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Palatability of Norway spruce needles infected with *Lophodermium piceae* to larvae of two sawfly species

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Highlights

- We describe the first experiment including both endophyte inhabited and endophyte free intact needles of Norway spruce for examining the possible effect of endophytes on the palatability of needles to insect larvae.
- The extent of needles consumed by neither of two sawfly species, *Neodiprion sertifer* and *Gilpina pallida,* differed depending on endophyte presence.

Abstract

Unequivocal evidence on the antagonistic effects of endophytic fungi associated with woody plants against insect herbivores has been documented in only a few cases so far. Experimental evidence of the significance of needle endophytes to coniferous trees has remained scant because it is difficult to obtain trees with needles free of endophytes that could be used as comparable controls for trees infected with endophytes. Previously we reported a new methodology to get Norway spruce (*Picea abies* (L.) Karst.) saplings without needle endophytes and to inoculate them with a needle endophyte *Lophodermium piceae* (Fuckel) Höhn. Here we describe the first trial where spruce saplings with and without needle endophytes were provided as substrate for insect larvae. We transferred larvae of two sawfly species, *Neodiprion sertifer* Geoffroy and *Gilpina pallida* Klug, to the seedlings. Even though the main host of these sawfly species is not Norway spruce, but Scots pine (*Pinus sylvestris* L.), they are also known to occasionally feed on spruce. In this experiment the larvae did not develop to pupae with the provided spruce substrate but consumed measurable amounts of needles. No significant difference was found between the extent of needles consumed by either of the two sawfly species in inoculated and uninoculated saplings.

Keywords *Gilpinia pallida*; *Neodiprion sertifer*; antagonism; fungi; herbivory; palatability
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1 Introduction

Probably all conifers harbour diverse endophytic fungi in their foliage (Carroll et al. 1977; Carroll and Carroll 1978; Petrini and Carroll 1981; Sieber 1988; Hata and Futai 1996; Hoffman and Arnold 2008) but the significance of these fungi to the host tree is still largely unknown. Several correlative studies suggest antagonistic effects of some endophytic fungi against insect herbivory on coniferous trees (Diamandis 1981; Lasota et al. 1983; Carroll 1988, 1995). Thus far experimental evidence has only been obtained with a rugulosin-producing endophyte *Phialocephala scopiformis* T. Kowalski & Kehr where its presence in *Picea glauca* Moench (Voss) trees impaired the growth of eastern spruce budworms (Miller et al. 2008; Quiring et al. 2020). *P. scopiformis* can be inoculated by injecting macerated mycelial solution into spruce twigs because the fungus is apparently able to colonise nearby needles (Sumarah et al. 2005; Sumarah et al. 2008). This so-called wound-inoculation method would probably not be successful with most Norway spruce needle endophytes because the fungal community of Norway spruce twigs differs largely from that of needles (Sieber 1988, 1989; Müller and Hallaksela 2000). This suggests that they colonise only small restricted tissue parts and/or are unable to penetrate the needle petiole.

Norway spruce (*Picea abies* (L.) Karst.) is a widespread conifer species native in Eurasian boreal forests and its needles are commonly infected by the endophytic fungus *Lophodermium piceae* (Fuckel) Höhn (Sieber 1988; Müller and Hallaksela 1998). Whether the relation between *L. piceae* and its host tree is a parasitic or mutualistic is still unsolved. This question is intriguing not only because Norway spruce has a high economic significance in its wide distribution area, but also because this conifer is rather resistant against needle-feeding insects. Large scale defoliation of Norway spruce by arthropods has so far not been reported in northern Europe. Spruce sawflies like *Pristiphora abietina* Christ and *Gilpina hercyniae* Hartig, and mites like *Nalepella haarlovi* Boczeck var. *picea-abietis* Löyttyniemi occur in Finland but do not cause serious damage in Norway spruce forests (Löyttyniemi 1972; Viitasaari and Varama 1987; Uotila and Kankaanhuhta 2003). The polyphagous nun moth *Lymantria monacha* L. has caused severe damages to Norway spruce forests in Central Europe (Bejer 1988; Liska and Srutka 1998) but in Finland this insect has occurred only sporadically and inconspicuously in southern coastal forests, until recently when a few small outbreaks have been reported (Fält-Nardmann et al. 2018).

Instead, for instance on Scots pine, the other major conifer tree in northern Europe, considerable outbreaks by sawfly species like *Diprion pini* L., *Neodiprion sertifer* Geoffroy, *Gilpina pallida* Klug and *Acantholyda posticalis* Matsumura occur from time to time (Christiansen 1970; Hanski 1987; Nevalainen et al. 2010, 2015; Voolma et al. 2016; Neuvonen and Viiri 2017).

Interestingly, the endophytic community of Scots pine needles is not dominated by a single fungal species while needles of Norway spruce are dominated by one species (Millberg et al. 2015). Often over 90% of Norway spruce needles are infected by endophytic fungi (Barklund 1987; Sieber 1988; Müller and Hallaksela 1998; Lehtijärvi and Barklund 2000) and generally one species, *L. piceae,* accounts for over 90% of the endophytic community (Sieber 1988; Müller and Hallaskela 1998). Individual infections of *L. piceae* are restricted to very small tissue areas as demonstrated by electron microscopic investigations (Suske and Acker 1987, 1989) and up to 34 infections have been isolated from single green needles (Müller et al. 2001). This restricted infection habit means that wound-inoculation of twigs with this fungus would probably not result in colonization of nearby needles similarly as with *P. scopiformis* mentioned above. The infections by *L. piceae* in needles are persistent and can exist in a dormant mode for at least six years within the needles (Müller et al. 2021).

Production of antifungal and herbicidal secondary metabolites is a common feature among plant endophytes (Schulz et al. 1999; Miller et al. 2008; Tanney et al. 2018; Jia et al. 2020). During a comprehensive screening program for biologically active secondary metabolites Schulz et al. (1999) detected production of herbicidally active substances three times more frequently among plant endophytes than among soil isolates. Endophytes can also increase needle resistance by triggering the host to produce antagonistic substances against fungal infections (Zhao et al. 2022).

We recently reported a method for eradicating endophytic fungi from needles of Norway spruce saplings (Müller and Hamberg 2021). In that publication we also described how endophyte-free intact needles can be inoculated with *L. piceae* in an outdoor experiment where the saplings were confined within plastic bags to prevent infections by ambient airborne spores. Availability of both endophyte-inhabited and endophyte-free saplings enables to carry out herbivory experiments with various needle feeding arthropods. Using this method, we aimed to investigate whether infection of Norway spruce needles with *L. piceae* affects their palatability to sawfly larvae.

2 Material and methods

2.1 Spruce saplings

Eighteen Norway spruce saplings, six of each of three clones (T4658, T4748 and T5134 derived from rooted cuttings and generated at Luke from trees in Central Finland in 2006) were heat treated for eradication of needle endophytes (Müller and Hamberg 2021). Then the saplings were transplanted to a shady garden site outdoors in Helsinki in October 2008 in groups of three seedlings per group including one sapling of each clone. Each sapling group was covered with a polyethylene bag (0.25 mm) to prevent fungal infections from the ambient air. Thereafter half of the sapling groups were inoculated with *L. piceae* and the rest were used as controls. Inoculation was achieved by placing naturally shed, highly infected needles on a metal sieve (mesh size 5 mm) that was suspended above each group of three saplings (Müller and Hamberg 2021).

Three sapling groups (in total nine saplings) were inoculated repeatedly during 2010–2012 using brown needles obtained from the ground underneath mature Norway spruce trees heavily infected with *L. piceae* as described by Müller and Hamberg (2021). The rest of the saplings (nine saplings) were not inoculated and served as controls. In summer 2012 the saplings, now at the age of 6 years and a height of 50–65 cm, were considered big enough for a palatability experiment. The inoculation success was assessed from needles of both the inoculated and control saplings each year in November. For this assessment needles of the three youngest age classes were taken (three needles per age class and sapling, i.e., 3 needles × 3 age classes × 3 saplings per group × 3 sapling groups, i.e., in total 81 needles) as described previously (Müller and Hamberg 2021). These needles were then surface sterilized, cut into ca. 2 mm sections that were incubated in water agar. Hyphae outgrowing from the needle sections were isolated and a randomly chosen batch of these isolates were identified based on their ITS sequences as described earlier (Müller and Hamberg 2021). The current year needles of inoculated saplings were infected in 2012 with *L. piceae* to a reasonably high level of 50%.

After the palatability experiment all seedlings were cut at their stem base and dried at 105 °C for three days whereafter dry weight was determined.

2.2 Palatability of spruce needles to pine sawfly larvae

In years 2011 and 2012, we did not find enough larvae of insect species feeding primarily on Norway spruce. Instead, larvae of two pine sawfly species, *N. sertifer* and *G. pallida*, were available in high numbers in pure Scots pine and mixed Scots pine – Norway spruce stands. Both *N. sertifer* and *G. pallida* are common pests in Scots pine forests of northern Europe but occasionally they feed also on needles of nearby Norway spruces (Pschorn-Walcher 1982). Thus, we tested palatability of endophyte-infected and endophyte-free needles of Norway spruce saplings to the larvae of these two insect species.

Pine branches supporting *N. sertifer* eggs were collected from a pine forest in Puumala, eastern Finland. They were incubated in the laboratory until the sawfly eggs hatched. Larvae of *G. pallida* were obtained from a rearing experiment initiated with larvae originating from a pine forest in Asikkala, southern Finland. Both sawfly species were reared in 10 litre plastic containers covered with nylon netting as described by Lindstedt et al. (2006). Larvae were fed twice a week with fresh pine branches until they were transferred to the spruce saplings.

Before transferring the larvae to twigs of the experimental saplings, the polyethylene bags covering the saplings were removed and each sapling was confined with a metal netting (height 70 cm, diameter 25 cm, mesh size 5 × 10 cm) and a nylon fabric (mesh size 1 mm) to prevent larvae from moving from one sapling to another (Fig. 1). The nylon fabric was tied around the

Fig. 1. Before transferring sawfly larvae to each sapling, the saplings were confined by a metal netting and a white nylon fabric to prevent larvae from moving from one sapling to another during the feeding experiment.

stem base of the sapling and 2 dl of bryophytes was filled to the stem base within the nylon fabric. On June 14th 2012 second and third instar larvae of *N. sertifer* were transferred to twig tips of 6-year-old Norway spruce saplings (50–65 cm high, detailed preparative treatment described in Müller and Hamberg 2021). Seven saplings (four *L. piceae* -inoculated and three non-inoculated control saplings) received 22 larvae each, in groups of 9–12 larvae per twig tip. Second and third instar larvae of *G. pallida* were transferred on August 13th 2012 to 8 saplings (four *L. piceae* -inoculated and four non-inoculated control saplings). Each sapling received 30 larvae, in groups of 10 larvae per twig tip (Fig. 2a).

Defoliation by the larvae was measured in September 14th, 2012 by measuring the twig length (cm) where all needles had disappeared (Fig. 2b). At the same time, the bryophytes were also removed and checked for possible pupae of sawflies.

Fig. 2. Norway spruce shoot a) with larvae of *Gilpina pallida* feeding on needles and b) after feeding.

2.3 Statistical analyses

The effect of *L. piceae* infection on palatability of Norway spruce twigs to larvae of *N. sertifer* ($n = 7$ saplings) and *G. pallida* ($n = 8$ saplings) was investigated with a linear model (LM) in *stats* package separately for each sawfly larvae species. (R Core Team 2017). A separate analysis was done using combined data of both larvae species. Our response variable, the extent of damage caused by a sawfly species along a twig (cm), was square root transformed to meet normality assumptions (checked by using residual plots). As an explanatory variable we used a factor describing whether a twig was infected with *L. piceae* or not: 1 = sapling was not infected, 2 = sapling was infected.

3 Results

Even though larvae of *N. sertifer* and *G. pallida* started to feed eagerly on needles of the spruce saplings, palatability of the provided needles to these two sawfly species turned out to be low regardless of the presence of endophytic infections by *L. piceae*. The larvae of *N. sertifer* disappeared within two weeks from the saplings and those of *G. pallida* had disappeared on September 13th (one month after their introduction to the saplings) when the saplings were finally inspected. No pupae were found in the bryophytes litter placed below the saplings. However, during their foraging on the saplings the larvae caused measurable needle loss. The extent of needle loss (twig length where needles were eaten) did not differ significantly between inoculated and non-inoculated saplings (LM, $t_5 = 790$, $p = 0.465$ and $t_6 = –0.660$, $p = 0.534$ for *N. sertifer* and *G. pallida*, respectively, Table 1). When data obtained with the two sawfly species were combined, the difference of needle loss between the endophyte infected and the non-infected saplings (4.3 and 4.2 cm/sapling, respectively) was negligible and statistically insignificant (LM, $t_{13} = 0.289$, $p = 0.777$). The larvae fed only on the current year needles 50% of which were found to be infected by *L. piceae* in inoculated saplings while uninoculated needles showed no infections (Müller and Hamberg 2021). The mean dry weight of the sapling shoots after the palatability experiment was 44, 45 and 69 g for clones T4658, T4748 and T5134, respectively. Variation between saplings was high and the standard deviation varied among clones from 44% to 69% compared to the mean weight of saplings of each clone.

Table 1. Palatability of Norway spruce needles to larvae of the pine sawflies *Neodiprion sertifer* and *Gilpina pallida*. Palatability was measured as twig length where all needles had been eaten. Mean values for eaten twig length are presented. Eaten twig length did not significantly differ between *Lophodermium piceae* inoculated and non-inoculated saplings.

Treatment	Number of saplings		Average twig length eaten (cm) per sapling (StDev)		*t*	*p*
	Inoculated with *Lophodermium piceae*	Non-inoculated	Inoculated with *Lophodermium piceae*	Non-inoculated		
Larvae of *Neodiprion sertifer*	4	3	5.0 (6.4)	2.0 (3.5)	0.790	0.465
Larvae of *Gilpina pallida*	4	4	3.5 (0.6)	5.9 (4.3)	–0.660	0.534
Control[a]	1	2	0.0	0.0	-	-
Results for *N. sertifer* and *G. pallida* combined	8	7	4.3 (4.2)	4.2 (4.2)	0.289	0.777

[a] = no sawfly larvae added

4 Discussion

Presence of the endophytic fungus *L. piceae* did not affect palatability of the needles to the two sawfly species tested in this experiment. Even though larvae of the two sawfly species are occasionally observed feeding also on Norway spruce needles, in this experiment, palatability of spruce needles to these sawfly larvae turned out to be low and therefore, the obtained evidence is not strong. However, the experimental set up used in this study provides an approach enabling similar tests to be done with other arthropod herbivores and pathogens.

The number of spruce saplings could have been higher in this experiment. The high standard error values for the twig lengths eaten (Table 1) and the standard deviation values for the sapling shoot dry mass of each clone at the end of the experiment indicate a high variation between replicate saplings. Thus, even when using clonal spruce saplings considerably higher replicate numbers should be used in order to identify small effects. Some improvement in aiming to reduce variation between replicate saplings could probably be achieved by initially selecting similar looking cuttings for the experiment.

This experiment should be repeated with other sawfly species specialised to Norway spruce. One challenge in these experiments is rearing of their larvae for application to the experimental trees. Relevant species include sawflies *P. abietina* and *G. hercyniae* and mites like *N. haarlovi* because they are native in northern Europe and their moderate to low occurrence could be a sign of sensitivity to *L. piceae* which is so common in needles of Norway spruce (Sieber 1988; Müller et al. 2019). The needle-feeding nun moth *L. monacha* can cause large outbreaks in spruce forests (Bejer 1988; Liska and Srutka 1998) and could therefore be expected to be restricted by other factors than occurrence of *L. piceae* in the spruce needles.

Declaration of openness of the research materials

The research material is available from the author on reasonable request.

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Authors’ contributions

Michael Müller designed and performed the field experiment and wrote the original draft manuscript. Martti Varama provided the sawfly larvae. Leena Hamberg made the statistical analyses. All authors have participated in manuscript preparation, and read and agreed to this revised version of the manuscript.

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