described in approximately 1000 plant species ranging over 93 families (Koptur 1992). There is increasing evidence for the defensive function of these nectar secreting glands (Bently 1977; Koptur 1992; Heil *et al.* 2001). They are generally thought to be catering for ants (Bently 1977), but they may also help sustaining other predators (Wooley *et al.* 2007) and parasitoids (Röse, Lewis & Tumlinson 2006). EF nectar-tracking ants (Stephenson 1982) and nectar-satiated parasitoids (Röse *et al.* 2006) stay longer in herbivore-occupied patches and attack more herbivores, suggesting that plants with increased EF nectar production could attract or retain more "bodyguards", thereby receiving greater protection against herbivores (Ness 2003). Reductions in herbivory have been associated with increased production of EF nectar in several plant species. This has been demonstrated in *Vicia sativa* (L.) (Koptur 1989), *Ricinus communis* (L.), *Gossypium herbaceum* (L.) (Wäckers *et al.* 2001), and *Phaseolus lunatus* (L.) (Heil 2004). In addition,

researchers have reported an increase in the overall number of EF nectaries following artificial leaf damage (Mondor & Addicott 2003; Mondor, Tremblay & Messing 2006; Pulice & Packer 2008).

Though the potential role of multispecies interactions in shaping the evolution of EF nectaries has been demonstrated (Rudgers & Gardener 2004), selection acting on EF nectary traits may extend beyond the simple mutualism via the tri-trophic food chain of plants-herbivores-enemies and involve mutualistic associations with microorganisms harboured by the plant. Effects derived from this different type of mutualism, in which plants are frequent partners (Barbosa, Krischik & Jones 1991), are still unclear (but see Laird & Addicott 2007).

Endophytic fungi are an important, yet relatively unexplored group of microorganisms asymptotically colonizing plants (Wilson 1995). Their interactions with host plants occur along a continuum and range from parasitic to mutualistic (Schulz & Boyle 2005). Although many studies have focused on the role these endophytic organisms play in increasing host resistance to herbivores (Carroll 1988, 1991; Clay 1988; Faeth 2002) and pathogens (Giménez *et al.* 2007), the view of endophytes as defensive mutualists has mainly stemmed from studies of seed-borne fungal endophytes benefiting their grass hosts as “acquired plant defences” (Cheplick & Clay 1988). In contrast to this unique and less frequent group of clavicipitaceous endophytes (see Clay 1988 and Breen 1994 for more details), the non-clavicipitaceous endophytic fungi are much more diverse and colonize a wide variety of plant tissues in virtually every host plant examined to date (reviewed by Schulz & Boyle 2005; Zhang, Song & Tan 2006). These horizontally transmitted endophytes, mostly allied with Ascomycetes (Carroll 1991), are thought to promote “inducible defences” as proposed by Carroll (1988, 1991). Regardless of which group they belong to, the role of fungal endophytes in plant-insect and plant-pathogen interactions is receiving increasing attention because of their potential use in pest control (Giménez *et al.* 2007; Backman & Sikora 2008; Kuldau & Bacon 2008; Mejia *et al.* 2008; Vega *et al.* 2008).

Fungal endophytes belonging to the genus *Acremonium* are among the unspecialized, widespread soil-borne fungi that are horizontally transmitted via spores and form less

intimate associations with their host plants (Gams 1991). Endophytes of this genus, which are predominantly restricted to the root systems of host plants, significantly influence plant-insect relationships (Vidal 1996; Dugassa-Gobena, Raps & Vidal 1998; Raps & Vidal 1998; Jallow, Dugassa-Gobena & Vidal 2004).

Broad beans (*Vicia faba* L., Fabaceae) sometimes produce one, but most often zero or two EF nectaries per leaf pair. These large, dark purple nectaries are located on the light-green stipules at the base of leaf petioles (Mondor & Addicott 2003). *Vicia faba* is also a common secondary host for the black bean aphid, *Aphis fabae* Scopoli (subsp. *fabae*) (Homoptera: Aphididae) (Dixon 1977). By manipulating the presence/absence of the soil-borne endophytic fungus *Acremonium strictum* in *V. faba* roots and inflicting aphid damage at a specific time and location, we tested the hypothesis that endophytic inoculation induces EF-mediated indirect defences by altering EF rewards. The present study is the first to simultaneously determine variable responses in both EF nectar and nectary traits. We followed the temporal patterns of EF nectar production and the number of EF nectaries by repeated sampling at fixed intervals. Furthermore, the effect of the endophyte inoculation on aphid life history traits was investigated.

## **Materials and methods**

### PLANTS, INSECTS, AND FUNGAL CULTIVATION

Broad bean seedlings (cultivar Hangdown Grünkernig, Gevo GmbH, NORTMOOR/OSTFR.) were grown in a greenhouse chamber. Two-week-old plants were individually transplanted into plastic pots (11 cm diameter) containing a mixture of soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta) and sand (4:1 ratio). Plants were irrigated regularly and fertilized once each week with NPKMg (15:10:15:2, COMPO GmbH, Münster).

Several adult females of *A. fabae* were collected from a permanent stock culture and reared for two parthenogenetic generations on young uninfested *V. faba* plants in a growth chamber at 20°C, 65±5% RH, and a photoperiod of 16L: 8D. Synchronized virginoparae (max. 24 h after imaginal moult) were used for the experiments.

A strain of *A. strictum* from DSMZ-GmbH, Braunschweig, was maintained in the laboratory on 0.3% malt extract agar (MEA). A spore suspension was prepared by adding a piece of malt extract agar containing fungus mycelia to an autoclaved 0.3% malt extract broth (same as MEA but without agar). This liquid culture was kept on a shaker at 23°C and 100 RPM for 12 days to ensure fungal sporulation. After vacuum filtering, the spore concentration in a drop of the culture was measured under the microscope in a Thoma counting chamber (64 × 0.025 mm<sup>2</sup>, chamber height 0.1 mm).

#### EXPERIMENTAL SET-UP AND DESIGN

Five days after transplanting, half the plants were watered with 50 ml of a spore suspension containing 10<sup>6</sup> *A. strictum* spores/ml, and the remaining control plants were watered with the same volume of the culture filtrate, which was fungus free. Five days post-inoculation, single plant replicates of inoculated and non-inoculated plants near the five-leaf stage were used in all experiments. Experiments were planned with two main factors in a full-factorial, repeated-measures design. The first factor (endophyte) was the inoculation of selected plants with *A. strictum* with two levels; inoculated (E+) and non-inoculated (E-). The second factor (aphid) was *A. fabae* infestation and also had two levels; *A. fabae*-infested (A+) and *A. fabae*-free (A-). Thus, four treatment combinations (E+A+, E+A-, E-A+, and E-A-) were produced, with ten individual plants randomly assigned to each. At the start of the experiments (day 0), a clip-on cage (3.5 cm diameter) was attached to the third leaf of all (E+A+) and (E-A+) plants. Ten virginoparae were confined to each clip-on cage and allowed to deposit nymphs. Fourteen hours later (1800-0800), all mother aphids were removed, leaving twenty newly born nymphs per clip-on cage. All experiments were carried out in a controlled environment at 20±2°C, 50±10% RH, and a 16L: 8D photoperiod.

#### EF NECTAR PRODUCTION (*Temporal dynamics of total EF nectar production*)

EF nectar per leaf pair was collected, using 5-µl micropipettes with 1-µl divisions, and then combined to permit determination of the total EF nectar production per plant. Collection of EF

nectar was carried out at 48 h intervals, starting on the day aphid damage was inflicted (day 0). On this day, nectar was collected just before application of the clip-on cages. Nectar was collected until day 10, thereby creating a repeated measures factor (i.e., date).

#### NUMBERS OF EF NECTARIES AND PLANT GROWTH PARAMETERS

Plant height, number of expanded leaf pairs, number of immature leaf pairs, and number of EF nectary pairs on each plant were recorded before attaching the clip-on cages on day 0. Seven and 10 days later, the same plant traits were assessed. Pre-treatment values were then subtracted from post-treatment values to quantify the change ( $\Delta$  change) in each character.

#### LIFE HISTORY TRAITS of *A. fabae*

The effect of *A. strictum* inoculation on *A. fabae* fitness was examined by following the life history traits of the twenty nymphs. Individuals were monitored daily and removed once they reached adulthood, leaving a single adult per clip-on cage for evaluation of fecundity. The birth weight ( $W_b$ ), adult weight ( $W_{ad}$ ), development period (number of days from birth to beginning of first reproduction) ( $d$ ), relative fecundity (number of offspring produced per day for ten days) ( $RF$ ), and mortality percentage ( $M\%$ ) were recorded. From these data, the intrinsic rate of natural increase ( $r_m$ ) was calculated using the formula of Wyatt & White (1977);

$$r_m = (0.738 \times \ln(Md)) / d$$

Where  $d$  is the development period and  $Md$  is the number of nymphs born in the period from  $d$  to  $2d$  from birth. The relative growth rate ( $RGR$ ) was also calculated using the equation of Scriber & Slansky (1981);

$$RGR = \Delta W / (W_x \times d)$$

Where  $\Delta W$  is the weight gained (adult weight – birth weight),  $W_x$  is the mean of adult weight plus birth weight divided by 2, and  $d$  is the development period.

#### ESTABLISHMENT OF *A. strictum*

At the end of the experiment, four to five plants were randomly selected from each of the four treatments. Roots of these plants were thoroughly washed, frozen in liquid nitrogen, and temporarily stored at -20°C until colonization by the endophyte was determined. Endophyte colonization (presence/absence) was determined for each treatment by real-time polymerase chain reaction (RT-PCR). To extract endophyte DNA, root samples were thawed and pulverized to fine powder in liquid nitrogen using a pestle and mortar. Root powder (100 mg) was then dispersed in 1 ml CTAB buffer containing 2 µl mercaptoethanol and 1µl proteinase K, following a variant of the CTAB method (Murray & Thompson 1980) simplified by Stewart & Via (1993) and modified by Brandfass & Karlovsky (2006). Following DNA extraction, RT-PCR was run to amplify and quantify the fungal colonization in the roots of *A. strictum*-inoculated and non-inoculated plants.

#### STATISTICAL ANALYSES

The RT-PCR data (quantity of *A. strictum* DNA extracted from roots) were analyzed using one-way ANOVA after checking the assumptions for normality and homogeneity of variance. A post hoc test was then performed using Tukey's Honestly-Significant-Difference to identify which differences were significant. As previous studies (e.g. Wäckers & Wunderlin 1999; Wäckers *et al.* 2001; Laird & Addicott 2007) showed that date has a significant effect in inducing EF nectary traits, repeated-measures two-way ANOVA was performed to analyze the temporal dynamics of EF nectar production with endophyte inoculation and aphid infestation as the main factors. ANOVA planned comparison test (orthogonal contrast) was then used to compare average nectar production among treatments within each sampling date. Bonferoni adjustment was carried out to correct for the  $\alpha$ -level in case of multiple comparisons. To determine the effect of the treatments on the number of EF nectaries, the change in number of EF nectary pairs per change in number of expanded leaf pairs was used as the dependant variable (i.e.,  $\Delta\text{EFnectary} / \Delta\text{ExpLvs}$ ). This variable directly assesses the trade-off between the plant's physiological investment (nectary production) and the area

to be defended (number of expanded leaves). Repeated-measures two-way ANCOVA was carried out to control for variation in the dependant variable that is associated with plant growth correlates by removing this variation from the error variance and thus making true differences in EF-mediated responses due to the treatments easier to detect (Steel & Torrie 1980). A linear regression model was used to test the correlation between the change in EFN nectary numbers and the change in the other plant characters. The following three covariates were included simultaneously: change in plant height, change in number of expanded leaf pairs, and change in number of immature leaf pairs. Average change in EF nectary number among treatments within each sampling date was compared using ANOVA planned contrasts with Bonferoni adjustment. Sets of one-way ANOVA were used for *A. fabae* life history traits, except repeated-measures one-way ANOVA was used for the relative fecundity (*RF*). All analyses were carried out using SYSTAT for Windows, version 12 (SYSTAT 2008).

## **Results**

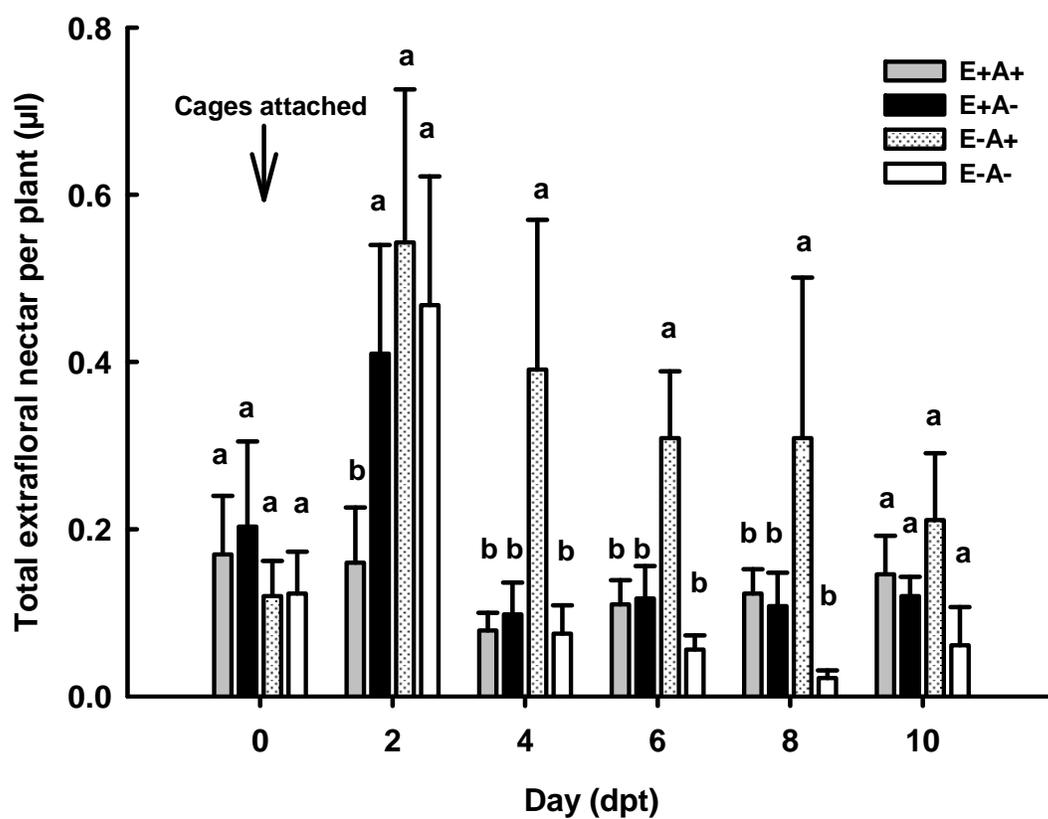
### ESTABLISHMENT OF *A. strictum*

Quantification of fungal colonization in different root zones using RT-PCR confirmed that *A. strictum* growth was significantly restricted to roots of inoculated plants ( $F_{3,36} = 13.163$ ;  $P < 0.000$ ; one-way ANOVA). All inoculated *V. faba* plants were successfully colonized by the endophyte, whereas non-inoculated plants were endophyte-free.

### EF NECTAR PRODUCTION (*Temporal dynamics of total EF nectar production*)

*Vicia faba* plants assigned to different treatments did not differ in baseline EF nectar production before being fed upon by *A. fabae*. Within 48h of the onset of feeding by the aphids, total nectar production per plant significantly increased in all treatments except in the treatment combining *A. fabae* infestation and *A. strictum* inoculation (E+A+), where EF nectar production was significantly reduced ( $F_{1,32} = 9.461$ ;  $P < 0.004$ ; repeated-measures two-way ANOVA) (Fig. 1). Repeated-measures two-way ANOVA showed a significant effect of sampling date ( $F_{1,32} = 4.976$ ;  $P < 0.033$ ). The induced increase in nectar production was

found only for a short period in *A. strictum*-inoculated plants (E+A-) ( $F_{1,32} = 4.959$ ;  $P < 0.038$ ). On the other hand, aphid infestation (E-A+) significantly prolonged the increase in total EF nectar production ( $F_{1,32} = 4.672$ ;  $P < 0.033$ ). Significant differences were found between E-A+ plants and the remaining treatments on 4 days post aphid damage ( $F_{1,32} = 4.788$ ;  $P < 0.036$ ; ANOVA planned contrast test with Bonferoni adjustment). From this day onwards, increased nectar production persisted solely in *A. fabae*-infested plants, while nectar secreted by plants in all other treatments decreased to below constitutive levels prior to inflicting the aphid damage (Fig. 1).



**Fig. 1** Total EF nectar production (mean  $\pm$  SE) per *V. faba* plant measured at 48 h intervals. Clip-on cages containing the aphid *A. fabae* were applied after EF nectar was measured on day 0 and removed 14 hours later (dpt= days past treatment with aphids). Different letters above columns indicate significant differences among treatments ( $P \leq 0.05$ ; planned contrast test with Bonferoni adjustment after repeated-measures two-way ANOVA).

## EF NECTARY NUMBERS AND PLANT GROWTH PARAMETERS

Two covariates significantly influenced EF nectary number. The change in EF nectary production was significantly and positively associated with the change in height (linear regression model;  $F_{1,28} = 4.546$ ;  $P < 0.040$ ) and with the change in numbers of immature leaf pairs ( $F_{1,28} = 12.771$ ;  $P < 0.001$ ). However, owing to the absence of a correlation with the change in numbers of expanded leaf pairs ( $F_{1,28} = 1.040$ ;  $P = 0.314$ ), this covariate was removed before running the final analyses (Table 1).

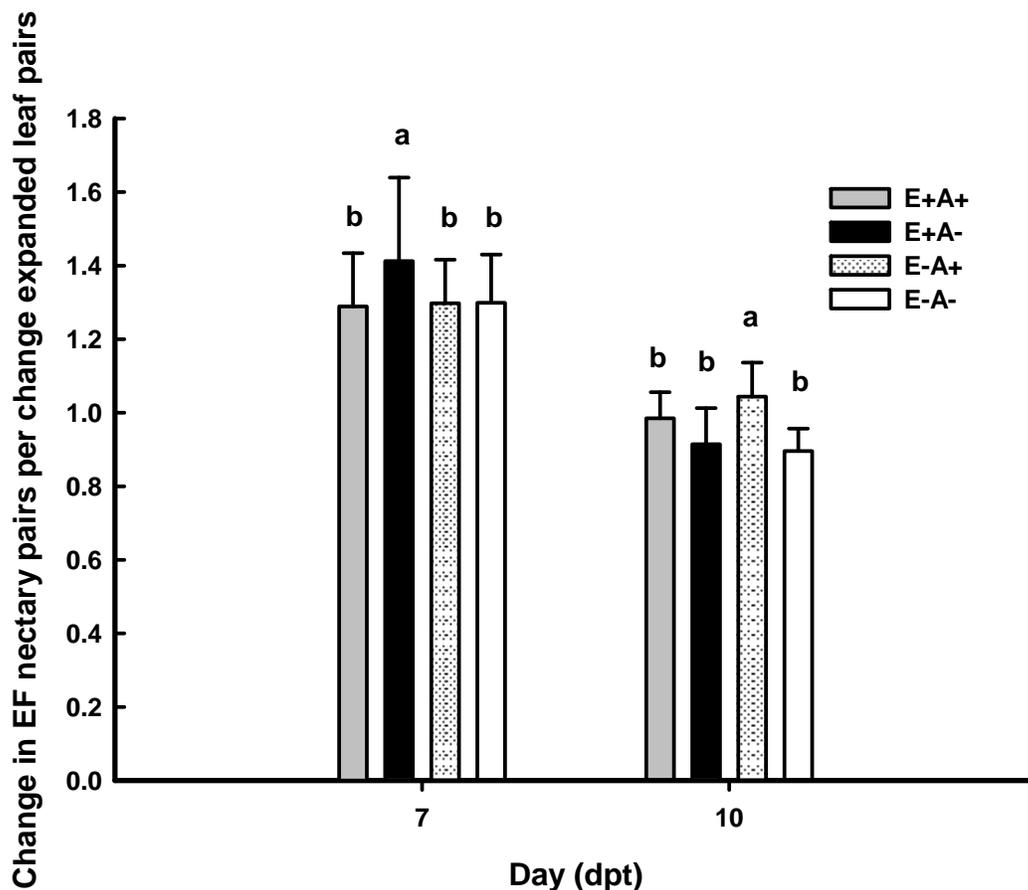
**Table 1.** Repeated-measures ANCOVA of the effects of endophyte inoculation and/or aphid infestation on the change in number of EF nectary pairs per the change in number of expanded leaf pairs in *V. faba*. ( $\Delta$  indicates the degree of change in the trait over 7 and 10 days following aphid infestation)

Source of variation	$F^a$	$P$
Endophyte	0.006	0.937
Aphid	0.021	0.885
Date	3.558	0.070
Endophyte×Aphid	0.301	0.588
Endophyte×Date	<b>8.550</b>	<b>&lt; 0.007</b>
Aphid×Date	2.952	0.097
Endophyte×Aphid×Date	0.077	0.784
$\Delta$ in Plant Height (7dpt)	0.014	0.906
$\Delta$ in Plant Height (10dpt)	0.231	0.635
$\Delta$ in Immature Leaves (7dpt)	0.277	0.603
$\Delta$ in Immature Leaves (10dpt)	0.299	0.589

<sup>a</sup>  $F$  value with 1 and 28 degrees of freedom

Plants produced significantly more EF nectaries (i.e.,  $\Delta EF_{nectary} / \Delta ExpLvs$ ) only in response to *A. strictum* inoculation (E+A-) ( $F_{1,28} = 8.550$ ;  $P < 0.007$ ; repeated-measures two-way ANCOVA) (Table 1; Fig. 2). Most interestingly, however, endophyte inoculation was only significant when date was involved (Table 1). Seven days after aphid introduction, *A. fabae* feeding did not increase EF nectary numbers in *A. strictum*-inoculated (E+A+) ( $F_{1,28} = 0.301$ ;  $P = 0.588$ ) or in *A. strictum*-free plants (E-A+) ( $F_{1,28} = 0.021$ ;  $P = 0.885$ ). However, 10 days following feeding by aphids, the rate by which plants produced EF nectaries was significantly increased in *A. fabae*-infested, non-inoculated plants (E-A+) ( $F_{1,28} = 7.432$ ;  $P < 0.011$ ; within

treatment effect) and significantly decreased in *A. strictum*-inoculated, *A. fabae*-free plants (E+A-) ( $F_{1,28} = 5.077$ ;  $P < 0.032$ ; within treatment effect) (Fig. 2).



**Fig. 2** Change in number of EF nectary pairs per change in number of expanded leaf pairs ( $\Delta\text{EFnectary} / \Delta\text{ExpLvs}$ ) (mean  $\pm$  SE) in *V. faba* over 7 and 10 days following aphid infestation (dpt= days past treatment with aphids). Different letters above columns indicate significant differences among treatments ( $P \leq 0.05$ ; planned contrast test with Bonferoni adjustment after repeated-measures two-way ANOVA).

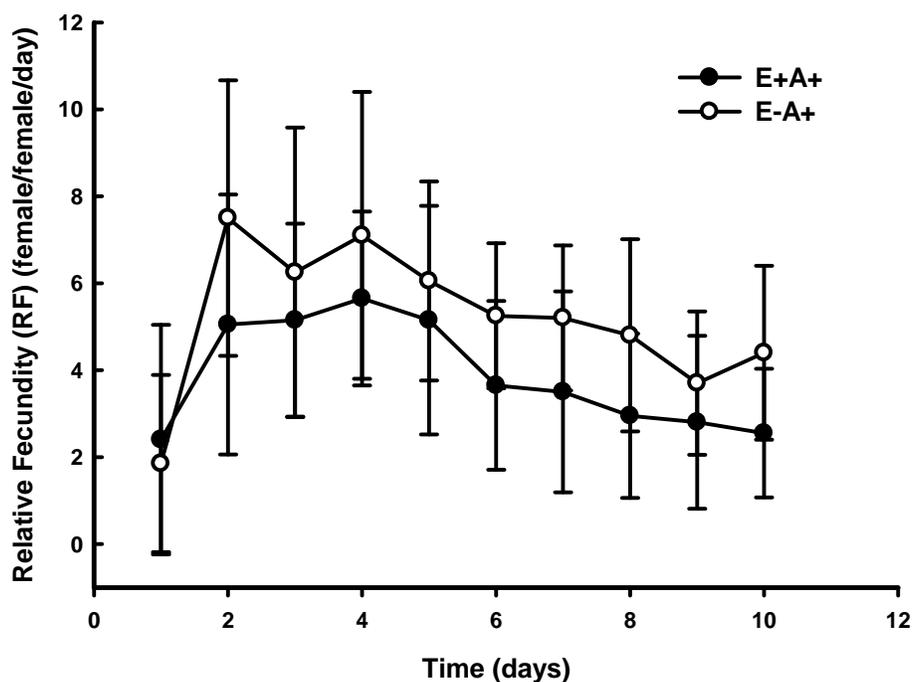
#### LIFE HISTORY TRAITS OF *A. fabae*

Relative fecundity (**RF**) was the only fitness parameter showing significant differences between E+A+ and E-A+ treatments ( $F_{1,18} = 5.649$ ;  $P < 0.029$ ; repeated-measures one-way ANOVA; Table 2). Inoculation with *A. strictum* reduced aphid relative fecundity, because *A. fabae* virginoparae laid more nymphs on endophyte-free plants (Fig. 3). The intrinsic rate of natural increase ( $r_m$ ) of aphids was less, but not significantly so, on endophyte-inoculated plants than on endophyte-free plants ( $F_{1,18} = 3.517$ ;  $P < 0.077$ ; one-way ANOVA) (Table 2).

**Table 2.** Calculated variances (derived from sum of squares in ANOVA) of fitness indices and fitness components of *Aphis fabae* on both endophyte-inoculated (E+A+) and endophyte-free (E-A+) host plants.  $W_b$ , birth weight (mg);  $W_{ad}$ , adult weight (mg);  $M\%$ , Mortality %;  $d$ , development period;  $1/d$ , development rate;  $RF$ , relative fecundity;  $r_m$ , intrinsic rate of natural increase (fem/fem/d);  $RGR$ , relative growth rate. The sample size is shown in parentheses.

Character	E+A+ mean±s.e. (n)	E-A+ mean±s.e. (n)	test-statistic <sup>a</sup>	P
$W_b$	0.004±0.000 (20)	0.004±0.000 (20)	$F_{1,18} = 0.806$	0.381
$W_{ad}$	0.047±0.002 (15)	0.047±0.002 (16)	$F_{1,18} = 0.001$	0.975
$M\%$	19.255±0.027 (15)	19.225±0.034 (16)	$F_{1,18} = 0.467$	0.503
$d$	8.800±0.200 (15)	8.400±0.163 (16)	$F_{1,18} = 2.400$	0.139
$1/d$	0.114±0.003 (15)	0.119±0.002 (16)	$F_{1,18} = 2.339$	0.144
$RF$	<b>3.885±0.457 (15)</b>	<b>5.210±0.320 (16)</b>	$F_{1,18} = 5.649$	<b>&lt;0.029</b>
$r_m$	0.101±0.022 (15)	0.143±0.007 (16)	$F_{1,18} = 3.517$	0.077
$RGR$	0.101±0.002 (15)	0.105±0.002 (16)	$F_{1,18} = 1.759$	0.201

<sup>a</sup> F value with 1 and 18 degrees of freedom.



**Fig. 3** Relative fecundity (RF) of *Aphis fabae* reared on endophyte-inoculated (E+A+) and endophyte-free (E-A+) host plants: number of offspring produced per virginopara per day for 10 days (mean ± SE) ( $F_{1,18} = 5.649$ ;  $P < 0.029$ ; repeated-measures one-way ANOVA).

## **Discussion**

Several studies have shown that herbivory causes plants to produce more EF nectar (Koptur 1989; Wäckers *et al.* 2001; Ness 2003) and more nectaries (Mondor & Addicott 2003; Mondor *et al.* 2006; Pulice & Packer 2008). Our results demonstrate for the first time a complex response in the temporal patterns of EF nectar production and the number of EF nectaries of broad bean plants treated with an endophytic fungus, either alone or in combination with an aphid. Endophytic inoculation induced a significant short-term increase in total EF nectar production and a prompt higher ratio of EF nectaries per expanded leaf. On the other hand, aphid infestation significantly prolonged the increase in total EF nectar production and delayed the increase in EF nectary number. When plants were simultaneously inoculated with endophyte and infested with aphids, however, both EF traits were significantly reduced.

The marked difference in induction of EF-mediated defences of endophyte-inoculated plants in absence and presence of herbivory may reflect differences in the costs and benefits of offering these rewards under different circumstances. With respect to the costs of producing EF nectar, Wäckers *et al.* (2001) showed that the amount of sugar excreted in EF nectar by damaged castor leaves corresponded to 1% of the leaf's daily assimilate production. Even though this cost may seem small on a per day basis, the cumulative cost could be substantial over the total period of plant growth. Whereas the absolute and/or relative costs of producing EF nectaries as opposed to EF nectar are less clear (Rosenzweig 2002), the costliness of producing these structures is indicated by the fact that some plant species have lost EF nectaries in ecosystems lacking mutualistic ant species (Bentley 1977). Moreover, damaged plants may produce additional EF nectaries only when nutrient levels increase (Mondor *et al.* 2006). Given that nectar production is costly, the production of additional nectaries is likely to be energetically expensive as well. In addition to the direct (physiological) costs of EF nectar/nectary production, offering this food reward is likely to entail potential indirect (ecological) costs via interactions involving other species (reviewed by Strauss *et al.* 2002).

Together, direct and ecological costs of EF traits may constrain the production of EF nectar and EF nectaries when costs outweigh benefits.

Upon herbivory, *A. strictum*-inoculated *V. faba* plants, already bearing fitness costs imposed by nourishing the endophyte colonizing their roots (Saikkonen *et al.* 2004; Schulz & Boyle 2005), may face further negative effects in terms of seed production and other fitness correlates. If EF nectary traits were induced, these plants might be overburdened with costs of producing EF rewards, already shown to be exacerbated by the presence of herbivory (Rutter & Rausher 2004). On the other hand, if endophytic inoculation induces alternative defence mechanism(s) upon herbivory, then EF rewards used to attract mutualistic bodyguards might be considered redundant and unnecessarily costly. This trade-off between different forms of defence would seem particularly reasonable when *A. fabae* life history traits are considered. Aphid individuals exhibited lower performance indices on endophyte-inoculated plants, mainly in terms of relative fecundity. This indicates that *A. strictum* altered the physiology of plants in response to *A. fabae* herbivory resulting in reduced aphid fitness. Moll & Vidal (1995) reported changes in the amino acid content in the phloem sap of *A. strictum*-inoculated plants. Dugassa-Gobena, Raps & Vidal (1996) found that inoculating tomato plants with *A. strictum* altered the sterol profile both qualitatively and quantitatively, which can negatively affect the performance of insects (Sivapalan & Gnanapragasam 1978; Richter, Adam & Vorbrodt 1987). In addition to changes in the nutritional chemistry of plants, resource limitation or sink competition might also act upon aphids feeding on these plants. We hypothesize that nutritional sinks induced by both organisms (insect and fungus) colonizing different parts of the plant will give rise to intra-plant, interspecific competition, the impacts of which will depend on the availability of resources (Larson & Whitham 1997). Given this scenario, an induction of EF rewards by herbivory might disturb the finely tuned mutual balance of antagonism between the endophyte and the host plant, largely depending on the tolerance of each partner to the surrounding biotic and abiotic environment (Schulz & Boyle 2005). If this interaction becomes imbalanced, the cryptic endophyte may turn into a plant pathogen, ultimately leading to host defence responses against the endophyte itself. To

maintain the fragile balance of antagonism safeguarding its survival and the health of its host, *A. strictum* might reduce EF rewards offered on aphid-infested plants and only induce them on aphid-free plants. However, is such a damage-dependent defence strategy (Mondor *et al.* 2006) induced in intact plants?

Heil & Kost (2006) reported that volatile organic compounds (VOCs) primed EF nectar secretion in lima bean plants (*Phaseolus lunatus* L., Fabaceae): exposure to such volatiles caused yet undamaged *P. lunatus* plants to increase their EF nectar production. Conceivably, EF nectaries may facilitate “plant-plant” interactions, especially among plants that share or compete for natural enemies of herbivores (Rudgers & Gardener 2004). Such priming effect was evident in aphid-free plants, either with or without *A. strictum* inoculation. However, when endophyte inoculation increased EF rewards in aphid-free plants, there was a significant effect of date, with plants producing most of their EF nectar and nectaries 2 and 7 days after *A. fabae*-infested plants had aphid cages attached, respectively. Given the importance of EF nectaries for *Vicia faba*-ant interactions (Katayama & Suzuki 2004) and assuming that mutualistic ants are analogous to defensive secondary compounds as proposed by Janzen (1966) and Rehr, Feeny & Janzen (1973), there should be a well-developed rapidly induced response syndrome in tightly evolved ant-plant systems, especially when risk of herbivory is increased. The temporal pattern found in *A. strictum*-inoculated plants could in fact help in optimizing indirect defence by concentrating the recruitment of antagonists (bodyguards) only at the time of attack (Heil *et al.* 2000; Wäckers *et al.* 2001).

The slower rate at which these plants produced EF rewards later on could also be due to the absence of mutualistic partners. Rudgers (2004) and Rutter and Rausher (2004) showed that when ant visitors were experimentally excluded, plants minimized allocation of resources to EF nectaries. A similar response was reported for the production of food bodies by *Piper cenocladum* to attract *Pheidole bicornis* mutualistic ants (Risch & Rickson 1981). The diminishing rate of increase in EF rewards in endophyte-inoculated plants might also be explained by a *plateau in benefit*, suggesting that additional benefits would unlikely accrue if

EF rewards were increased beyond a certain range of values. There is little evidence, however, for such a plateau (Rutter & Rausher 2004). Our results also show that *A. strictum*-inoculated plants did invest more in nectary numbers than in nectar production, which supports the hypothesis that increasing the visual display might be more effective and adaptive than increasing the resources from existing nectaries (Mondor & Addicott 2003). This hypothesis seems particularly plausible in *V. faba*, where nectaries are visually conspicuous and the most common mutualistic partners, ants, use visual cues in foraging (David & Wood 1980).

The failure to increase EF-mediated defences in endophyte-inoculated plants being fed upon by aphids does not mean that *A. strictum* cannot induce EF-mediated defences in conjunction with other forms of defence. Below, we offer two explanations for the lack of induction of EF defences in endophyte-inoculated *A. fabae*-infested plants. First, although plant-ant relationships involving EF nectaries are often regarded as examples of mutualism (Bently 1977; Ness 2003; Rudgers 2004), the interaction sign (mutualism or parasitism) seems to change when ants are tending Homoptera (Oliver, Cook & Leather 2007). When *V. faba* plants were parasitized by *Aphis craccivora*, ant attraction by EF nectar decreased with an increasing number of ant-tended aphids on the plant because ants were more attracted to the honeydew than to the EF nectar (Sakata & Hashimoto 2000; Katayama & Suzuki 2003), and this high attractiveness facilitated the exclusion of herbivorous insects, except aphids, by ants (Suzuki, Ogura & Katayama 2004). Oliver *et al.* (2007) also demonstrated that the positive effect of attendance on aphids by the ant *Lasius niger* reduced the fitness of *A. fabae*-infested plants. They further suggested that costs of ant attendance in *V. faba* plants are unlikely to be offset by other beneficial agents that also visit EF nectaries (e.g., parasitoids). These results, coupled with the fact that sap-sucking insects often vector plant pathogens (reviewed by Buckley 1987), strongly beg the question whether the prolonged increase in EF nectar production and in the induction of EF nectaries 10 days after *A. fabae* feeding in endophyte-free plants was a worthwhile investment. Second, several studies have found that induced resistance increased as the damage on the plant increased (Henderson &

Holloway 1942; Haukioja & Neuvonen 1987; Karban 1987). Although there is no evidence of a damage threshold that must be exceeded before EF traits are induced, Inouye & Taylor (1979) reported that EF nectar production varied with intensity of herbivore pressure, and Mound (1962) showed that the increase in nectar secretion following the attack by sucking insects was positively correlated with increased infestation levels. This suggests that EF-mediated defences should probably be thought of as a graded rather than an on/off response, and that different levels of damage to the plants would translate into variations in costs and benefits of mutualistic interactions via the rewards offered.

From a cost/benefit perspective, mutualisms have been thought to possess “conditional outcomes”, which may vary with the biotic and abiotic setting (Bronstein 1994). Endowed with a high degree of phenotypic plasticity in EF nectary traits (Rudgers 2004), plants can adjust allocation to EF traits as cued by environmental factors, so that benefits are maximized and production costs are minimized (Moran 1992). Still quite unpredictable, however, is whether EF-mediated responses can be completely shifted when multiple mutualists are distantly involved (i.e., endophytic fungi colonizing the roots and the mutualistic insects visiting the shoots). Bronstein (1994) predicted that mutualisms in which a third species is intimately involved are more likely to show conditional outcomes than other forms of mutualism. Conceivably, costs and benefits of mutualisms, involving beneficial insects (e.g., ants) defending reward-producing plants (e.g., EF nectary-bearing plants) will shift with the identity and abundance of other associates (e.g., endophytes). Adding a further dimension of conditionality to such interactions is the creative phenotypic plasticity through which the endophytic influence is expressed. By varying levels of herbivory and soil nutrients, Faeth & Fagan (2002) experimentally showed that the costs and benefits of harbouring symbiotic endophytes in grasses changed the outcome of the endophyte-plant mutualism.

Taken together, the variation in endophyte-mediated EF response patterns, as reported here, may come as little surprise when considering how dynamic and context-dependent both interacting partners (i.e., endophyte and EF nectaries) are. However, further investigations of the interactions between different endophytes, EF nectary plants, and herbivores, under

different environmental conditions, should give more insight on how EF-mediated defences are moulded by the endophyte mutualists.

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## **Resource-based trade-off in multiple mutualisms: can nutrient availability shift the outcomes of multi-species interactions?**

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## Summary

- The idea that multispecies interactions range from mutualistic to antagonistic at various ecological scales of conditions (e.g. presence of other species and/or abiotic factors) has only been considered recently, hence we know very little about how individuals balance the competing demands of multiple mutualisms.
- We investigated a four-way interaction consisting of a host plant (*Vicia faba*) bearing extrafloral (EF) nectaries, a fungal endophyte (*Acremonium strictum*), an insect herbivore (*Helicoverpa armigera*), and nutrient availability.
- Following herbivory, the level of variation in offering two EF rewards (nectar volume and nectary number) in the absence of endophyte infection was only slightly affected by nutrient addition; whereas EF rewards of endophyte-infected plants responded to nutrient addition in a more complex way depending on herbivore damage. Increasing nutrient supply increased the extent of root colonization with *A. strictum* and alleviated the negative effects of herbivory on plant fitness in both endophyte-infected and endophyte-free plants. Several measured parameters of insect fitness were improved by nutrient addition on endophyte-free plants, but were less responsive on endophyte-infected plants.
- We suggest that plants regulate multiple mutualisms (as well as other resource-demanding functions) in response to variation in resource availability so as to attain a favourable cost/benefit ratio.

**Key words:** cost/benefit framework, extrafloral-mediated defences, fungal endophytes, *Helicoverpa armigera*, host-endophyte interactions, multiple mutualisms, multi-species interactions, resource availability

## Introduction

Mutualisms are thought to be mediated through the production and consumption of resources among interacting species (Holland *et al.*, 2005). As such resource production (generally considered the costs of mutualism) could otherwise be allocated to growth or reproduction, mutualists are predicted to minimize these investments costs (Holland *et al.*, 2009). While the costs of mutualism are increasingly recognized for their role in the ecology and evolution of mutualistic interactions, they remain less well understood than the benefits of mutualism (Bronstein, 2001).

The idea that multi-species interactions can range from mutualistic to antagonistic at various ecological scales of conditions (e.g. presence of other species and/or abiotic factors; Bronstein, 1994; Bronstein & Barbosa, 2002; Neuhauser & Fargione, 2004) has only been considered recently, hence we know very little about how individuals balance the competing demands of multiple mutualisms (Mack & Rudgers, 2008). For mutualistic interactions, most experiments have manipulated only one mutualist or functional group of mutualists, potentially overlooking interactions among species that confer different types of benefits (Stachowicz & Whitlatch, 2005). Besides, although manipulation of environmental factors that affect the costs and benefits of mutualisms can aid in understanding the dynamics of multi-species interactions (Bronstein, 1994), prior work has largely been conducted under constant environmental conditions (but see Mack & Rudgers, 2008).

The widespread occurrence of endophytic fungi, which live within host plant tissues without causing any visible symptoms of disease (Wilson, 1995), in almost all plants (Rodriguez *et al.*, 2009) has prompted numerous investigations into the ecological significance of these microorganisms as mediators of multitrophic interactions (reviewed in Hartley & Gange, 2009). The association between fungal endophytes and their host plants is generally considered a mutualistic one (but see Faeth, 2002; Faeth & Fagan, 2002; Jani *et al.*, 2010); since plants provide the fungi with nutrition as well as protection from external environmental

stresses (Schulz & Boyle, 2005) and receive, in turn, increased resistance to insect herbivores and plant pathogens (Giménez *et al.*, 2007) in addition to abiotic stresses (Kuldau & Bacon, 2008) by the endophytes. Although the beneficial effects of endophytic fungi presumably counterbalance any costs to the host of supporting a heterotrophic symbiont, potential metabolic costs to hosts may only appear in resource-limited conditions (Cheplick *et al.*, 1989; Ahlholm *et al.*, 2002; Saikkonen *et al.*, 2004).

Whereas the effect of nutrient availability on the mutualistic interactions among endophytic fungi and their host plants has been well investigated within the clavicipitaceous endophytes that are limited to some cool- and warm-season grasses (reviewed in Saikkonen *et al.*, 2006), such effect has not been explored for the more ubiquitous non-clavicipitaceous endophytes in plants other than grasses (see Rodriguez *et al.*, 2009 for the latest review on fungal endophytic classes). The horizontally-transmitted non-clavicipitaceous endophytes, which are extremely diverse and colonize a wide variety of plant tissues in virtually every host plant examined to date (Schulz & Boyle, 2005; Zhang *et al.*, 2006), are thought to benefit their hosts by promoting inducible defences (Carroll, 1988; 1991). However, Jaber & Vidal (2009) recently reported that a root-colonizing endophyte belonging to this group (*Acremonium strictum* Gams) directs plant resources in herbivore-damaged plants away from extrafloral nectaries (hereafter referred to as EF nectaries) to the endophyte sink in plant roots, despite the advantage of these nectaries as a form of inducible indirect defence in plants (Mondor & Addicott, 2003; Pulice & Packer, 2008). EF nectary-bearing plants mediate arthropod-plant protective mutualism by recruiting plant defenders (e.g. ants, predatory mites, wasps, ladybird beetles, etc.; reviewed in Heil, 2008). These plants also influence the effectiveness of their indirect defence by changing the amount and quality of rewards, to which the nectary-visiting arthropods (ants; as most frequently cited) can quickly respond (Heil & Mckey, 2003). In addition, damaged plants with high nutrient levels are able to produce more EF rewards than plants that are nutrient-limited (Mondor *et al.*, 2006). We therefore expect that abundant

nutrient levels would alleviate costs of both sheltering endophytes and offering EF nectary rewards.

In this study, we investigated a four-way interaction consisting of a host plant *Vicia faba* L. (Fabaceae) bearing EF nectaries, an endophytic root fungus *A. strictum*, an insect herbivore *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae), and nutrient availability. We experimentally manipulated the presence of the root endophyte and established different fertilizer levels to explore how the common host plant would balance mutualists (i.e. endophyte and EF-recruited arthropods) that confer similar protection benefits following *H. armigera* herbivory under variable resource levels. Here, we did not examine the effects of EF rewards on protective arthropods (especially ants) deterrence of herbivory (for more details on the importance of EF rewards for *V. faba*-ant interactions; see Katayama & Suzuki, 2004). We rather aimed to test the following hypotheses: 1) sheltering and nourishing endophytes impose fitness costs on their host plants (Saikkonen *et al.*, 2004; Schulz & Boyle, 2005), 2) herbivory can reduce resource availability and subsequently have indirect impact on plant fitness in terms of growth and reproduction (Koptur *et al.*, 1996), 3) costs of inducing EF-mediated traits are exacerbated by herbivory (Rutter & Rausher, 2004) particularly in endophyte-colonized plants (Jaber & Vidal, 2009), and 4) the magnitude of 1), 2), and 3) depends on the amount of available resources. The following questions were specifically addressed: Is endophyte colonization in inoculated plants dependent on the amount of available resources? How do two EF nectary traits (nectar volume and nectary number) respond to interactions among endophyte, herbivore and nutrient availability? Do these interactions alter some parameters of plant fitness? Finally, how do *H. armigera* life history parameters (i.e. immature performance) respond to endophyte-plant-nutrient availability interactions?

## Material and Methods

### Study species

*Vicia faba* L. (cv. Hangdown Grünkernig, Gevo GmbH, Nortmoor, Germany) plants were grown in a greenhouse chamber. In *V. faba*, conspicuous, ant-attended EF-nectaries (Engel et al., 2001) are produced on the stipules that grow in pairs at the base of leaf petioles (Mondor & Addicott, 2003). Each stipule pair can bear none, one, or two EF nectaries; but >99% of the stipule pairs in this experiment bore two EF nectaries. Two-week-old plants were individually transplanted into plastic pots (11 cm diameter) with a mixture of non-sterile soil (Fruhstorfer T25 Erde, Hawita Gruppe GmbH, Vechta, Germany) and sand (1:1 ratio).

A strain of *A. strictum* (DSMZ-GmbH, Braunschweig, Germany) was maintained in the laboratory on 0.3% malt extract agar (MEA). Re-isolations have been used throughout the last years to ensure viability of the fungus. Liquid malt extract agar medium (0.3%) was autoclaved at 120°C for 20 minutes. To prepare the spore suspension, a piece of malt extract agar containing fungus mycelia was added to the autoclaved media. The suspension was kept on a shaker (at 23°C and 100 RPM) for 12 days to guarantee fungal growth and sporulation.

*H. armigera* was selected as the herbivore, based on the findings that feeding on *A. strictum*-inoculated *V. faba* plants had a strong influence on this insect's fitness parameters in a previous study (Jaber & Vidal, 2010). The egg masses 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 14L: 10D photoperiod until hatching. Neonate larvae were reared on standard bean flour based artificial diet for *Helicoverpa spp.* (Teakle, 1991) until the second larval instar stage. Early second instar larvae were transferred from the artificial diet to leaves of *V. faba* plants (non-treatment plants) for habituation. Only larvae which successfully moulted to the third instar stage on *V. faba* plants were used in the experiment.

### Experimental set-up

Five days after transplanting, plants were randomly assigned to one of twelve treatment combinations which were randomly distributed among blocks arranged along a single

greenhouse bench. Twice each week, blocks were randomly rotated on the bench. There were 12 replicates per treatment combination (n=12). The experimental design was 2×2×3 factorial with two endophyte infection groups (E+, E-), two herbivory levels (H+, H-), and three nutrient levels (F++, F+, F-).

To prepare the fungal inoculum for the endophyte treatment, spore concentration in a drop of the suspension (after vacuum filtering) was measured under the microscope in a Thoma counting chamber (64 × 0.025 mm<sup>2</sup>, chamber height 0.1 mm). Plants assigned to be inoculated (E+) were watered with 70 ml of spore suspension containing 10<sup>6</sup> *A. strictum* spores/ml and control plants (E-) were watered with the same volume of (fungus-free) culture filtrate. The inoculum density used here was found sufficient to colonize *V. faba* roots in previous studies (Jaber & Vidal, 2009, 2010).

Nutrient availability was altered by applying three fertilization treatments: fertilization twice each week (high nutrient level; F++), fertilization once each week (intermediate nutrient level, F+), and no fertilization (low nutrient level; F-). Fertilization treatments were initiated five days after *A. strictum* inoculation and continued throughout the duration of the experiment. 70 ml of a mixed fertilizer solution (15% N, 11% P, 15% K, 1% Mg, 0.1% Fe, 0.1% Mn, 0.04% Cu, 0.025% B, 0.005% Mo, 0.015% Zn, Compo GmbH, Münster, Germany) was added to each pot of plants assigned to be fertilized, while non-fertilized plants received the same amount of tap water.

Three days after initiating the fertilization treatments (eight day following *A. strictum* inoculation), a clip-on cage was attached to the third leaf of all plants assigned to the herbivory treatment. A single early third-instar *H. armigera* larva was introduced into each clip-on cage on (H+) plants while cages on (H-) plants remained empty. Each larva was moved to the next leaf nearly before consuming all leaf material within the cage and kept on the plant until pupation. All work was carried out in a controlled environment at 22 ± 2°C, 65 ± 10% RH and a photoperiod of 14L : 10D.

### EF-mediated defence responses

EF nectar per leaf pair was collected using 5  $\mu$ l micropipettes with 1  $\mu$ l divisions and the collected volume was calculated based on the proportion of the pipette filled. Nectar from all nectary pairs on each plant was pooled to permit determination of the total EF nectar production per plant. EF nectar collection commenced at the start of all treatments (immediately before *A. strictum* inoculation). Recording continued before fertilization, before herbivory infliction, and thence was carried out at 72 h intervals until 12 days past herbivory (dph). Using this recording range, it was possible to determine the onset of a potential induction in nectar production in response to each treatment as well as its rate of decline. We were unable to apply a similar recording range to EF nectary numbers, as these two forms of EF defence (nectar and nectary) operate on very different temporal scales (with nectar induction being relatively rapid compared to nectary induction; Mondor *et al.*, 2006). Therefore, number of EF nectary pairs on each plant was recorded before applying any of the treatments (starting immediately before *A. strictum* inoculation). The number was recorded again seven and fourteen dph. Pre-treatment values were then subtracted from past-treatment value to quantify the change ( $\Delta$ ) in the number of EF nectary pairs.

### Other plant responses

Plant height, number of expanded leaf pairs, and number of immature leaf pairs on each plant were recorded before applying any of the treatments (as with the abovementioned EF nectary pairs number). Seven and fourteen dph, the same plant traits were assessed and pre-treatment values were subtracted from past-treatment values to quantify the change ( $\Delta$ ) in each character. Time of first open flower (days to flowering) was also recorded for each plant. At the end of the experiment, the aboveground biomass of all plants was harvested at ground level and oven-dried to constant weight at 70°C for a week in order to obtain the dry shoot

weight. A C/N analysis was then performed to examine the total carbon and nitrogen content of the shoots in different treatment combinations. Dried shoot biomass was ground with a swing mill grinder (Siebtechnik, Mühlheim, Germany). Three-mg samples of finely-milled shoot material were weighed and analyzed using a C/N elemental analyser (Vario EL III, Elementar, Hanau, Germany).

### Insect responses

*H. armigera* third-instar larval weight was individually measured immediately before introducing the larvae into the clip-on cages and again five days later (at the fifth-instar stage), in order to calculate the relative growth rate (RGR) according to Farrar *et al.* (1989) as follows:  $RGR = \text{biomass gained (mg fresh weight)} / [(\text{fresh weight at third-instar stage} + \text{fresh weight at fifth-instar stage}) / 5] \times 5 \text{ (days)}$ . The larvae were checked twice daily for molting and survival until pupation. The freshly formed pupae were individually weighed. Data recorded at the end of this part were the RGR, the larval period, the pupal weight, and the pupal period.

### Effectiveness of the endophyte inoculation

At the end of all experiments, nine plants were selected from each of the 12 treatment combinations. Roots of these plants were thoroughly washed, frozen in liquid nitrogen and temporarily stored at -20°C until the verification of endophyte colonization. Detection and quantification of endophyte colonization were determined for each treatment combination by real-time polymerase chain reaction (RT-PCR). To extract the endophyte DNA, root samples were thawed and pulverized to fine powder in liquid nitrogen using a pestle and mortar. Root powder (100 mg) was then dispersed in 1 ml CTAB buffer containing 2 µl mercaptoethanol and 1 µl proteinase K following a variant of the CTAB method (Murray & Thompson, 1980), simplified by Stewart & Via (1993) and modified by Brandfass & Karlovsky (2006).

Following DNA extraction, RT-PCR was run to amplify and quantify the fungal colonization in the roots of plants with regard to different treatment combinations.

### Statistical analyses

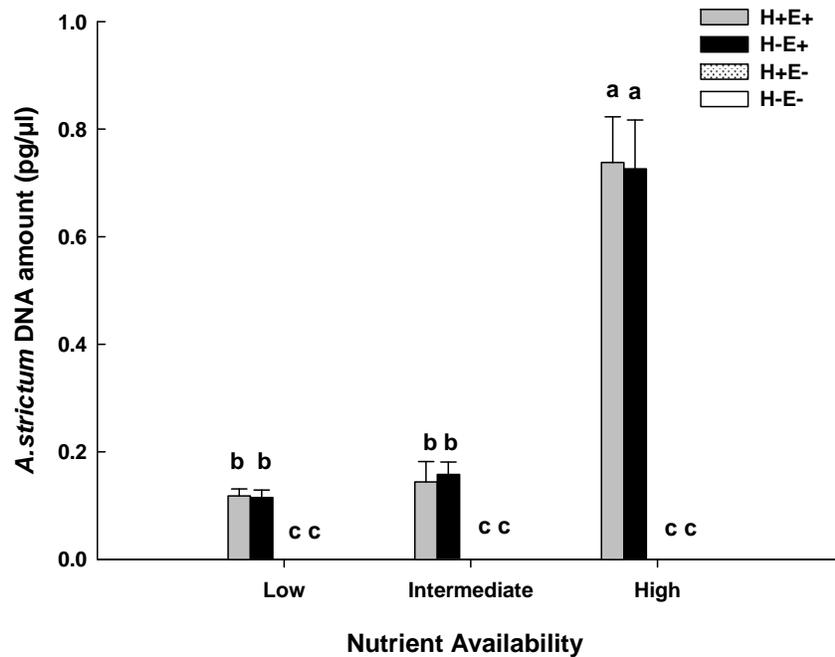
SYSTAT 12 for Windows (SYSTAT, 2008) was used for the statistical analyses. Raw data met assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). The RT-PCR data were analyzed using three-way ANOVA (with endophyte inoculation, herbivory and nutrient availability as the main factors) and Fisher's least significant difference (protected LSD). The responses of nectar production to endophyte infection, herbivory, and nutrient availability were examined with a repeated measures four-way ANOVA (GLM procedure) with endophyte infection, herbivory, nutrient availability, and date as the main factors. Fisher's protected LSD test was then used to compare average nectar production among treatment combinations within each sampling date. To calculate the differences among treatments with regard to the EF nectary number, the change in number of EF nectary pairs per change in number of expanded leaf pairs was used as the dependent variable (i.e.,  $\Delta\text{EFnectary} / \Delta\text{ExpLvs}$ ; see Jaber & Vidal, 2009). A repeated-measures four-way ANCOVA with endophyte infection, herbivory, nutrient availability, and date as main factors was carried out to control for variation in the dependent variable associated with plant growth correlates. A linear regression model was used to test for correlation between changes in EFN nectary numbers and other plant characters. The following three covariates were included simultaneously: change in plant height, change in number of expanded leaf pairs, and change in number of immature leaf pairs. We used separate factorial three-way ANOVAs (GLM procedures) to test for differences in the following dependent variables: change in plant height, change in number of expanded leaf pairs, change in number of immature leaf pairs, days to flowering, the shoot dry weight, C concentration, N concentration, and C/N ratio based on the factors of endophyte infection, herbivory, and nutrient availability. Bonferroni

correction for multiple testing (as modified by Simes, 1986) was carried out in order to control for the experiment-wide error. Fisher's protected LSD test was then used to separate treatment combinations. Finally, sets of two-way ANOVAs (with endophyte infection and nutrient availability as main factors and with Bonferroni correction for multiple testing) were used for *H. armigera* response variables except the RGR. Two-way ANCOVA (with endophyte and nutrient availability as main factors and the initial fresh weight of third-instar larvae as a covariate) was used for the RGR parameter to correct for any bias due to differences in initial larval weight (Raubenheimer & Simpson, 1992). Differences between treatment means were then compared using Fisher's protected LSD test.

## Results

Establishment of *Acremonium strictum* in inoculated plants and the effect of nutrient availability on endophyte colonization

RT-PCR of root extracts showed that *A. strictum* colonization was significantly restricted to the roots of inoculated *V. faba* plants; whereas non-inoculated plants were *A. strictum*-free ( $F_{1, 96} = 223.225$ ,  $P < 0.0001$ ; three-way ANOVA). Quantification of the fungal DNA by RT-PCR also detected a significant two-way interaction between endophyte colonization and nutrient availability ( $F_{2, 96} = 80.247$ ,  $P < 0.0001$ ). Increasing the available nutrients from low to high levels significantly increased *A. strictum* concentration in the roots of inoculated plants; only at the highest level of nutrient availability (Fisher's protected LSD test,  $P < 0.05$ ; Fig. 1).



**Fig. 1.** Real-time polymerase chain reaction (RT-PCR) analysis of DNA extracted from roots of *Vicia faba* plants in different treatment combinations. The (mean  $\pm$  SE) of *Acremonium strictum* DNA found in *Vicia faba* DNA is expressed as (pg/ $\mu$ l). Different letters above columns indicate significant differences ( $P \leq 0.05$ ; Fisher's protected LSD test after three-way ANOVA).

Responses of two EF nectary traits to the interactions among endophyte, herbivore, and nutrient availability

Sampling date significantly affected the total production of EF nectar, resulting in a hump-shaped response at each nutrient level ( $F_{5, 72} = 4.403$ ,  $P = 0.001$ ; Table 1; Fig. 2). EF nectar production was significantly increased in endophyte-infected plants independent of nutrient availability or herbivory ( $F_{1, 72} = 4.140$ ,  $P = 0.036$ ; Table 1; Fig. 2). On the other hand, nutrient availability had a more variable effect on inducing nectar production in endophyte-infected plants, alone ( $F_{2, 72} = 15.542$ ,  $P < 0.0001$ ; Table 1) and in response to herbivory ( $F_{2, 72} = 5.638$ ,  $P = 0.005$ ; Table 1), as compared to endophyte-free plants (Fig. 2). Prior to *H. armigera* herbivory, total nectar production was significantly increased in endophyte-infected

plants, but not in endophyte-free plants, with increased nutrient level (Fisher's protected LSD test after repeated-measures four-way ANOVA,  $P < 0.05$ ; Fig. 2). Whereas the increased nectar production in response to herbivory was not significant in endophyte-infected plants at low nutrient level (Fig. 2A); it was so at intermediate nutrient level for endophyte-infected herbivore-free plants (Fig. 2B) and at high nutrient level for endophyte-infected herbivore-damaged plants (Fisher's protected LSD test,  $P < 0.05$ ; Fig. 2C). On the other hand, *H. armigera* herbivory induced nectar production in endophyte-free plants irrespective of nutrient availability ( $F_{2, 72} = 3.471$ ,  $P = 0.067$ ; Table 1; Fig. 2).

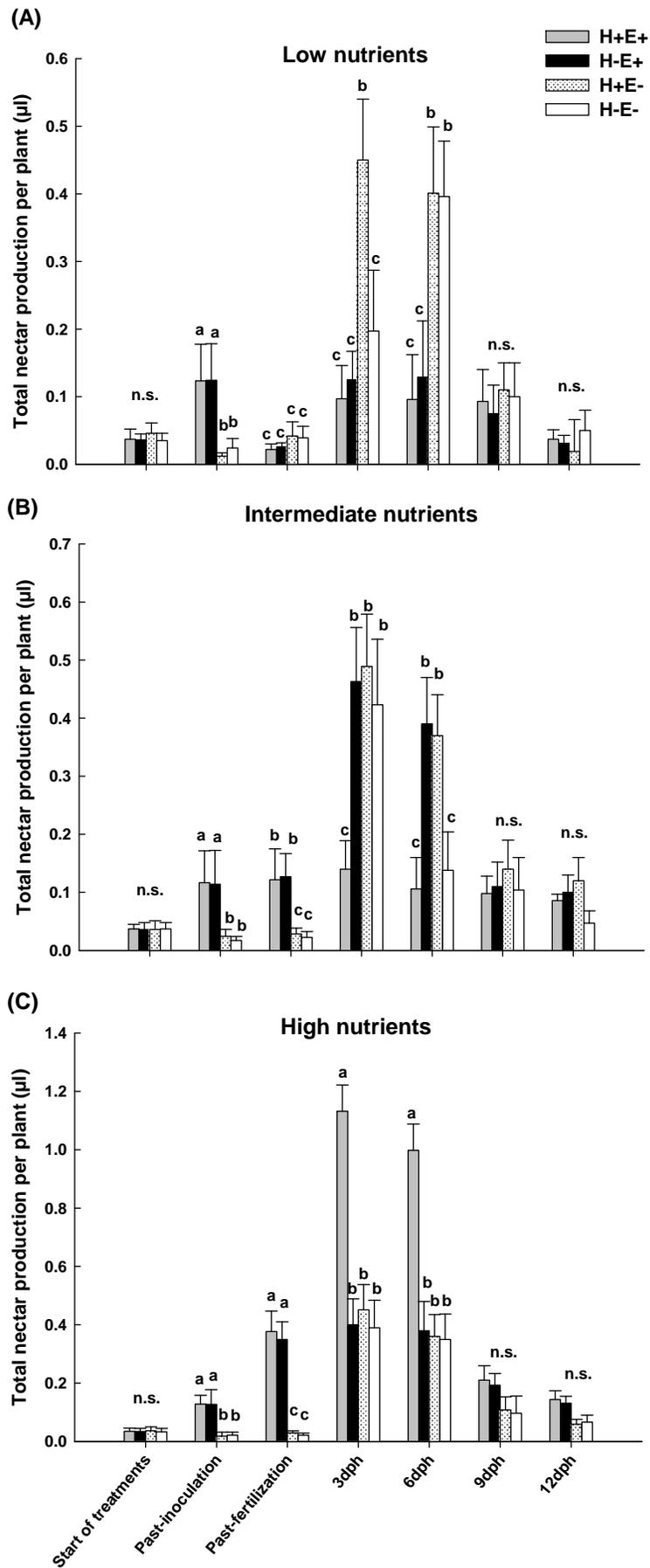
**Table 1.** Effects of endophyte infection, herbivory, nutrient availability, and date on the total EF nectar production and the change in number of EF nectary pairs per the change in number of expanded leaf pairs ( $\Delta\text{EFnectary}/\Delta\text{ExpLvs}$ ) in *V. faba* plants.

Source	Total EF nectar production <sup>a</sup>			$\Delta\text{EFnectary}/\Delta\text{ExpLvs}$ <sup>b</sup>		
	df	F	P	df	F	P
Endophyte (E)	1	4.140	<b>0.036</b>	1	0.827	0.365
Herbivory (H)	1	4.561	<b>0.034</b>	1	2.953	0.088
Nutrient availability (N)	2	7.554	<b>0.001</b>	2	50.508	<b>&lt;0.0001</b>
Date (D)	5	4.403	<b>0.001</b>	1	0.043	0.836
E × H	1	0.141	0.708	1	16.967	<b>&lt;0.0001</b>
E × N	2	15.542	<b>&lt;0.0001</b>	2	17.010	<b>&lt;0.0001</b>
E × D	5	1.013	0.416	1	1.091	0.298
H × N	2	3.471	0.067	2	1.220	0.299
H × D	5	0.766	0.578	1	2.154	0.145
N × D	10	1.595	0.125	2	1.037	0.358
E × H × N	2	5.638	<b>0.005</b>	2	3.374	<b>0.038</b>
E × H × D	5	1.694	0.147	1	0.633	0.428
E × N × D	10	1.049	0.412	2	0.583	0.560
H × N × D	10	0.724	0.700	2	0.521	0.595
E × H × N × D	10	2.168	<b>0.030</b>	2	1.349	0.263
$\Delta$ in plant height 7dph <sup>c</sup> (covariate)	-	-	-	1	0.009	0.999
$\Delta$ in plant height 14dph (covariate)	-	-	-	1	0.031	0.861
$\Delta$ in expanded leaves 7dph (covariate)	-	-	-	1	2.395	0.124
$\Delta$ in expanded leaves 14dph (covariate)	-	-	-	1	2.026	0.157
Error df			72			116

<sup>a</sup> Repeated-measures four-way ANOVA

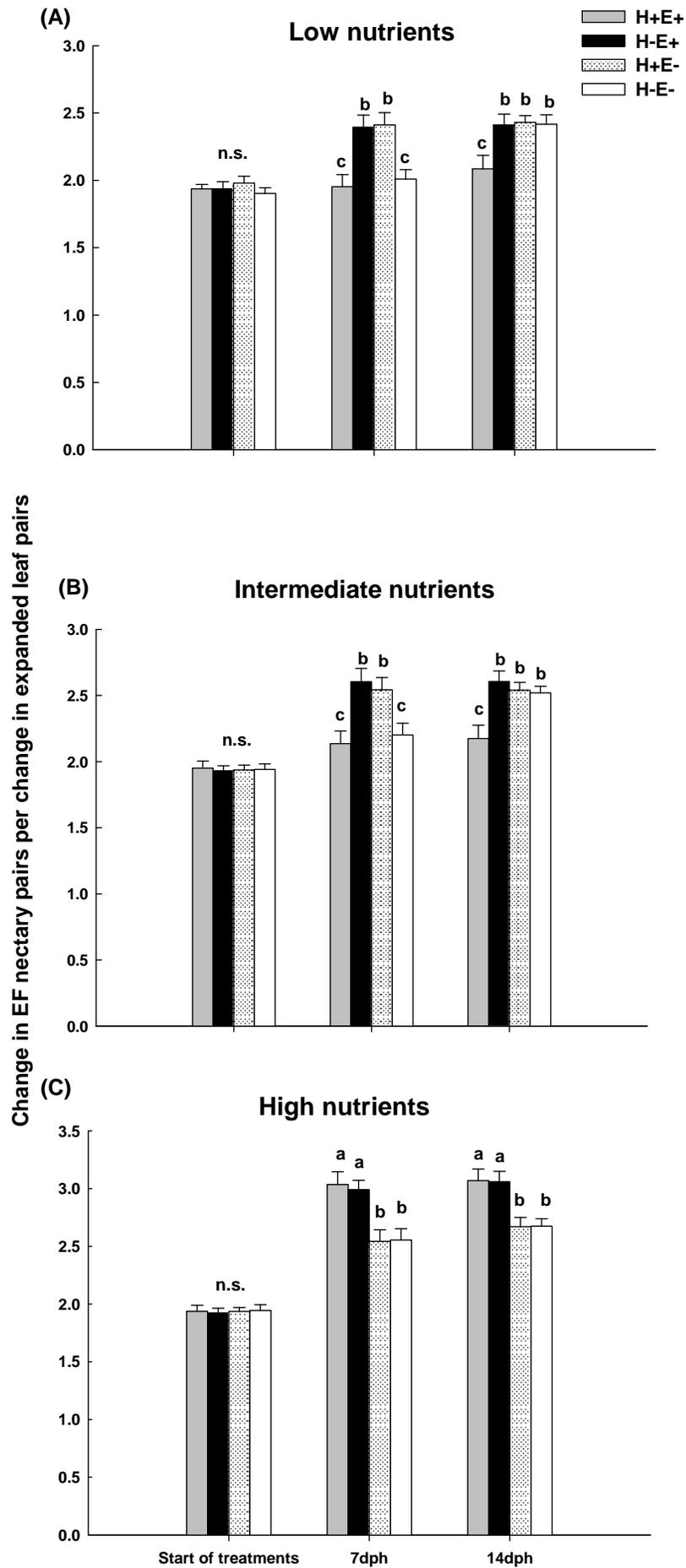
<sup>b</sup> Repeated-measures four-way ANCOVA

<sup>c</sup>  $\Delta$  indicates the degree of change in the trait over 7 and 14 days past herbivory (dph)



**Fig. 2.** Total EF nectar production (mean  $\pm$  SE) of *V. faba* plants in response to endophyte infection, herbivory, nutrient availability, and date. Nectar collection commenced immediately before endophyte infection via *A. strictum* inoculation (i.e. start of all treatments). Recording continued before inducing variability in nutrient availability via fertilization (i.e. past-inoculation), before infliction of *H. armigera* herbivory (i.e. past-fertilization), and thence was carried out at 72 h intervals until 12 days past herbivory (dph). **(A)** low nutrient availability; **(B)** intermediate nutrient availability; and **(C)** high nutrient availability. Different letters denote significantly different treatment combinations among nutrient levels within each sampling date ( $P \leq 0.05$ ; Fisher's protected LSD test after repeated-measures four-way ANOVA).

Only two plant characters were found to significantly influence EF nectary production. The change in EF nectary number was significantly and positively correlated with the change in plant height (7dph:  $F_{1, 142} = 4.391$ ,  $P = 0.038$ ; 14dph:  $F_{1, 142} = 3.921$ ,  $P = 0.050$ ; linear regression model) and the change in number of expanded leaf pairs (7dph:  $F_{1, 142} = 8.094$ ,  $P = 0.005$ ; 14dph:  $F_{1, 142} = 105.93$ ,  $P < 0.0001$ ; linear regression model). These two characters were, thus, used as covariates when running the final ANCOVA analysis for EF nectary production (Table 1). In contrast to total EF nectar production, endophyte infection induced the production of EF nectaries (i.e.,  $\Delta\text{EFnectary} / \Delta\text{ExpLvs}$ ) more than herbivory, resulting in a significant endophyte  $\times$  herbivory interaction ( $F_{1, 116} = 16.967$ ,  $P < 0.0001$ ; Table 1; Fig. 3). Although in a less similar fashion, nutrient availability interacted with endophyte infection in absence ( $F_{1, 116} = 17.010$ ,  $P < 0.0001$ ; Table 1) and presence of *H. armigera* herbivory ( $F_{1, 116} = 3.374$ ,  $P = 0.038$ ; Table 1) as in the case of EF nectar production. Following herbivory, the increase in EF nectary production was not significant in endophyte-infected herbivore-damaged plants at low and intermediate nutrient levels; at both of which only endophyte-infected plants (free of herbivore damage) showed a significant increase in nectary production (Fig. 3A, B). At high nutrient level, however, endophyte-infected plants produced significantly more EF nectaries (irrespective of herbivore damage; Fig. 3C), resulting in a less



**Fig. 3.** Change in number of EF nectary pairs per change in number of expanded leaf pairs ( $\Delta\text{EFnectary}/\Delta\text{ExpLvs}$ ) (mean  $\pm$  SE) of *V. faba* plants. Number of EF nectary and expanded leaf pairs on each plant was recorded before applying any of the treatments, and again seven and fourteen days following the infliction of *H. armigera* herbivory (dph= days past herbivory). **(A)** low nutrient availability; **(B)** intermediate nutrient availability; and **(C)** high nutrient availability. Different letters denote significantly different treatment combinations among nutrient levels within each sampling date ( $P \leq 0.05$ ; Fisher's protected LSD test after repeated-measures four-way ANCOVA).

pronounced interaction term between endophyte infection, herbivory, and nutrient availability than in the case of EF nectar production (Table 1). Nutrient availability, in absence of endophyte infection, had no significant effect on the production of EF nectaries following herbivory ( $F_{2, 116} = 1.220$ ,  $P = 0.299$ ; Table 1; Fig. 3); as a significant increase in EF nectary production in response to herbivory was found in endophyte-free plants at low nutrient level and did not significantly change with increased nutrient availability (Fig. 3).

Responses of plant fitness parameters to the interactions among endophyte, herbivore, and nutrient availability

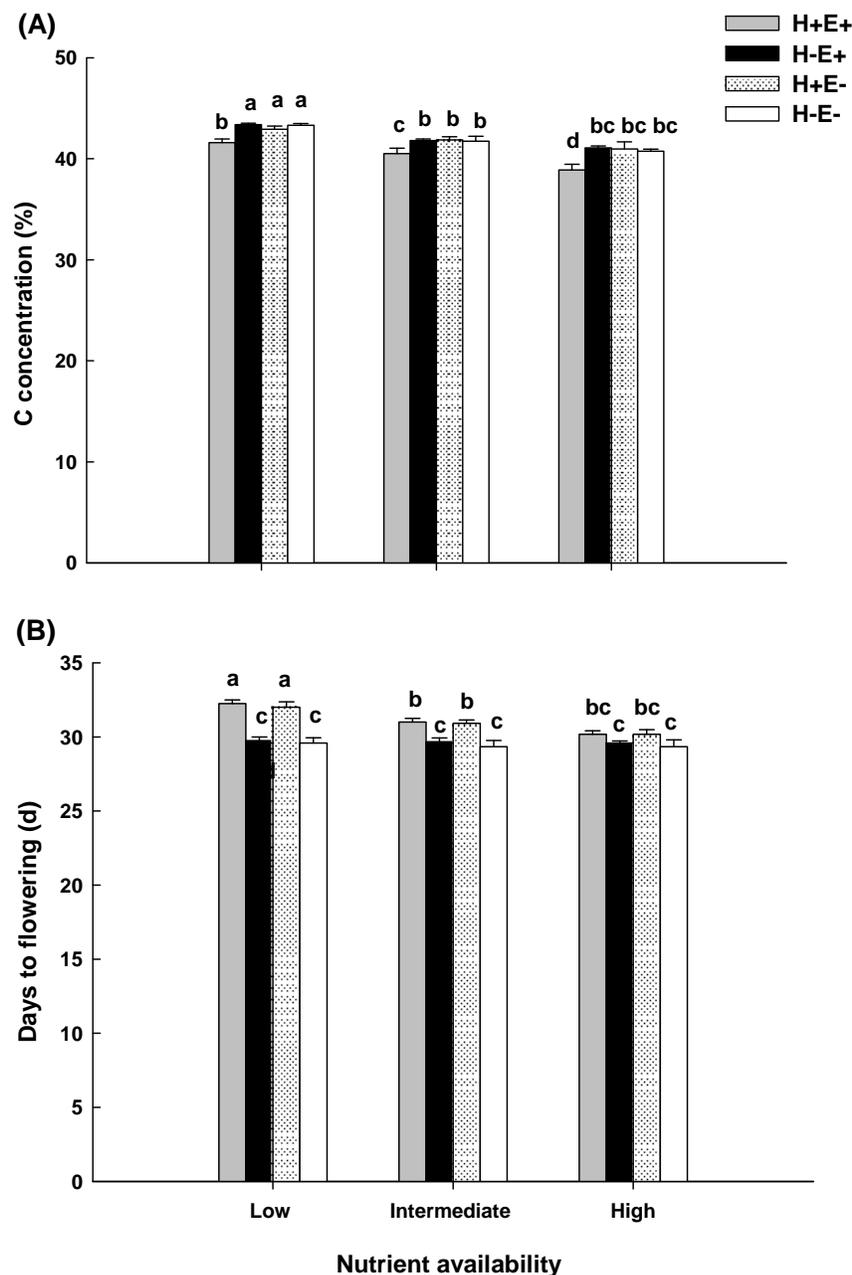
There were no significant main or interactive effects of endophyte, herbivory, nutrient availability on plant growth during the course of the experiment (i.e. the degree change in measured plant traits; Table 2). By the end of the experiment however, herbivore-damaged plants had a significantly lower shoot dry weight ( $F_{1, 132} = 74.747$ ,  $P < 0.0001$ ; Table 2). Conversely, a significant increase in shoot dry weight in response to increased nutrient availability was found in all treatments ( $F_{2, 132} = 5.130$ ,  $P = 0.007$ ; Table 2). Increasing nutrient availability also resulted in a significant decrease in foliar C concentration, an increase in foliar N concentration, and a decrease in foliar C/N ratio (Table 2). However, only the rate by which the foliar C concentration was decreased differed significantly among plants assigned to different treatments ( $F_{1, 108} = 18.374$ ,  $P < 0.0001$ ; Table 2) and was consistently

**Table 2.** Three-way ANOVA ( $F$  and  $P$  values) for the effects of endophyte infection, herbivory, and nutrient availability on *V. faba* fitness parameters.  $P$ -values are adjusted by Bonferroni correction for multiple testing.

Source	$df$	$\Delta^a$ in plant height		$\Delta$ in expanded leaves		$\Delta$ in immature leaves		Shoot dry weight		C concentration		N concentration		C/N ratio		Days to flowering	
		$F$	$P$	$F$	$P$	$F$	$P$	$F$	$P$	$F$	$P$	$F$	$P$	$F$	$P$	$F$	$P$
Endophyte (E)	1	1.401	0.239	0.719	0.398	0.584	0.446	1.258	0.246	12.208	<b>0.001</b>	0.179	0.673	0.696	0.406	0.930	0.337
Herbivory (H)	1	1.849	0.176	1.283	0.259	0.065	0.799	74.747	<b>&lt;0.0001</b>	21.970	<b>&lt;0.0001</b>	1.428	0.235	0.029	0.866	67.799	<b>&lt;0.0001</b>
Nutrient availability (N)	2	2.968	0.055	2.464	0.089	1.108	0.333	5.130	<b>0.007</b>	47.975	<b>&lt;0.0001</b>	110.756	<b>&lt;0.0001</b>	137.039	<b>&lt;0.0001</b>	11.385	<b>&lt;0.0001</b>
E $\times$ H	1	1.401	0.239	1.087	0.299	0.003	0.959	0.736	0.392	18.374	<b>&lt;0.0001</b>	1.703	0.195	0.201	0.655	0.138	0.711
E $\times$ N	2	0.294	0.745	0.063	0.939	0.381	0.684	0.098	0.907	0.268	0.765	0.347	0.707	0.660	0.519	0.022	0.978
H $\times$ N	2	0.366	0.694	0.030	0.970	0.439	0.646	0.286	0.752	0.798	0.453	0.155	0.856	0.202	0.817	7.330	<b>0.001</b>
E $\times$ H $\times$ N	2	1.588	0.208	0.229	0.796	0.096	0.909	0.028	0.972	0.952	0.389	0.501	0.608	0.184	0.832	0.088	0.916
Error $df$		132		132		132		132		108		108		108		132	

<sup>a</sup>  $\Delta$  indicates the degree of change in trait

lower in *A. strictum*-inoculated, *H. armigera*-damaged plants as compared to the remaining treatments at each nutrient level ( $P < 0.05$ ; Fisher's protected LSD test after three-way ANOVA with Bonferroni correction for multiple testing; Fig. 4A). *H. armigera*-damaged plants flowered significantly later than herbivore-free plants; irrespective of endophyte infection ( $F_{1, 132} = 67.799$ ,  $P < 0.0001$ ; Table 2; Fig. 4B). Significant advancement in flowering of herbivore-damaged plants, but not of herbivore-free plants, was attained by increasing nutrient availability ( $F_{2, 132} = 7.330$ ,  $P = 0.001$ ; Table 2); again regardless of endophyte infection (Fig. 4B).



**Fig. 4.** Effects of endophyte infection, herbivory, and nutrient availability on *Vicia faba* fitness parameters (mean  $\pm$  SE). Only fitness parameters with significant interactions in response to treatments are shown. **(A)** carbon concentration (% in three-mg samples of shoot dry matter) and **(B)** days to flowering (d). Different letters above columns denote significant differences among treatment combinations ( $P \leq 0.05$ ; Fisher's protected LSD test after three-way ANOVA with Bonferroni correction for multiple testing).

#### Responses of *H. armigera* fitness parameters (immature performance) to interactions among endophyte, plant, and nutrient availability

Endophyte infection significantly reduced all measured parameters of insect fitness (Table 3; Fig. 5). *H. armigera* larvae reared on endophyte-infected plants suffered significantly reduced growth rate ( $F_{1, 65} = 27.797$ ,  $P < 0.0001$ ; Fig. 5A) and pupal weight ( $F_{1, 66} = 41.246$ ,  $P < 0.0001$ ; Fig. 5A), and significantly prolonged larval ( $F_{1, 66} = 75.028$ ,  $P < 0.0001$ ; Fig. 5B) and pupal period ( $F_{1, 66} = 106.747$ ,  $P < 0.0001$ ; Fig. 5D) as compared to those reared on endophyte-free plants. Nutrient availability had, by contrast, a positive effect on the performance of *H. armigera* larvae (Table 3); although this was only significant for larvae reared on endophyte-free plants ( $P < 0.05$ ; Fisher's protected LSD test after two-way ANOVA with Bonferroni correction for multiple testing; Fig. 5). Increased nutrient availability on endophyte-free plants significantly increased the larval growth rate ( $F_{2, 65} = 3.811$ ,  $P = 0.027$ ; Fig. 5A) and the pupal weight ( $F_{2, 66} = 5.445$ ,  $P = 0.006$ ; Fig. 5C). It also resulted in a highly significant advancement of larval development ( $F_{2, 66} = 9.822$ ,  $P < 0.0001$ ; Fig. 5B) and adult emergence ( $F_{2, 66} = 9.939$ ,  $P < 0.0001$ ; Fig. 5D). Even though nutrient addition did not unduly improve larval performance under endophyte infection, larval growth rate and pupal weight of insects reared on endophyte-infected plants at high nutrient level were comparable to those of insects reared on endophyte-free plants at low nutrient level ( $P < 0.05$ ; Fisher's protected LSD test; Fig. 5A, C).

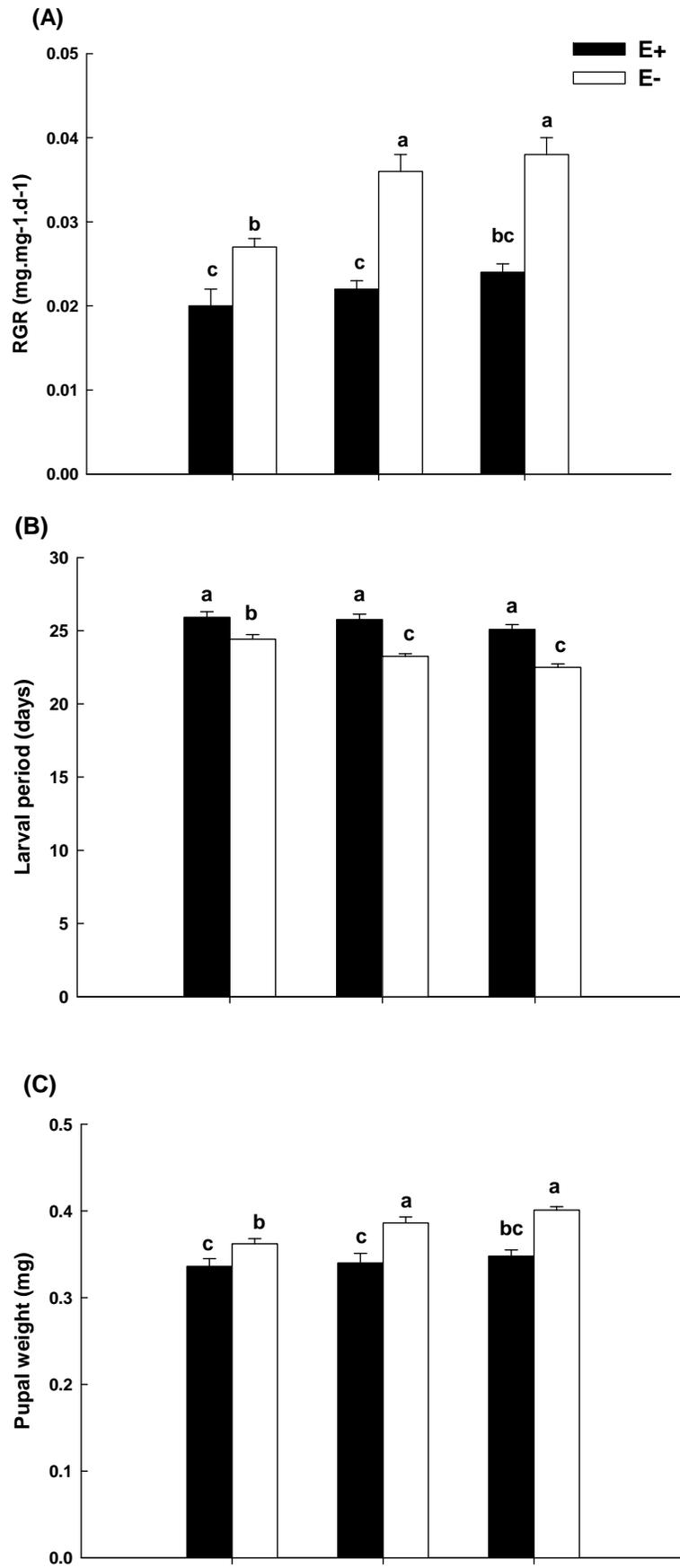
**Table 3.** Effects of endophyte infection and nutrient availability on *H. armigera* fitness parameters. *P*-values are adjusted by Bonferroni correction for multiple testing.

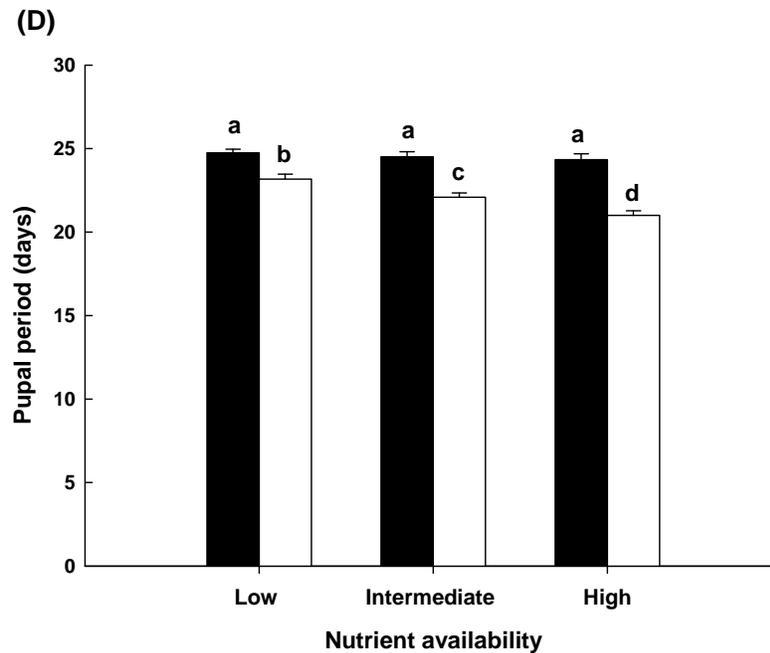
Parameter	Endophyte infection (E)			Nutrient availability (N)			E × N			Larval initial weight (covariate)			Error <i>df</i>
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	
RGR <sup>a, b</sup>	1	27.797	<0.0001	2	3.811	0.027	2	1.314	0.276	1	2.775	0.101	65
Larval period <sup>c</sup>	1	75.028	<0.0001	2	9.822	<0.0001	2	1.887	0.160	-	-	-	66
Pupal weight <sup>c</sup>	1	41.246	<0.0001	2	5.445	0.006	2	1.589	0.212	-	-	-	66
Pupal period <sup>c</sup>	1	106.747	<0.0001	2	9.939	<0.0001	2	4.563	0.014	-	-	-	66

<sup>a</sup> RGR = relative growth rate

<sup>b</sup> Two-way ANCOVA with Bonferroni correction for multiple testing

<sup>c</sup> Two-way ANOVA with Bonferroni correction for multiple testing





**Fig. 5.** Effects of endophyte infection and nutrient availability on *H. armigera* fitness parameters (mean  $\pm$  SE). **(A)** relative growth rate (RGR) ( $\text{mg}\cdot\text{mg}^{-1}\cdot\text{d}^{-1}$ ); **(B)** larval period (days); **(C)** pupal weight (mg); and **(D)** pupal period (days). Different letters above columns denote significant differences among treatment combinations ( $P \leq 0.05$ ; Fisher's protected LSD test after two-way ANOVA with Bonferroni correction for multiple testing; two-way ANCOVA was used for RGR).

## Discussion

To our knowledge, our work is the first to quantify the responses of two traits (i.e. nectary number and nectar volume) of the ant-attended EF nectaries to interactions among endophyte, herbivory, and nutrient availability; which opens a new dimension of applying the cost/benefit framework to multiple mutualisms. Prior to infliction of *H. armigera* herbivory, total nectar production of *V. faba* plants was significantly increased in response to *A. strictum* inoculation. Considering that production of EF rewards is damage-dependent (Mondor *et al.*, 2006), it was rather surprising that fungal endophyte infection induced nectar production in absence of damage as well. Root colonization with fungal endophytes has been shown to induce different forms of host plant defence reactions, such as mechanical defences (e.g. Benhamou &

Garand, 2001; Narisawa *et al.*, 2004) and synthesis of defence metabolites (e.g. Schulz *et al.*, 1999; Mucciarelli *et al.*, 2003). Such induced host defence responses (similar to those limiting colonization by pathogens) are presumably only initially activated to limit colonization of the fungal endophytic invader, resulting in a balance of antagonisms between the host and the fungus as hypothesized by Schulz & Boyle (2005). Therefore, endophyte-host interactions do not exclude fungal virulence (enabling infection) and plant defences (preventing development of diseases; Schulz & Boyle, 2005; Kogel *et al.*, 2006); and active host defence reactions (including EF-mediated defences) might have been triggered by the initial invasion of the fungus. Induction of EF traits (e.g. nectar volume: Koptur, 1989; Wäckers *et al.*, 2001; nectary number: Mondor & Addicott, 2003; Pulice & Packer, 2008; changes in nectar composition: Smith *et al.*, 1990; Ness, 2003) is hitherto mainly cited as a defensive plant response to herbivory and has never been previously linked to microbial infection (but see de la Fuente & Marquis, 1999).

Following herbivory, the level of variation in offering EF rewards in the absence of endophyte infection was minimally affected by nutrient availability; most likely because the requirements for inducing EF defences under such circumstances were met even at low nutrient level. This finding, however, poorly reconciles with that of Mondor *et al.* (2006) who reported that EF rewards are resource-dependent. On the other hand, EF defences of endophyte-infected plants responded to nutrient addition in a more complex fashion; depending on herbivore damage. Low nutrient availability limited EF defences in endophyte-infected herbivore-damaged plants, but not in their damage-free counterparts (on which EF nectar and nectary were induced due to the “priming effect”; sensu Heil & Kost, 2006). However, this disparity in offering EF rewards between damaged and damage-free endophyte-infected plants vanished at high nutrient levels, especially in terms of EF nectary production (which might be an adaptive approach of increasing visual display more than increasing resources from existing nectaries; Mondor & Addicott, 2003). These results lend

credence to the premise that the marked difference in induction of EF-mediated defences of endophyte-infected plants in absence and presence of herbivory may reflect differences in the costs and benefits of offering these rewards under different circumstances (Jaber & Vidal, 2009). Mechanisms driving the negative effects of endophyte infection on EF rewards in herbivore-damaged plants at low nutrient conditions may include competition for limited resources offered by their shared host and/or spatial proximity of resource allocation in the interactions among plants and multiple mutualists.

Cheplick *et al.* (1989) demonstrated that, especially under conditions of low nutrient supply, endophyte infection might incur a “metabolic cost” due to competition between the host and the endophyte for a limited supply of resources; corroborating the suggestion of a substantial endophyte sink from prior studies (Thrower & Lewis, 1973; Smith *et al.*, 1985). Also, several studies (reviewed in Schulz, 2006) showed that when the host plant is stressed and the balanced antagonism between the endophytic fungus and the host is tilted in favour of the fungus; the same endophyte that under certain conditions interacts mutualistically with its host may become pathogenic. However, mutualistic interactions have more frequently developed between endophytic fungi and the roots, because roots (serving as a natural carbon sink of the plant) are in close contact with the environment harbouring these microorganisms and can supply dual- as well as multi-organism symbioses with nutrients (Schulz & Boyle, 2005). Sink strength is a product of its activity and a function of resource availability (Herms & Mattson, 1992). If resources are absorbed by plants in an approximation to the rate of their supply and driven by gradients of concentration, increased competition for these resources may occur when many sinks are developing together, e.g. the root-restricted endophyte (Jaber & Vidal, 2010) and the foliar EF nectaries (i.e. “sugar valves”; see Bently, 1977 and Wäckers *et al.*, 2001). In this case, sinks closer to resources would be expected to benefit first. At low resource conditions, the endophyte sink might not only be strengthened by the spatial proximity to allocate resources from the soil to the endophyte residing in plant roots; but also

by herbivore attack. An intra-plant inter-specific competition between the endophyte and the herbivore, colonizing different plant parts and competing for essential resources supplied by their shared host plant, has been suggested as the potential mechanism underpinning the negative effects of *A. strictum* (Jaber & Vidal, 2009, 2010) and *Acremonium alternatum* (a closely related species; Raps & Vidal, 1998) on several insect herbivores. Accordingly, the herbivore-induced endophyte sink may stimulate increased nutrient uptake from the rest of the plant and therefore receive priority use of limited resources in stressful conditions (in keeping with the “fragile” balanced antagonism of the endophyte-host interactions; Schulz & Boyle, 2005). In addition, the endophyte (already residing in plant roots) may have temporal priority of resource allocation relative to protective arthropods (yet to be EF-recruited). If the herbivore-induced endophyte sink in plant roots is sufficiently strong to impinge on the resources available for other plant functions (including EF defences), limited resources will be diverted to one pathway at the cost of the other resulting in a spatial partitioning of resources. The degree to which partitioning of resources in endophyte-infected herbivore-damaged plants, and the consequent decoupling of endophyte- and EF-mediated defences, will depend on the availability of the resources shared.

For organisms faced with a limited supply of resources for growth and reproduction, defence related trade-offs can be expected because these processes will compete with each other for nutrients that are within the plant and thus available for allocation (Herms & Mattson, 1992; Mole, 1994). Although this idea of “trade-offs” in defences is widely accepted, empirical evidence is scarce. Rehr *et al.* (1973) reported a negative relationship between chemical defence (cyanogenic glycosides) and pugnacious ant mutualists (mediated through foliar nectaries and nutritive structures) in an inter-specific study of *Acacia*, and suggested that maintenance of both ant and chemical defences places unnecessary metabolic burden on the plant. Björkman & Anderson (1990) also reported trade-offs among several defence-related traits in an intra-specific study of *Rubus bogotensis*. A similar trade-off between plant growth

and defence has been framed by Herms & Mattson (1992) who suggested that allocation of resources by plants to chemical and structural defences diverts resources from production of vegetative and reproductive structures. In our study however, a trade-off between plant growth and defence was not evident in *A. strictum*-free nor in *A. strictum*-infected plants; probably due to the decoupling of endophyte- and EF-mediated defences (in the latter) when the available nutrient base was limiting resource allocation to both defences. Induction of EF-mediated defences (which entails a suite of direct and indirect costs; reviewed in Strauss *et al.*, 2002) in endophyte-infected herbivore-damaged plants at conditions of low nutrient availability might have not only restricted the commitment of resources to the endophyte sink, but also to other resource-demanding plant functions (e.g. growth, maintenance, reproduction) known to be highly constrained by low availability of resources (Herms & Mattson, 1992). Accordingly, low nutrient endophyte-infected plants had to reduce resource allocation to EF traits following herbivory in order to maintain similar growth and development patterns as compared to endophyte-free plants. The growth and reproduction parameters measured in our study provide support for this interpretation. Shoot biomass and the onset of flowering were not altered by endophyte infection, even at conditions of low nutrient availability; which is in contrast to some studies (e.g. Cheplick *et al.*, 1989; Ahlholm *et al.*, 2002; Faeth & Sullivan, 2003). Reports on the effects of endophyte infection on host plant growth are, however, notoriously inconsistent and have been mainly obtained from endophyte-grass systems focusing on a few host genotypes or a few specific cultivars (Cheplick, 2007; but see Hesse *et al.*, 2004). On the other hand, both of the plant parameters measured here were depressed by herbivory, while increasing resource availability from low to high nutrient levels alleviated the negative effects of herbivory on these parameters of plant fitness in endophyte-infected and endophyte-free plants similarly.

Herbivory is considered one of the two dominant biotic forces (the second is competition) that affect plant fitness and interact with resource availability to result in fitness trade-offs

associated with different resource allocation patterns in different environments (Stearns, 1976). In the current study, herbivory caused substantial tissue loss and thus presented a strong force acting on the plants. Losses to herbivory were greater (though not significantly so) in endophyte-infected plants due to compensatory consumption triggered by a lower quality of *A. strictum*-infected tissues (Jallow *et al.*, 2004). Increasing nutrient supply improved host plant quality by increasing foliar nitrogen. However, this nutrient-mediated increase in foliar nitrogen was accompanied with a decrease in foliar carbon; which was greatest in endophyte-infected herbivore-damaged plants. Apparently, nutrient addition had strengthened the herbivore-induced endophyte sink that partitioned a larger amount of assimilated carbon to the roots and eventually caused less accumulation of carbon in the foliar plant tissues. Even though not measured here, a higher root C/N ratio might have well occurred in the roots of herbivore-damaged endophyte-infected plants due to the endophyte sink competition. This postulation is supported by the highly significant negative correlation between the foliar C concentration and the amount of endophyte in the roots of inoculated plants among increased nutrient levels ( $F_{1, 106} = 13.231$ ,  $P < 0.0001$ ; linear regression model; data not shown). Increasing nutrient supply was found to increase the extent of root colonization with *A. strictum*, which does not agree with Rasmussen *et al.* (2007) who reported a reduction in the concentration of *Neotyphodium lolii* in infected perennial ryegrass under both increased nitrogen supply and high sugar cultivar. The authors, however, concluded that the negative impact of nutrient supply on fungal (and alkaloid) concentration found in their study appear counterintuitive; but in keeping with the recent new perspectives of the controversial nature of host/endophyte mutualism and that the growth of the endophyte is under continual and dynamic control by the host. Nutrient addition in our study also decreased the foliar C/N ratio of plants on the whole, which is consistent with the carbon/nutrient balance hypothesis (CNB); predicting that increased nutrient uptake in fertile soils decreases the C/N ratio within the plant (Bryant *et al.*, 1983).

The better performance of insects reared on endophyte-free plants at high nutrient level is probably due to the decreased level of foliar carbon within these plants, potentially inhibiting the production of the C-based secondary metabolites (e.g. condensed tannins and phenolics, of which intermediate to high concentrations are reported from *V. faba*; Berger *et al.*, 2003) as growth receives allocation priority (also in line with the CNB theory). This is further corroborated by the highly significant positive relationship found between larval growth rate and the foliar carbon concentration and the significant negative relationships found between the latter and the larval and pupal developmental periods (linear regression analyses; data not shown). The improved insect performance on endophyte-free plants with increased nutrient availability could also be attributed to the increased level of nitrogen (as insects are usually nitrogen-limited; Mattson, 1980), but regression analyses did not support this premise (data not shown). On the other hand, the measured parameters of insect fitness were less responsive to increased nutrient availability when larvae were reared on endophyte-infected plants. This could ostensibly be due to a qualitative and quantitative change in phytosterols (i.e. allelochemicals known to influence the feeding, growth and development of insects) caused by *A. strictum* infection (Dugassa-Gobena *et al.*, 1996), in addition to a resource shunt to the endophyte as nutrient availability to the host plant increases. Such variability in phytosterols within endophyte-infected plants may have deleterious effects on the growth and development of herbivorous insects (Bernays, 1993). Yet at high resource levels, the endophyte-triggered reduction in plant quality would likely be buffered by changes in nutrient supply as shown by our results.

To recap, endophyte-infected plants (following herbivory) bearing (1) costs of resources lost to the endophyte residing in the roots (exacerbated due to herbivore-induced sink competition), (2) costs of resources lost to the herbivores (exacerbated due to herbivore compensatory consumption), and (3) costs of maintaining similar fitness patterns to endophyte-free plants (compliant with the endophyte-host balanced antagonism) may face a

two-edged sword in their EF defences against herbivores at conditions of limited resources: induction of EF traits would decrease attack rates by herbivores through recruiting natural enemies; but could also jeopardize their cost/benefit framework if costs outweigh benefits. Conceivably, trade-offs between defence strategies are likely to take place under such circumstances. When resources are available in abundance, EF traits of endophyte-infected herbivore-damaged plants may divert nutrient reserves accumulated beyond the requirements of the endophyte sink and the plant physiological processes and thus coexist as a complementary; rather than a competing defence alternative. This enforces the adaptive phenotypic nature of EF-defence traits. Plants display phenotypic plasticity which may enable them to assume the most adaptive phenotype in a particular environment in order to buffer the effects of spatial and temporal variation in resource availability (Herms & Mattson, 1992). By the same token, plants may regulate the activity of their EF traits in order to attain a favourable cost/benefit ratio (Bently, 1977).

There are several important caveats to note. First, our understanding of the internal resource base on which different defence traits trade-off is far from being accurate. We generally assume that what is in the environment is also available internally (based on the premise that plants absorb resources in an approximation to the rate of their supply). In this way, plant trade-offs have been considered as a reflection of the environmental micro-economics, which is the basis for conducting fertilizer and enrichment experiments (Mole, 1994) and of which our study (as well as many others; e.g. Cheplick *et al.*, 1989; Cheplick, 2007; Mack & Rudgers, 2008) have no direct analysis. Besides, the short time scale and the lack of estimate or control over other coexisting symbioses (e.g. Mycorrhizae and nitrogen-fixing bacteria) might compromise the generality of our conclusions. Also, including other unmeasured components of plant fitness (e.g. fruit and seed production) may have provided an insight to examining further how costs of harbouring endophytes and herbivory (alone and simultaneous) might influence a wider range of plant functions. However, results from our

study emphasize that the more we are able to simultaneously consider multiple plant partners, the more we may be able to broaden our understanding of the context under which organisms adapt and evolve.

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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 to 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 investigation and whether they would translate into a subsequent generation of the herbivore.
4. *A. 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 artificial diet.
5. *A. 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 to 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.

**Keywords:** *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, since 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 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 unspecialized 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-colonized 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 colonizing plant roots (Hartley & Gange, 2009). In contrast to

both foliar and root endophytes colonizing herbaceous and woody plants, the root-inhabiting mycorrhizae (especially the vesicular-arbuscular mycorrhizae, 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 mycorrhizal fungi; Gange, 2007 for the most recent review of insect-mycorrhiza interactions).

Among the unspecialized root-colonizing 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 Heliiothinae (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, Sequeira, & Short, 2004). Johnson & Zalucki (2005) reported that larvae of generalist feeders do not behave in an equivalent manner on intact plants as compared to plant parts; most likely due to 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 to 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 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*

*V. 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 fertilized 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 malt extract agar media (0.3%) was autoclaved at 120°C for 20 minutes. 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 ( $64 \times 0.025 \text{ mm}^2$ , 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. The inoculum density used here was found sufficient to colonize plant roots in previous studies (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 five 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 14L: 10D photoperiod until hatching. Neonate first-instar larvae (hatching within 12 h) were later used as the first generation (F1) in experiment 1.

#### *Establishment of A. 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-sterilized root pieces and leaf discs. Surface sterilization 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 sterilized 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<sup>-1</sup> 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 H. armigera first generation (F1) to A. 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.

120 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, while the other half were offered leaf discs from E- plants. Leaf discs were replenished as necessary and filter papers were replaced by new ones every 48 h. Petri dishes of E+ and E- treatments were randomized inside an environmental-controlled climatic chamber (25°C, 60% RH and 14L: 10D 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; 5 larvae per plant). Due 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 experiment, some larvae attempted to escape the plants on which they were released and thus were found drowned in water. These larvae were excluded from the calculation of percent 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 days and 11 days after the beginning of the experiment. The relative growth rate (RGR) was calculated according to Farrar *et al.* (1989) as follows:  $RGR = \text{Biomass gained (mg fresh weight)} / [(\text{fresh weight at day 9} + \text{fresh weight at 11 day})/2] \times 2(\text{days})$ . 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 for 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 *H. armigera* second generation (F2) to *A. 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 14L: 10D photoperiod until hatching. Sixty four neonate larvae (n= 64) of the hatching sib group (full sib) from each treatment were reared on standard bean flour-based artificial diet (Teakle, 1991) and served as F2 generation. The life history parameters of the F2 generation were followed as described with 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). *A. 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 analyzed 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 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. Tukey-Kramer 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 randomization 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 Bonferonni correction for multiple testing. All analyses were performed using SYSTAT for Windows, version 12 (SYSTAT, 2008).

## **Results**

### *Establishment of A. 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). 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 *A. strictum* from roots and shoots of inoculated (E+) and control (E-) *V. faba* plants. Values within rows followed by different letters are significantly different ( $P < 0.0001$ ; logistic regression).

	<i>V. faba</i> roots		<i>V. faba</i> shoots	
	E+ plants	E- plants	E+ plants	E- plants
Samples	300	300	250	250
Samples with isolates	273	none	none	27
<i>A. strictum</i> isolates recovered	231	none	none	none
% <i>A. strictum</i> infection	77 <b>a</b>	0 <b>b</b>	0	0

*Experiment 1.*

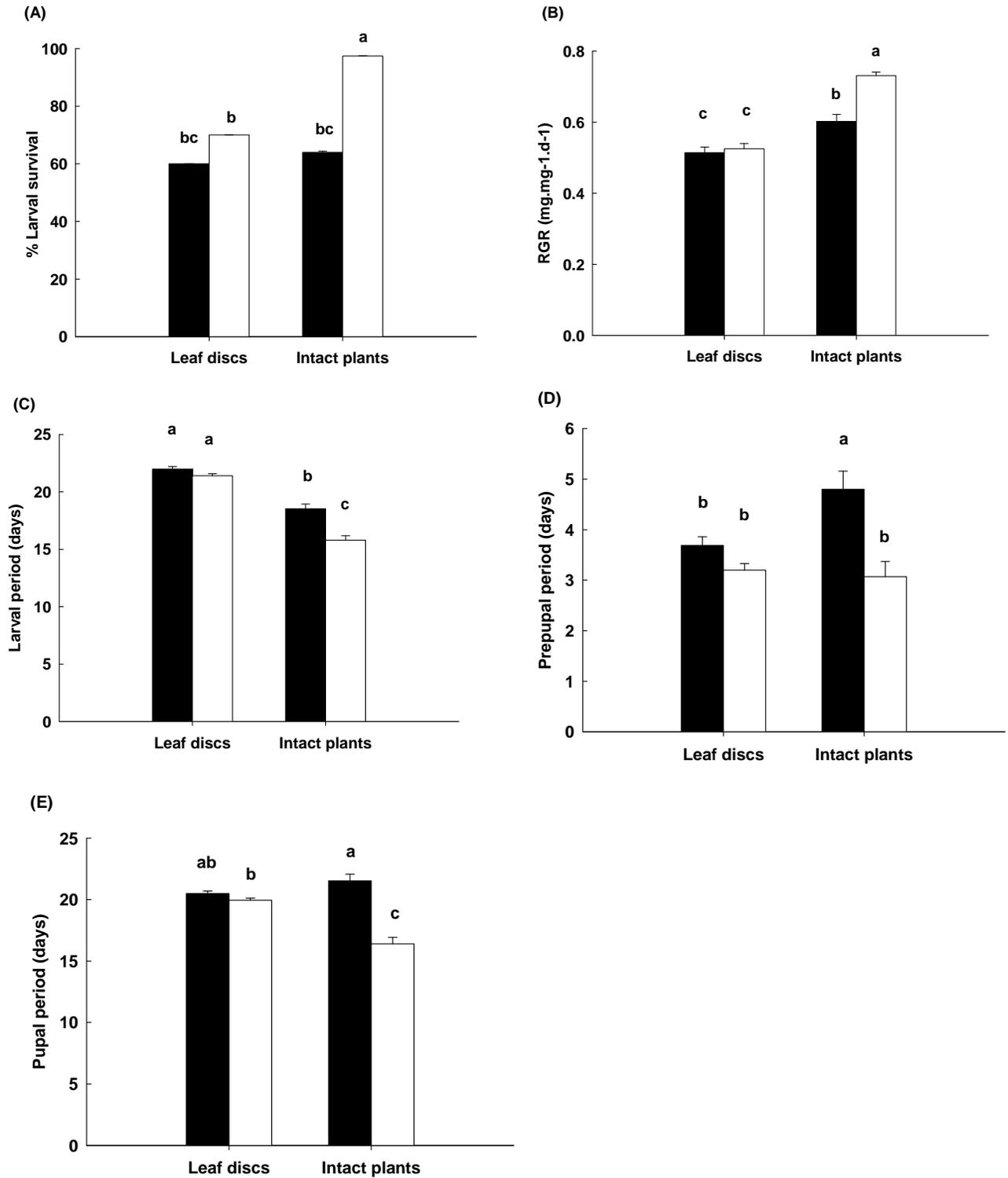
Both main factors, *endophyte infection* and *experimental setting*, had strong significant effects on the life history parameters of *H. armigera* F1 generation and there was a significant interaction between the two factors for all the sampled parameters except pupal weight (Table 2). *A. strictum* negative effects on *H. armigera* fitness were dependent on the experimental setting used (Fig. 1). 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

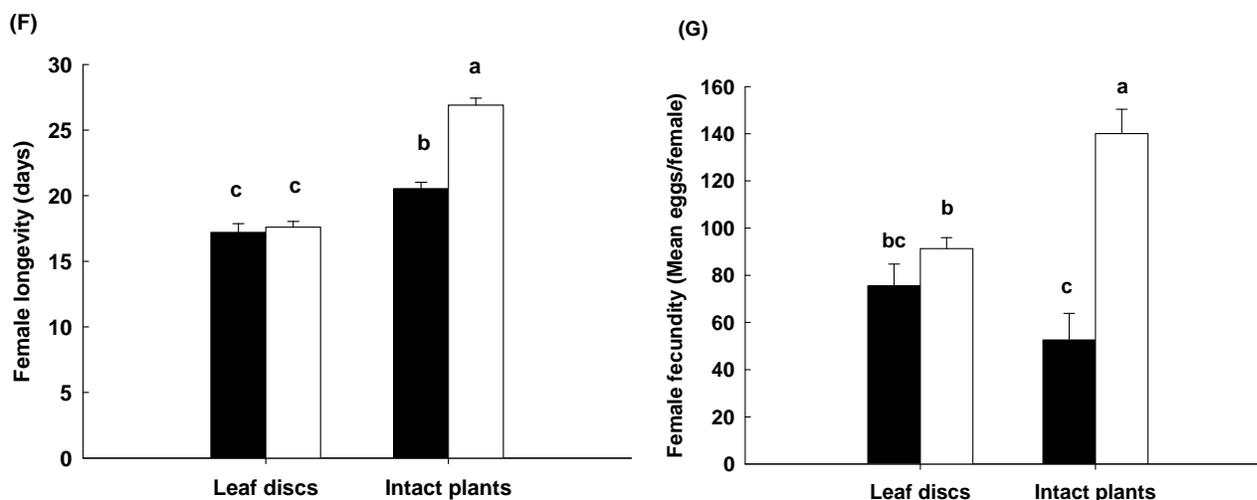
compared to their non-inoculated counterparts ( $P < 0.05$ ; Tukey-Kramer HSD test after two-way ANOVA with Bonferroni correction). None of these parameters differed between the E+

<i>H. armigera</i> life history parameter (F1 generation)	Endophyte infection (I)	Experimental setting (S)	I × S
% larval survival	***	***	***
Relative growth rate (RGR) (mg*mg <sup>-1</sup> *d <sup>-1</sup> )	**	***	***
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/♀)	***	*	***

**Table 2.** Two-way ANOVA ( $P$ -values) for the effects of endophyte infection (I) and experimental setting (S) on the life history parameters of *H. armigera* first generation (F1).  $P$ -values are adjusted by Bonferroni correction for multiple testing.

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). It was slightly larger on E+ treatment in both experimental settings though (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 to leaf discs (Fig. 1).



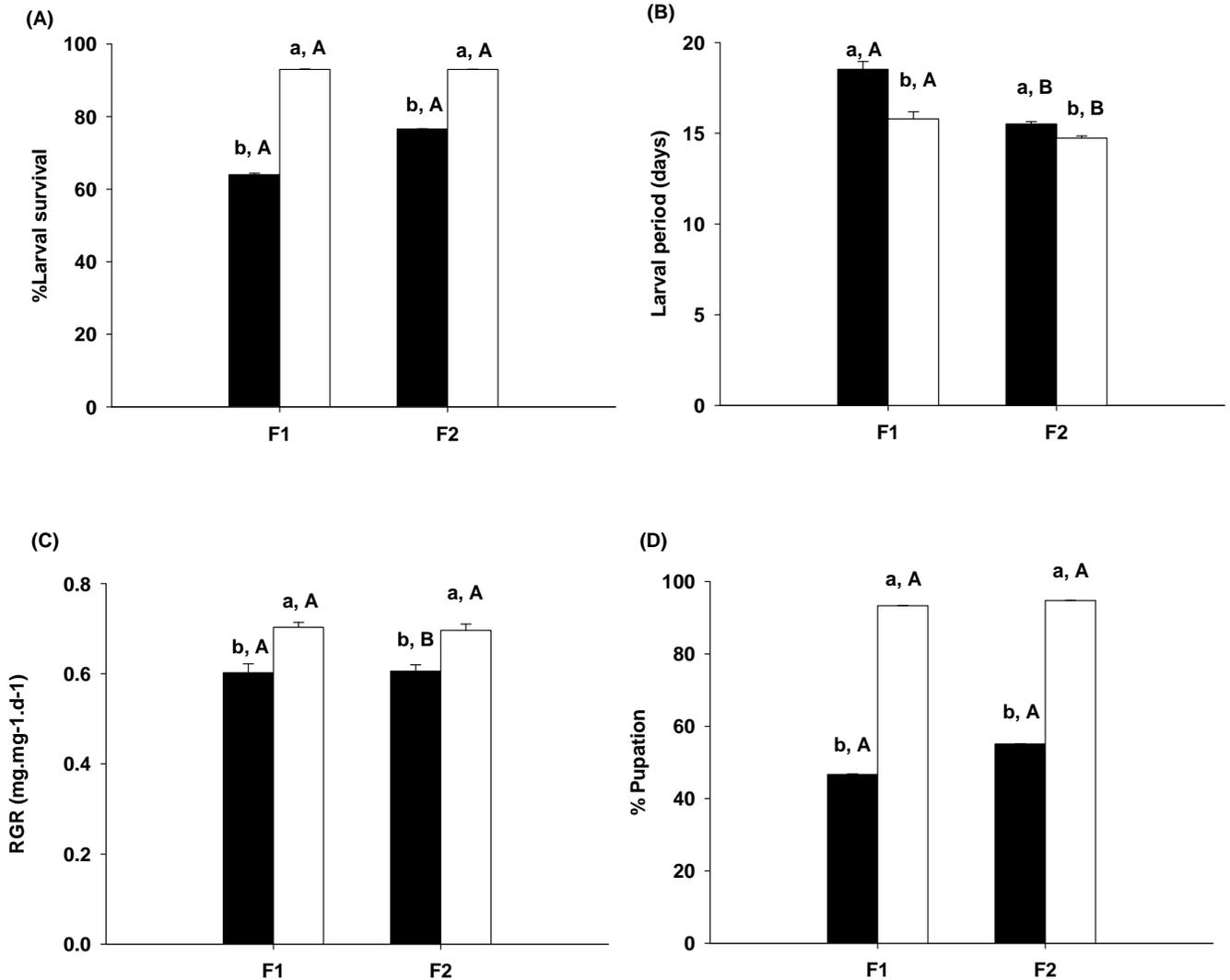


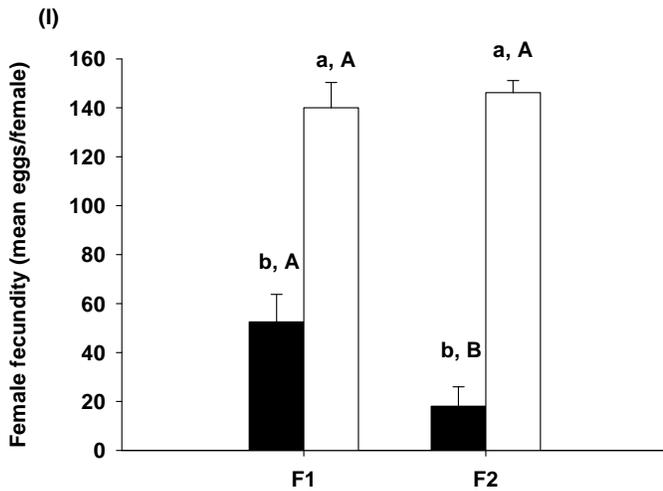
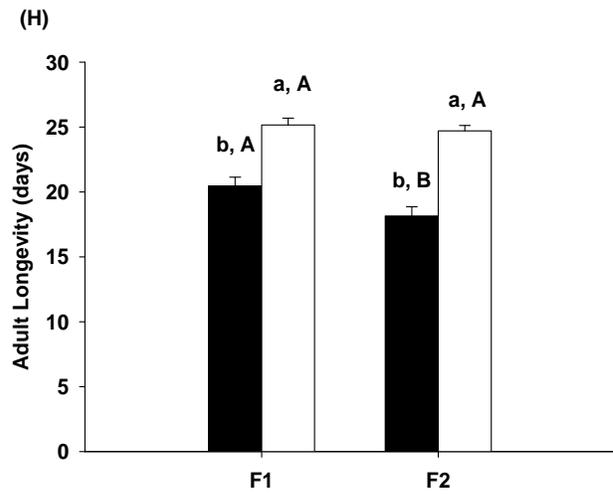
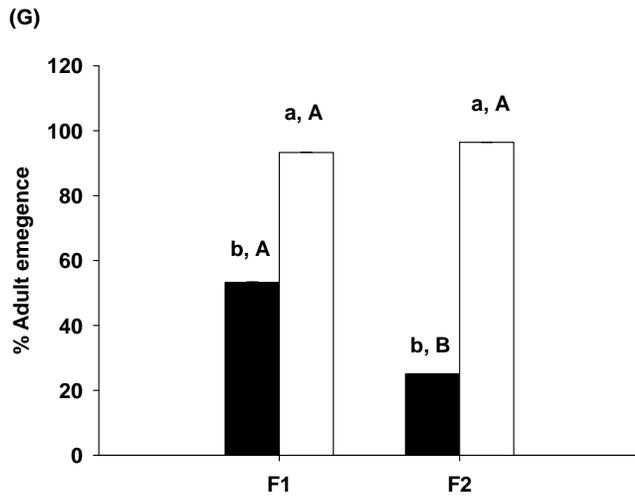
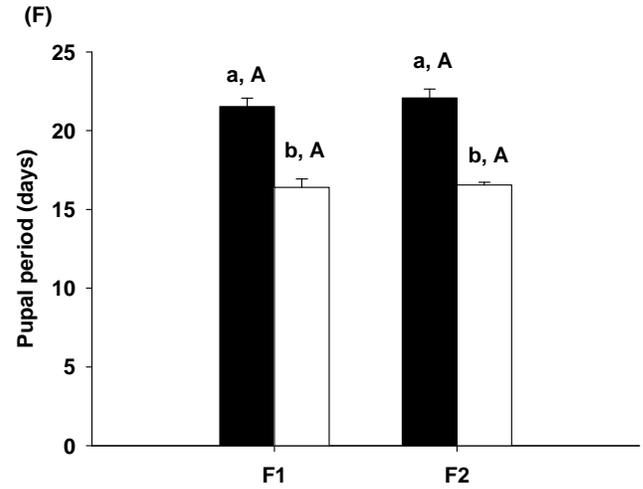
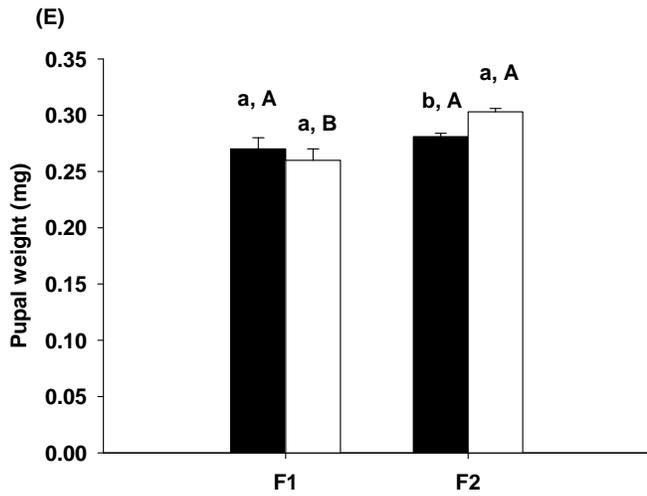
**Figure 1.** Effect of endophyte infection and experimental setting on the life history parameters of *H. armigera* first generation (F1; mean  $\pm$  SE). (A) % larval survival; (B) relative growth rate (RGR) ( $\text{mg}\cdot\text{mg}^{-1}\cdot\text{d}^{-1}$ ); (C) larval period (days); (D) prepupal period (days); (E) pupal period (days); (F) female longevity (days); and (G) female fecundity (mean eggs / female). Insects were either offered leaf discs of *A. 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 *V. faba* plants (E-). We used Tukey-Kramer 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 correction for multiple testing).

#### 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 significantly vary 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 to 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).





**Figure 2.** Life history parameters of two successive generations of *H. armigera* (mean  $\pm$  SE). F1: first generation reared on *A. strictum*-inoculated whole plants (E+; black bars) or non-inoculated plants (E-; white bars); F2: second generation reared on artificial diet after hatching from eggs laid by females of F1 generation (reared on E+ or E- plants; 1 female per treatment). (A) % larval survival; (B) larval period (days); (C) relative growth rate (RGR) ( $\text{mg}\cdot\text{mg}^{-1}\cdot\text{d}^{-1}$ ); (D) % pupation; (E) pupal weight (mg); (F) pupal period (days); (G) % 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 correction for multiple testing).

## 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 first documented evidence that endophyte-mediated negative effects on the 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.

*A. strictum* infection caused significant reductions in larval survival and growth rate, female longevity and fecundity, and a significant delay in moulting and eclosion of *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) as compared 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 due to significant differences between larvae reared on E+ plants as compared to leaf discs of E+ plants, but also to significant differences between those reared on E- plants as compared to leaf discs of E- plants. F2 generation larvae,

reared on 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 F2 generation as compared to 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* colonization is restricted to *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 might have been interrupted by cutting out leaf discs could account for the reduced insect fitness on inoculated plants as compared to 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, while 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 to 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 playing 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 (due to 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 to 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 ill-understood; but are often attributed to indirect and complex factors (Faeth & Hammon, 1997; Jallow *et al.*, 2004; Jallow *et al.*, 2008). *A. 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.R.J., unpublished data) could negatively affect the fitness of *H. armigera* larvae reared on E+ plants in comparison to 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) colonize 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-diversified horizontally-transmitted endophytes) that were reported to negatively impact plant growth (e.g. Schulz *et al.*, 1998; 1999; Hashimoto & Hyakomachi, 2001), *A. strictum* did not alter several measures of *V. faba* fitness after inoculation (L.R.J., unpublished 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 as 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 preys (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 as compared to larvae fed on E- plants (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 (though 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 F1 generation fed on E- intact plants displayed a significant further increase in the 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 hypothesize that the reduction in fitness parameters of insects reared on E+ intact plants in 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 & 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 and for very short time and thus failed to demonstrate an impact of endophytes on plant-herbivore interactions should be revisited. Also, results emerging from studies using 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 colonization 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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## Discussion

Plants are able to respond to herbivore attack by defensive mechanisms that are either “static” (i.e. constitutive) or “active” (i.e. inducible; Gatehouse, 2002). Inducible defences particularly allow plants to be phenotypically plastic (i.e. changing their phenotype in response to their environment; Dicke *et al.*, 2003) that may consequently allow them to minimize fitness costs of resistance (Karban & Baldwin, 1997; Cipollini, 1998; Cipollini *et al.*, 2003). The adaptiveness of phenotypic plasticity in terms of induced defence responses not only depends on environmental abiotic factors affecting the balance of biosynthetic and ecological costs and benefits of defence; but also on a plethora of biotic factors shaping the physiological, chemical, and molecular characteristics of plants in response to attack (Dicke & Hilker, 2003; Dicke *et al.*, 2003). Microorganisms can be important mediators of interactions between plants and macroorganisms (Barbosa *et al.*, 1991), and the role that symbiotic (e.g. Spiteller *et al.*, 2000; Pozo & Azcon-Aguilar, 2007; Kempel *et al.*, 2010) as well as pathogenic (e.g. Cardoza *et al.*, 2002; Rostas *et al.*, 2003) microorganisms may play in the induction process of plant defences against herbivores is just beginning to unfold. For example, Spiteller *et al.* (2000) suggested that the elicitors for the induction of plant volatiles by herbivory is a product of the endosymbiotic bacteria in the herbivore’s gut of which influence may be greater than currently appreciated. More recently, Kempel *et al.* (2010) showed that symbiosis of plants with arbuscular mycorrhizal fungi (AMF) is an important but overlooked trigger of induced resistance to herbivory. In my studies, I present an example on how another microbial symbiont (i.e. fungal endophyte) influences the induction of an indirect form of defence (i.e. the production of EF nectary rewards).

Endophytic colonization of the roots and herbivore infestation induced the production of two EF defence traits (EF nectar volume and EF nectary number) in *V. faba*; only when separately inflicted upon the plants. Both EF rewards were, on the other hand, significantly reduced in plants simultaneously colonized with the fungal endophyte and infested with the herbivore;

which was predicted (from a cost/benefit perspective) as a trade-off between the endophyte- and the EF-mediated defences. However, the patterns in which these rewards are induced against herbivory in endophyte-colonized plants seem to be context-dependent. Different levels of nutrient availability were found to affect the herbivore-induced production of EF rewards in the presence of *A. strictum*; which is consistent with Dicke *et al.* (2003) who premised that the ability of symbiotic microorganisms to influence the dynamics of inducible indirect plant defences against herbivory is in concert with changes induced by abiotic factors. Apparently, as long as the resource base available to *A. strictum*-colonized plants (following herbivory) is enough to feed the costs for the herbivore-induced EF reward production and the costs imposed by the herbivore-derived endophyte sink in the roots; the mutualistic tri-trophic interaction (via EF rewards) will be promoted alongside the mutualistic endophyte-host interaction (via the endophyte-mediated negative effects on the herbivore). In that case, both endophyte- and EF-mediated defences will act *synergistically* (as slow-growing herbivores resulting from the endophyte-derived reduced food digestion and conversion efficiencies will be more exposed to natural enemies than fast-growing herbivores; the slow-growth-high-mortality hypothesis; see Clancy & Price, 1987; Lill & Marquis, 2001) and host plants to both mutualists (endophytes and natural enemies) may ultimately wreak havoc on their herbivore attackers with the deployment of both defences. Should the available resources fall short of satisfying the demands of both defences (and mutualists), plants will actively downscale their EF rewards (which could be misused or counterproductive under certain circumstances) in favor of keeping their mutualistic interaction with the endophyte stable (i.e. preventing it from turning parasitic) and thus uncouple their endophyte- and EF-mediated defences. Taking into account the conditions under which the attraction of natural enemies of herbivores (via induction of plant volatiles or food rewards) do not necessarily benefit the plants (ably discussed in van der Meijden & Klinkhamer, 2000), such synthesis seems intuitively appealing as well.

Ecologists have long understood that the mechanisms responsible for plant defences (including direct defences, indirect defences, and tolerance) incur metabolic trade-offs that could result in fitness costs (Baldwin *et al.*, 2001). Although some studies have revealed that the costs of induced indirect defences are not as high as those of induced direct defences (e.g. Haltitschke *et al.*, 2000; Heil *et al.*, 2000), fitness costs for induced resistance (in general; reviewed in Heil & Baldwin, 2002) can still arise from processes both internal and external to the plant; including costs of allocation of fitness-limiting resources to defence traits, indirect ecological or “environmental” costs, costs related to trade-offs with other defences, and costs resulting from negative influences on plants’ mutualists. An example of the last type of costs is the reduced size and number of root nodules in response to the chemical induction of pathogen resistance in alfalfa and broad bean (Martinez-Abarca *et al.*, 1998; Heil, 2000). Yet from an evolutionary perspective, natural selection on plants will lead to maximizing fitness; which means optimizing the balance between the costs and benefits of defence(s) while reducing fitness loss due to damage. Within the cost/benefit context, if EF rewards are expensive (in terms of production as well as ecological costs; Bently, 1977; Wäckers *et al.*, 2001; Strauss *et al.*, 2002; Mondor *et al.*, 2006); one may expect natural selection *against* the induction of these rewards in circumstances where the cost/benefit ratio is compromised on the whole plant level (e.g. conditions of limited resources that when allocated to defence could not be used for growth and other fitness-relevant processes; see Herms & Mattson, 1992 for more insights on *the growth differentiation balance hypothesis*). Consequently, plants (via phenotypic plasticity through which induced responses are expressed) would reduce investment in EF defence traits that might ultimately lead to fitness costs. Downregulation in the induction of EF rewards (accommodating protective insect mutualists yet to be summoned) under low resource conditions may simultaneously free up the resources required for the enophyte sink; and thus optimize the cost/benefit framework for the finely-tuned host-endophyte mutualistic interaction (accommodating the fungal endophytic mutualist already

residing in the plant's roots; see Schulz & Boyle, 2005 for more details on the hypothesis of *the fragile balance of antagonisms in host-endophyte interactions*).

Even though results from my studies allow tantalizing glimpses of the hitherto unaddressed herbivore-induced EF defence responses in biotic interactions, the processes (on the physiological and/or molecular levels) underlying the complicated metabolic coordination through which endophyte-colonized plants seem to tailor their herbivore-induced EF responses under variable environmental conditions remain elusive. A limited mechanistic understanding of these processes could possibly be derived from the assumption of resource limitation, which must be coped with by controlled shifts in metabolic resource flows from primary metabolism to defence (for insight on further explanations of how trade-offs arise; see Ballhorn *et al.*, 2008). Corresponding to this interpretation and to the hypothesis formulated by Janzen (1966), plants should avoid “superfluous costs” resulting from redundant defences (see also the corroborating prediction given by Heil, 2001). Supporting evidence for this hypothesis comes from studies showing reduced chemical anti-herbivore defence of myrmecophytic plants (i.e. plants that are well-defended against herbivores and pathogens by the action of their mutualistic ants; e.g. Rehr *et al.*, 1973; Seigler & Ebinger, 1987; Heil *et al.*, 1999; Dyer *et al.*, 2001; but also see Heil *et al.*, 2002; Webber & Mckey, 2009). In addition, the activation of induced defence responses does clearly entail a complex reorganization of the plant metabolism in order to reduce potential fitness costs within the context of an “overall defence strategy” of the host plant (Baldwin *et al.*, 2001). For example, studies in the *Nicotiana attenuata-Manduca sexta* system have revealed that the tailoring of induced direct and indirect defence responses is part of a large transcriptional reconfiguration of the host plant (elicited in part by the herbivore oral secretion) that is coordinated to realize fitness benefits (e.g. Halitschke *et al.*, 2000; Kahl *et al.*, 2000; Hermsmeier *et al.*, 2001; Schittko *et al.*, 2001).

If microbes (e.g. endosymbiotic bacteria in the herbivore gut; Spiteller *et al.*, 2000; arbuscular mycorrhizal fungi associating with plants roots; Pozo & Azcon-Aguilar, 2007; Kempel *et al.*, 2010; fungal endophytes colonizing host plants; studies presented here) are indeed involved in the production of the elicitors that plants use to recognize herbivory and call for help from the third trophic level, then the complexity of a tri-trophic network of interactions would increase by adding a fourth trophic level. In that case, shifts in gene-expression patterns (which is largely flexible for inducible defences; Dicke *et al.*, 2003) will allow shifts in the investment in different herbivore-induced EF response patterns of plants in microbial (e.g. endophytic) associations according to the available resource base so as to attain a favorable cost/benefit ratio; not only on the whole plant level, but also on the host plant-microbial symbiont level (e.g. Schulz & Boyle, 2005). However, trade-offs (such as the one presented here) based on a metabolic competition for limited resources between various defences and between those and other plant functions should not exclude the possibility for “*redundancy in defences*” (i.e. plants investing in multiple defences when possible; Romeo *et al.*, 1996); which might be necessary to avoid damage by a complex suite of herbivores as suggested by Steward & Keeler (1988). An alternative theory of “*complementary defence syndromes*”, that has been formalized more recently (Kursar & Coley, 2003; Agrawal & Fishbein, 2006), also emphasizes suites of defences rather than binary trade-offs. In fact, empirical evidence for the concept of trade-offs among different defence systems is not overwhelming; but most studies supporting or refuting defensive trade-offs (which could be difficult to detect; see Simms, 1992; Morris *et al.*, 2006) have so far been investigating trade-offs among direct chemical and morphological plant-based defences (reviewed in Koricheva *et al.*, 2004) or among direct and indirect plant-based defences (e.g. Halitschke *et al.*, 2000; Kahl *et al.*, 2000; Ballhorn *et al.*, 2008). Less studies have investigated trade-offs in defensive strategies including inducible indirect plant defence traits (e.g. EF rewards, domatia, food bodies) mediating defensive mutualisms via the recruitment of protective natural enemies (e.g. Steward & Keeler, 1988;

Dyer *et al.*, 2001; English-Loeb & Norton, 2006), and no studies have ever explored trade-offs among different defensive mutualisms. To the best of my knowledge, studies presented here are the first to probe the concept of trade-off (or synergy) between EF- and endophyte-mediated defensive mutualisms.

My studies also offer a possible explanation for some of the mixed results observed when scrutinizing literature regarding endophyte-host plant-herbivore interactions. The effect of endophytic fungi colonization on herbivory might, at first glance, seem rather idiosyncratic; herbivores sometimes perform better on endophyte-colonized plants (e.g. Gange, 1996; Vicari *et al.*, 2002), sometimes worse (e.g. Raps & Vidal, 1998; McGee, 2002), and sometimes they are not affected by the endophyte colonization (e.g. Bazely *et al.*, 1997; Faeth & Hammon, 1997). Most of these and other studies on endophyte-plant interactions, besides paying no attention to other endosymbiotic microorganisms that would possibly be present and affect the interaction (but see Vicari *et al.*, 2002; Mack & Rudgers, 2008), have been carried out using plant parts instead of normal functioning intact plants. In one of my experiments however, the negative effects of endophyte infection on the herbivore fitness were more evident when larvae foraged freely on endophyte-inoculated intact whole plants than when offered leaf discs of inoculated plants. Such finding suggests that the effects of endophyte-plant interactions on herbivores “in the real world” may be difficult to assess accurately with the artificial experimental settings used in endophyte studies so far. Also, the long-term effect of endophyte-plant interactions on herbivory (potentially lasting across successive generations of the herbivore as shown by my results) has hitherto received very little investigation (e.g. Faeth & Hammon, 1997; Durham & Tannenbaum, 1998) and is still an open venue for future endophyte research.

Studies to date have only scratched the surface of knowledge on how bottom-up forces (such as nutrient availability and also microbial soil biota) can affect the composition of insect communities (including herbivores, carnivores, pollinators, etc.), and how this can

subsequently influence top-down forces (i.e. antagonists in general). However, research on these topics is starting to gain increasing interest and will certainly enhance our understanding of ecology and evolution “in the real world”.

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## Publications

**Jaber LR** & Vidal S (2009) Interactions between an endophytic fungus, aphids, and extrafloral nectaries: do endophytes induce extrafloral-mediated defences in *Vicia faba*? *Functional Ecology*, 23, 707-714

**Jaber LR** & Vidal S (2010) Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. *Ecological Entomology*, 35, 25-36

**Jaber LR** & Vidal S (submitted) Resource-based trade-off in multiple mutualisms: can nutrient availability shift the outcomes of multi-species interactions?

**Jaber LR**, Tefera T & Vidal S (in prep.) The effects of strain and inoculation method on the endophytic establishment of *Beauveria bassiana* (Ascomycota: Hypocreales) and its potential for insect biocontrol

Tefera T, **Jaber LR** & Vidal S (in prep.) Endophytic colonization of *Vicia faba* by *Beauveria bassiana* and the effect on mortality and growth of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

**Jaber LR**, Stahlmann H & Vidal S (in prep.) Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in broad bean and oilseed rape and its potential for insect biocontrol

Stahlmann H, **Jaber LR** & Vidal S (in prep.) Endophytic colonization of *Vicia faba* by *Beauveria bassiana* and its interactions with the rust pathogen *Uromyces vicia-fabae*

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## **PERSONAL DATA**

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## **LANGUAGES**

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- **Arabic:** Mother tongue
- **English:** Excellent user
- **German:** Good user

## **EDUCATION**

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- **1997-2001:** B.Sc. Jordan University of Science and Technology, Faculty of Agriculture, Department of Plant Production. Degree conferred 2001, with a cumulative average of **85.6** and a rating of **Excellent**
  
- **2002-2005:** M.Sc. University of Jordan, Faculty of Agriculture, Department of Horticulture and Plant Protection. Major in Entomology, area of specialization is integrated pest management (IPM). Thesis title **“Integrated Pest Management Program for Controlling *Capnodis carbonaria* and *C. tenebrionis* (Coleoptera: Buprestidae) In Irbid Governate”**. Degree conferred in February, 2005, with a cumulative average of **3.94** and a rating of **Excellent**
  
- **Since 2006:** PhD student in the Agricultural Entomology Section, Department for Crop Sciences, Georg-August-University Goettingen, Germany

## ***HONOR AWARDS and RESEARCH GRANTS***

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- Four times on the Honor Roll during B.Sc. studies with four Honor Awards **(1997-2001)**
- Four Assistantship Grants during M.Sc. studies **(2002-2005)**
- Funding of M.Sc. studies by the Arab Organization of Agricultural Development (AOAD) **(2002-2005)**
- Co-funding of M.Sc. studies by Bayer CropScience **(2002-2005)**
- Gottlieb Daimler-und Karl Benz-Stiftung / Daimler-Benz Scholarship (in the framework of the program „Research in Foreign Countries“) **(2006)**
- Funding of PhD studies by Deutscher Akademischer Austausch Dienst (DAAD) / German Academic Exchange Service **(2006-2010)**

## ***PUBLICATIONS***

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- **Jaber LR** & Vidal S (2009) Interactions between an endophytic fungus, aphids, and extrafloral nectaries: do endophytes induce extrafloral-mediated defences in *Vicia faba*? ***Functional Ecology***, 23, 707-714
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- Tefera T, **Jaber LR** & Vidal S (in prep.) Endophytic colonization of *Vicia faba* by *Beauveria bassiana* and the effect on mortality and growth of *Helicoverpa armigera* (Lepidoptera: Noctuidae)
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## TALKS

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- **DGaaE Entomological Conference** (March 2009, Göttingen)
- **Max-Planck Institute for Chemical Ecology** (June 2009, Jena)
- **Multitrophic Interactions Workshop** (March 2010, Göttingen)

## TEACHING EXPERIENCE

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- **Instructor:** Biological control and Biodiversity (Georg-August University Goettingen, 2008-2010)

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## **PROFESSIONAL SERVICE**

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- **Reviewer:** Journal of Applied Entomology (manuscripts reviewed = 2)

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## **GRADUATE STUDENT TRAINING**

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- **H. Stahlmann:** MSc (Aug 2009 – Jun 2010)
- **L. Verano Irazu:** MSc (Oct 2009 – Feb 2010)