
Volume 9 June 2014
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FUNGAL ECOLOGY 9 (2014) 61–64

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Short communication

Endophytic fungi of grasses protect other plants from aphid herbivory

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ARTICLE INFO

Article history:
Received 1 July 2013
Revision received 2 December 2013
Accepted 17 December 2013
Available online 12 March 2014
Corresponding editor:
James White Jr

Keywords:
Associational protection
Defensive mutualism
Neotyphodium
Protecting symbiont
Symbiosis
Volatile emission

ABSTRACT

Microbial plant symbionts are widely spread in nature changing the way their hosts interact with their environment. *Neotyphodium* fungal endophytes (Clavicipitaceae) are grass symbionts known by their production of alkaloids, toxic for herbivores. Here, we hypothesized that endophytes confer associational protection to non-host plants and change emission of host volatile-compounds. In an outdoor mesocosm experiment, we examined aphid infestation on *Trifolium repens* plants, in response to *Neotyphodium occultans* presence in neighbouring *Lolium multiflorum*. Contrasting levels of *Rhizobium* were generated in *T. repens* plants considering that symbiosis with N_2-fixers would influence herbivore abundance. Aphid infestation increased responding to symbiotic status of *T. repens* plants, when surrounded by non-symbiotic *L. multiflorum* plants. Endophytes modified the volatile imprints of *L. multiflorum* plants measured by an electronic nose, suggesting a potential mechanism to benefit neighbours. This associational protection constitutes a new mutualism trait of grass-endophyte symbiosis, becoming a non-private benefit available to other community partners.

Symbiotic interactions are wide spread phenomena in nature. Historically, these interactions have awakened curiosity even considering the fact that microbial symbionts are common, abundant and diverse inhabitants of plant species. Recent studies suggest that multiple symbionts with different niches change the way in which their partners interact with non-symbiotic neighbours, consumers and other microbial symbionts (Vicari et al., 2002; Omacini et al., 2006, 2012; Kempel et al., 2009; Schausberger et al., 2012).

Vertically-transmitted endophytes (*Neotyphodium* spp., Clavicipitaceae) although less acknowledged than other plant symbionts, live within aerial tissues of many cool-season grasses without forming stromata (Clay and Schardl, 2002; Omacini et al., 2012). These fungal symbionts are viewed as defensive mutualists because the synthesis of bioactive alkaloids in symbiotic plants has been shown to negatively affect invertebrate and vertebrate herbivores (Clay and Schardl, 2002). Despite the fact that alkaloids and their protection can be transferred to a hemiparasitic plant attached to a host (Lehtonen et al., 2005), associational herbivore protection of neighbouring plants mediated by endophytes has not yet been studied.

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http://dx.doi.org/10.1016/j.funeco.2014.01.004

In this study, we hypothesized that legumes which generally coexist with grasses in grasslands and pastures, gain protection from invertebrate herbivores in patches dominated by endophyte symbiotic plants. Additionally, this endophyte-mediated protection can be modified by *Rhizobium* presence, given that symbiosis with nitrogen fixing bacteria influences herbivore abundance on legumes (Sprent, 2007; Dean et al., 2009). To test these hypotheses, we performed an outdoors experiment in mesocosms (pots of 0.30 m diameter and 0.20 m depth) in which four *Trifolium repens* plants were grown surrounded by 16 *Lolium multiflorum* plants (legume-grass mixed mesocosms) or 20 *L. multiflorum* were grown alone (grass pure stands). Plants of *T. repens* and *L. multiflorum* were grown presenting contrasting proportions of symbiotic individuals with *Rhizobium leguminosarum* or *Neotyphodium occultans*, respectively. Four months after sowing, in late spring, natural infestation by aphids was reported in both plant species. Presence of aphids was recorded in 10 *T. repens* leaves and 10 *L. multiflorum* tillers, randomly selected from each mesocosm. Furthermore, to test if endophyte presence induces a change in the array of volatile-compounds emitted in the neighbourhood we used an artificial nose to measure the volatile emission of four patches dominated by non-symbiotic and four patches dominated by symbiotic *L. multiflorum* plants (E− and E+ patches, respectively) randomly distributed in the same experimental field.

The experiment consisted of 24 grass-legume mixed mesocosms and 24 pure grass stands. In both types of mesocosms, we sowed *L. multiflorum* plants from a population with <10 % (E−) or 95 % (E+) symbiotic individuals. Besides, in mixed mesocosms, *T. repens* plants presented contrasting *Rhizobium* symbiotic status (R− or R+). From the combination of *L. multiflorum* symbiotic status (E− or E+) and *T. repens* symbiotic status (R− or R+) we obtained four treatments that were repeated six times. *T. repens* seeds were inoculated with a commercial inoculant (12 g^{-1} of seeds) exclusively containing *R. leguminosarum* biovar *trifolii* bacteria (Ribol, Rizobacter Argentina S.A.) to obtain R+ plants. By contrast, to obtain R− plants, seeds were inoculated with the same quantity of the product previously autoclaved (20 min, 121 °C) to destroy the bacteria. Seeds of E− and E + *L. multiflorum* plants were obtained from the F1 generation of plots (1 m^2) sown with seeds from one population with 95 % endophytic individuals, treated with the fungicide triadimenol (0.5 g pa 100 g^{-1} seeds) or not treated (see details in Omacini et al., 2006, 2009).

Total aphid infestation was analysed given that the number of each aphid species recorded (i.e. *Rhopalosiphum padi*, *Sipha maydis*, *Metopholophium festucae*) was very low and variable among experimental units to analyse each species separately. Analyses were performed with Linear mixed effect models (*lme*) with the package nlme (Pinheiro and Bates, 2009) using statistical software R (R-cran Project). First, in mixed mesocosms, aphid infestation was analysed separately in each plant species. The model included *T. repens* symbiotic status (R− and R+) and *L. multiflorum* symbiotic status (E− and E+) as fixed factors, without random factor. Then, aphid infestation was analysed for *L. multiflorum* including type of microcosm (pure stands vs. mixtures) and endophytic symbiotic status (E− and E+) as fixed factors. Normal distribution of data and homogeneity of variance was evaluated. When necessary, data were log-transformed prior to analysis. The differences between treatments were further evaluated by *post hoc* Tukey's honestly significant difference test (significance: $p < 0.05$). Data of volatile imprints obtained from the nose were ordinated through principal component analysis (PCA) using InfoStat software. Then, the position of each point in the first two principal components was analysed as a MANOVA to detect differences in the volatile imprint of low and high endophyte-level patches.

The percentage of legume leaves naturally infested by aphids depended on the symbiotic status of these plants and of their neighbours (*Rhizobium* × Endophyte: $F_{1,20} = 4.79$, $P = 0.04$). Symbiotic *T. repens* plants were highly protected from aphid herbivory only when surrounded by symbiotic *L. multiflorum* plants (Fig 1A, left panel). In non-rhizobial *T. repens* plants (R−), aphid infestation was very low in both E− and E+ mesocosms while in R+ plants it was about 26 % in E− mesocosms, decreasing to 8 % in E+ mesocosms. In mixed mesocosms, aphid infestation in *L. multiflorum* plants depended exclusively on their own symbiotic status (E: $F_{1,20} = 5.81$, $P = 0.02$, Fig 1A, right panel), which proved to be three times higher in E− than in E+. Surprisingly, the magnitude of these protections measured as the decrease in aphid natural infestation was similar for both plant species. It is noteworthy that aphid infestation was remarkably lower in *L. multiflorum* pure stands (E−: 13 % ± 1.47 %; E+: 1.6 ± 0.16 %) than in mixed mesocosms ($F_{1,44} = 5.52$, $P = 0.02$) suggesting that *T. repens* presence increased *L. multiflorum* aphid infestation, irrespective of the symbiotic status.

The negative impacts of *Neotyphodium* on invertebrate herbivores have been documented since last century (Clay, 1988), and alkaloids in host tissues have been suggested as the main mechanism (Bush et al., 1997; Wilkinson et al., 2000; Clay and Schardl, 2002; Faeth et al., 2002). However, the endophyte effect on herbivory on non-host plants within the community has been scarcely studied (Lehtonen et al., 2005; Omacini et al., 2009). What is more, this protection has never been documented in a neighbouring plant without alkaloids. As regards the role of *Rhizobium*, it is possible to assess that it promotes aphid infestation, but this promotion was disrupted by endophyte presence in another plant present in the same community. It is relevant, taking into account that *Rhizobium* usually increases N content and N_2-fixation increasing legume attractiveness (Sprent, 2007). However, as we did not measure changes in plant quality, we cannot disregard other changes induced by *Rhizobium* presence on patch morphological or chemical characteristics that can also modify herbivore abundance (Kempel et al., 2009, Dean et al., 2009).

Less studied than alkaloids, volatiles emission can also be modified by endophyte presence and must be considered in future experiments. Volatile imprints produced by patches with different endophyte status were different according to our PCA + MANOVA analyses (Fig 1B). The two principal components of the ordination explained 55 % and 22 % of variability in the emission of volatiles. Axis scores were tested using MANOVA and significant differences were found in the imprints of E+ and E− patches ($F_{2,5} = 8.8$, $P = 0.023$). In this sense, changes in herbivory due to volatile emission induced by symbionts were studied both for mycorrhizal fungi (Schausberger et al., 2012) and *Rhizobium* bacteria (Kempel

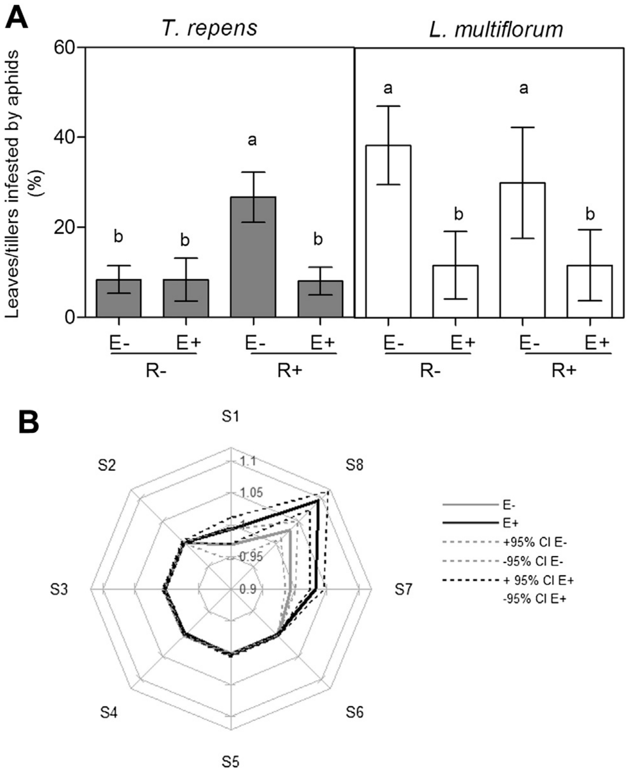

Fig 1 – (A) Leaves infested by aphids (%, mean + SE, *n* = 6) of non-inoculated (R−) or *Rhizobium*-inoculated (R+) *T. repens* plants (grey bars, left panel) growing in neighbourhoods conformed by low endophyte (E−) or high endophyte (E+) *L. multiflorum* plants. Tillers infested by aphids (%, means + SE, *n* = 6) of these *L. multiflorum* plants are shown in right panels (white bars). Different letters indicate significant differences between treatments (Tukey test, $P < 0.05$). Analyses were performed separately for each species. (B) Volatile emission imprints of low endophyte (E−, grey lines) or high endophyte (E+, black lines) *L. multiflorum* patches obtained from eight different sensors (from S1 to S8). Each axis represents a sensor while each vertex of the imprint is the corresponding average value (continuous line) and upper and lower 95 % confidence interval (+95 % CI and −95 % CI, dashed lines). Value equal to 1 implies that it is equal to pure air pattern.

et al., 2009). However, for *Neotyphodium* endophytes, only the volatile change in tall fescue and *Lolium perenne* has been described (Yue et al., 2001; Pańka et al., 2013) without knowing its implications on other interactions. Our results suggest that changes in the characteristics of the volatile emissions induced by endophyte presence may explain aphid selection of a patch both directly, through E+ plants volatile chemicals deterring herbivores or indirectly, inducing defences in *T. repens* (Agrawal, 1998; Kessler and Baldwin, 2001).

This study appears to be novel in showing that herbivore protection conferred to the host by fungal endophytes can be extended to a neighbouring plant, which supports our hypothesis. Furthermore, we detected that endophytes can modify volatile imprints of *L. multiflorum* plants, though it is uncertain if this is the mechanism behind aphid's behavioural avoidance of symbiotic *T. repens* plants. Counteracting effects of both symbionts on each host performance and interactions arising from them can have important consequences for ecosystem functioning. A wide range of new questions arise in relation to the forces structuring communities, as endophytic symbiosis can affect top-down and bottom-up controls by modifying complex interactions over the host plant neighbourhood (Chaneton and Omacini, 2007; Faeth and Shochat, 2010).

Taken together, our study suggests a new mutualism trait for the grass-endophyte symbiosis. Associational protection conferred by endophyte to the host grass is not a private benefit as it became available to other community partners in a pasture.

Acknowledgements

We are very grateful to Gonzalo Molina for his assistance in electronic nose utilization, and Beatriz Santos, Fernando Lattanzi, Marta Telesnicki, Luis I. Perez and Luciana D'Acunto for valuable comments on the results and the manuscript. We are also grateful to two anonymous reviewers whose comments increase the value of the manuscript. P. García Parisi was supported by a doctoral fellowship from CONICET (Argentina). This study was supported by University of Buenos Aires and by grants from CONICET-ANPCyT (PICT 1525).

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