Letters

Low synthesis of secondary compounds in the lichen *Lobaria pulmonaria* infected by the lichenicolous fungus *Plectocarpon lichenum*

Introduction

A lichen thallus may function as a self-sustaining micro-ecosystem including organisms from various kingdoms and taxonomic groups. In its simplest form, a lichen is an assemblage of one species-specific fungal host (the mycobiont), giving name to the particular symbiotic association, and fed by an associated eukaryotic and/or a prokaryotic photosynthetic partner (the photobiont). Together these partners sometimes form a lichen thallus in culture in the absence of additional organisms (Marton & Galun, 1976; Ahmadjian & Jacobs, 1983; Stocker-Wörgötter, 2001). Over the years, a number of additional associates have been identified. These include > 1800 lichenicolous fungi, defined as fungi living on lichens (Hawksworth, 1982; Lawrey & Diederich, 2003, 2016), various bacteria (Grube & Berg, 2009), and lately the discovery of symbiotic basidiomycete yeasts that contribute to lichen phenotype variation (Spribille *et al.*, 2016). The nature of associations with such additional partners apparently ranges from mutualistic symbiosis (e.g. bacteria involved in vitamin production and stress protection) to commensalism and parasitism (Lawrey & Diederich, 2003; Erlacher *et al.*, 2015). Others are saprophytic or start as parasites and then live as saprophytes after the demise of the host (Hawksworth, 1982). Yet, the knowledge on how lichenicolous fungi influence lichen fitness is fragmentary and incomplete. There is thus a need for more experimental field studies of growth and secondary metabolism in lichens associated with and without additional organisms.

Most lichens produce carbon-based secondary compounds (CBSCs) providing protection against various biotic and abiotic stressors such as high solar radiation, predators and pathogens (as reviewed by Lawrey, 2009; Solhaug & Gauslaa, 2012; Asplund & Wardle, 2017). Given the antimicrobial effect of many CBSCs, one may assume that they also deter some lichenicolous fungi. This may apply for generalist and opportunistic saprophytic fungi, whereas specialized lichenicolous fungi often tolerate their hosts' secondary compounds (Lawrey, 1993; Lawrey & Diederich, 2003). Some parasitic fungi may even degrade their hosts' secondary compounds (Lawrey *et al.*, 1999; Edwards *et al.*, 2017).

The lichens *Lobaria pulmonaria* and *L. scrobiculata* infected with highly host-specific *Plectocarpon* species have lower CBSC concentrations than sympatric thalli without infection symptoms (galls) (Merinero *et al.*, 2015a; Asplund *et al.*, 2016). However, we do not know whether these specialized lichenicolous fungi reduce CBSC concentrations in infected thalli, or simply inhabit poorly defended thalli of populations that typically display a wide variation in CBSC content (Vatne *et al.*, 2011). CBSCs in *L. pulmonaria* comprising the stictic acid complex serve as defence against lichen-feeding snails, but whether they also impair lichenicolous fungi is unknown (Asplund & Gauslaa, 2008). Because *Plectocarpon* species visibly modify only local parts of the otherwise healthy hosts' thalli by gall formation, they are considered commensalistic lichenicolous fungi (Ertz *et al.*, 2005). However, growth is an important driver of fitness, and a recent short-term growth chamber experiment showed that *Plectocarpon* spp. reduce the growth of *L. pulmonaria* and *L. scrobiculata* (Merinero & Gauslaa, 2018). Yet we do not know whether this pattern also occurs under long-term natural field conditions.

Here, we experimentally reduced the concentration of CBSCs in pairs of *L. pulmonaria* with and without galls of *Plectocarpon lichenum* and transplanted these in their original habitat for 7 months. We used this experimental set-up to study whether thalli infected with *P. lichenum* have lower synthesis of lichen CBSCs and/or lower lichen growth rates than thalli without galls. Because these functions influence lichen fitness, such experiments would allow a functional characterization of the interplay between these specialized lichenicolous fungi and their hosts.

Materials and Methods

We sampled the epiphytic lichen *L. pulmonaria* with and without *P. lichenum* galls in a mixed oak–beech forest in central Spain. Recent studies show that *Plectocarpon* is only localized to the gall and nearby lichen tissue (Bergmann & Werth, 2017), suggesting that asymptomatic thalli (or thalli without galls) are probably non-infected. For each thallus with *Plectocarpon* galls ($n = 18$), we collected a similar sized thallus without galls and with a similar number of reproductive structures (soralia) from the same tree. Each thallus was cut in two pieces of similar size. One piece was rinsed in 100% acetone for 40 min, and the control was not rinsed in acetone. This treatment non-destructively and substantially reduces the lichen CBSC concentration (Solhaug & Gauslaa, 2001; Asplund & Gauslaa, 2008).

We transplanted thalli on oak (*Quercus pyrenaica* Willd.) trunks in groups of four–two non-rinsed thalli (with and without *Plectocarpon* galls) together with their acetone-rinsed counterparts – for 201 d in their source site. By measuring CBSCs and biomass before and after the transplantation, we calculated change in CBSC relative to start values and relative growth rate (RGR). Detailed

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descriptions of the materials and methods are given in Supporting Information Methods S1.

Results and Discussion

The acetone treatment reduced the total CBSC concentration by *c.* 66% in healthy thalli. Thereby, there was no difference in CBSC concentration between acetone-rinsed healthy and infected thalli before the transplantation (Table S1). After the 201-d transplantation period, all individual CBSCs had significantly higher concentrations in the healthy acetone-rinsed thalli than in those with *Plectocarpon* galls. As such, healthy thalli with initially reduced CBSC concentration increased their total mean concentration of CBSCs by 62%, while infected thalli showed a slight reduction in CBSCs (Fig. 1a; Table 1). Low CBSC concentrations commonly occur in thalli infected by *Plectocarpon* (Merinero *et al.*, 2015a; Asplund *et al.*, 2016), but such observations *per se* do not exclude additional mechanisms for the low levels of CBSCs in infected thalli: *P. lichenum* may prefer thalli with low CBSC synthesis rates, which can be tested with, for example, inoculation experiments. However, this explanation is unlikely because the stictic acid-deficient chemotype of *L. scrobiculata* has the same infection rates of *Plectocarpon* as the normal chemotype (Merinero *et al.*, 2015a). Enzymes from some lichenicolous fungi can degrade their host's CBSCs, but this is probably uncommon among lichenicolous fungi (Mosbach & Ehrensvärd, 1966; Lawrey *et al.*, 1999), and we did not observe any degradation of CBSCs in non-acetone rinsed infected thalli during the 201-d experiment (Table S1). The low synthesis in infected thalli potentially increases the susceptibility of the host to pathogens. For instance, the lichenicolous fungi *Nectria parmeliae* can only grow in lecanoric acid-producing lichens after this compound has been degraded by a *Fusarium* fungus, or after removal of lecanoric acid by acetone (Lawrey, 2000). Unlike the high CBSC synthesis in healthy acetone-rinsed thalli during the transplantation period, there was a slight decrease in CBSC concentration for the healthy non-acetone rinsed thalli (Fig 1a; Table 1). This could have emerged as a dilution effect due to carbon fixation. For this reason, there was no significant difference in total CBSCs between healthy and infected non-acetone rinsed thalli at the end of the transplantation (Table S1).

Infected thalli hardly grew and some even lost biomass during the experiment, whereas healthy thalli gained 13% in biomass during the 201-d transplantation (Fig. 1b; Table 1), which is similar to earlier reported annual growth rates in healthy *L. pulmonaria* on oak trunks in the studied site (Merinero *et al.*, 2015b). In a previous 14-d growth chamber experiment under optimal growth conditions, thalli without galls grew twice as fast as thalli with galls (Merinero & Gauslaa, 2018). Thereby, the growth-reducing effect of *P. lichenum* on its lichen host is even stronger in natural field habitats than in the laboratory. The growth-reducing mechanisms of fungal parasites are poorly understood. Fungal parasites infecting vascular plants can damage the photosynthetic apparatus resulting in reduced growth rates (Agrios, 2005). However, Grube & de los Ríos (2001) argue that *P. lichenum* is a mycoparasite that degrades fungal hyphae, and thus acquires nutrients and carbon, without direct contact with the host's photobionts. Acetone rinsing did not significantly affect the RGR of the lichens (Fig. 1b; Table 1), consistent with the view that the acetone treatment does not lead to impaired carbon fixation or

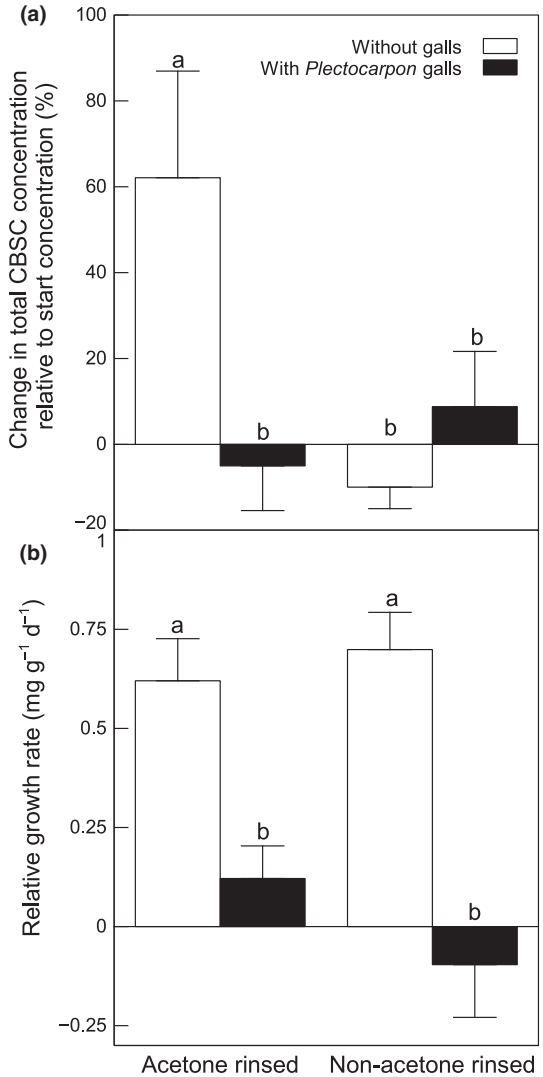

Fig. 1 (a) Change in total carbon-based secondary compounds (CBSCs) concentration relative to start concentration and (b) relative growth rate (RGR) in acetone rinsed vs non-rinsed (control) *Lobaria pulmonaria* with or without galls of *Plectocarpon lichenum* (mean + SE). Bars, within the same panel, topped with the same lowercase letter are not significantly different ($P < 0.05$) according to a Tukey's test.

Table 1 Two-way analysis of variance (ANOVA) testing for the effect of (1) acetone treatment and (2) presence vs absence of *Plectocarpon lichenum* on the relative change in carbon-based secondary compounds (CBSCs) and relative growth rate of *Lobaria pulmonaria*

	Relative change in CBSCs *F* (*P*)	Relative growth rate *F* (*P*)
Acetone treatment (A)	2.17 (0.147)	0.13 (0.719)
Plectocarpon (P)	2.76 (0.114)	**38.52 (< 0.001)**
A × P	**9.51 (0.003)**	2.00 (0.163)

df = 1, 53. Bold text denotes significant effects at $P < 0.05$.

other physiological malfunctioning in the lichen thalli. Likewise, Solhaug & Gauslaa (2001) found unchanged photosystem II efficiency (F_V/F_M) in *L. pulmonaria* after a few hours submersion in acetone. Thus it is unlikely that the 40-min acetone treatment used in this study affected the photobionts.

Conclusions

Our data suggest that *P. lichenum* adversely influences its lichen host by reducing CBSCs and growth. Our findings do not allow us to conclude whether the lack of CBSC synthesis is because *P. lichenum* specifically targets the host CBSC synthesis or if the reduced metabolism in general impedes the production of CBSCs. Nevertheless, this gall-forming lichenicolous fungus reduces its host's fitness and thus this association can hardly be considered commensalistic, as previously suggested (Ertz *et al.*, 2005). Severe *Plectocarpon* outbreaks thereby potentially affect the population structure of the lichen host by limiting its growth, and thus its competitive ability, and/or by increasing its susceptibility to additional lichenivore attack (Asplund *et al.*, 2016). Whether our findings represent a general pattern that also occur in other lichen hosts or with other lichenicolous fungi remains to be explored.

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Author contributions

J.A., S.M. and Y.G. planned and designed the research. J.A. and S.M. performed experiments and conducted fieldwork. J.A. analysed data and J.A., S.M. and Y.G. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Table S1 Concentrations of carbon-based secondary compounds in acetone rinsed and non-rinsed *Lobaria pulmonaria* with and without galls of *Plectocarpon lichenum*

Methods S1 Detailed description of materials and methods.

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Key words: carbon-based secondary compounds (CBSCs), fungal parasites, lichens, parasitism, relative growth rate (RGR), symbiosis.

***New Phytologist* Supporting Information**

Article title: Low synthesis of secondary compounds in the lichen *Lobaria pulmonaria* infected by the lichenicolous fungus *Plectocarpon lichenum*

Authors: Johan Asplund, Yngvar Gauslaa & Sonia Merinero

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The following Supporting Information is available for this article:

Table S1 Concentrations of carbon based secondary compounds (mean ± SE mg g^{-1}) in acetone rinsed and non-rinsed *Lobaria pulmonaria* with and without galls of *Plectocarpon lichenum*.

Methods S1 Detailed description of materials and methods

Table S1 Concentrations of carbon based secondary compounds (mean ± SE mg g-1) in acetone rinsed and non-rinsed (control) Lobaria pulmonaria with and without galls of Plectocarpon lichenum. Concentrations were measured both before and after the 201 days transplantation. Bold numbers represent significant differences (P<0.05) between infected vs healthy thalli according to a t-test.

	Pre transplantation			Post transplantation		
	Infected	Healthy	t (*P*)	Infected	Healthy	t (*P*)
Acetone rinsed						
Stictic	6.47±1.1	7.49±1.0	0.68 (0.504)	**5.62±0.8**	**10.13±1.31**	**2.94 (0.008)**
Constictic	1.86±0.5	2.13±0.5	0.37 (0.718)	**1.45±0.3**	**3.07±0.47**	**2.85 (0.009)**
Norstictic	0.78±0.1	0.7±0.1	0.54 (0.595)	**0.46±0.05**	**0.98±0.15**	**3.30 (0.005)**
Cryptostictic	0.22±0.03	0.32±0.05	1.65 (0.113)	**0.15±0.02**	**0.34±0.05**	**3.59 (0.002)**
Methyl norstictic	0.08±0.01	0.09±0.01	0.25 (0.804)	**0.06±0.01**	**0.09±0.01**	**2.71 (0.015)**
Total	9.35±1.7	10.70±1.5	0.56 (0.577)	**7.74±1.2**	**14.56±1.74**	**3.25 (0.004)**
Non-acetone rinsed						
Stictic	19.39±1.5	22.88±0.8	2.05 (0.054)	18.84±1.1	20.06±0.8	0.880 (0.388)
Constictic	**5.78±0.6**	**8.97±1.0**	**2.65 (0.014)**	6.14±0.6	7.89±0.9	1.69 (0.104)
Norstictic	**1.73±0.2**	**2.50±0.3**	**2.39 (0.024)**	1.80±0.2	2.29±0.2	1.87 (0.073)
Cryptostictic	**0.33±0.1**	**0.62±1.0**	**3.68 (0.001)**	**0.35±0.04**	**0.57±0.1**	**3.29 (0.003)**
Methyl norstictic	**0.22±0.02**	**0.32±0.03**	**2.80 (0.009)**	0.28±0.01	0.30±0.02	0.916 (0.368)
Total	**27.45±2.3**	**35.30±1.8**	**2.67 (0.013)**	27.40±1.7	31.11±1.6	1.58 (0.126)

Methods S1 Detailed description of materials and methods

We sampled *Lobaria pulmonaria* with and without *P. lichenum*-galls in a mixed forest of *Quercus pyrenaica* and *Fagus sylvatica* in central Spain (Montejo de la Sierra, Madrid, 41º06'44''N, 03º29'33''W; 1263 m a.s.l.; mean annual precipitation: 818 mm; mean temperature 9.5ºC; climate data from CLIMOEST; Sánchez-Palomares *et al.*, 1999) in July 2015. For each thallus with *Plectocarpon*-galls (n=18), we collected a similar sized thallus without galls and with similar amount of reproductive structures (soralia) from the same tree. In the lab, we cleaned the air-dry lichens from debris and cut each thallus in two pieces of similar size. One piece was a control, and the other was rinsed in 100 % acetone for 40 min. This treatment non-destructively and substantially reduces the lichen CBSC concentration (Solhaug & Gauslaa, 2001; Asplund & Gauslaa, 2008). To avoid evaporation during the CBSCs extraction we closed the containers with acetone (c 70 ml) with aluminium foil. Afterwards, thalli were left air dry for 24 h to ensure acetone evaporation. We cut a small part (c 20 mg) from all thalli in the four categories to quantify pre-transplantation CBSC concentrations. Before and after transplantation, we recorded the air dry mass (±0.1 mg) of all thalli. During these measurements, we weighted ten extra thalli; then oven-dried them at 70°C for 72 h to record their oven dry mass (DM). We calculated the DM of all transplants using the mass reduction factor of the sacrificed oven-dried thalli. DM (mean ± 1 SE) of *L. pulmonaria* with and without *Plectocarpon*-galls was 177.2 ± 6.9 and 238.6 ± 13,6 mg, respectively. Relative growth rate, RGR = $((\ln(DM_{end}/DM_{start})) \times 1000/\Delta t$ ($mg \cdot g^{-1} \cdot d^{-1}$), was quantified following Evans (1972). Δt was 201 days.

A transplantation unit consisted of four thalli sewn with polyester thread to a 25 × 25 cm mosquito net: two control thalli (with and without *Plectocarpon*-galls) together with their acetone-rinsed counterparts. We set the transplantation experiment in a fenced oak forest patch in the site where the lichens were collected. We stapled each net on the northern side of a tree trunk at 75 cm from the ground. Transplants stayed in the field from 30 Aug 2015 to 18 Mar 2016, comprising an important growing season for lichens in Mediterranean forests.

In order to quantify CBSCs after transplantation, each transplant was ground in a ball mill to a fine powder. Approximately 35 mg of the powder was extracted in acetone for three

successive 45 min periods. The combined extract was evaporated to dryness and dissolved in 1000 µl methanol. The extracted compounds were then quantified on a 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) including a 1,040-M diodearraydetector (Following Nybakken *et al.*, 2007). Separation was achieved on an ODS Hypersil 50 × 4.6 mm column. The injection volume was 10 µl and the flow rate was 2 ml min-1. Solvent A consisted of 0.25% orthophosphoric acid and 1.5% tetrahydrofuran in Millipore (Millipore, Billerica, Massachusetts, USA) water; solvent B was 100% methanol. The run started with 30% B. Within 15 min, solvent B was increased to 70% and further to 100 % the next 15 min, and then isocratically in 100% B for a further 5 min. At the end of the run, solvent B was reduced to 30% within 1 min, and the column was flushed with 30% B for 5 min before the next run. Individual compounds were analysed using UV-spectra and retention times. We identified five different depsidones (stictic, constictic, norstictic, cryptostictic and methyl norstictic acid) and these are all commonly found in this species. Collectively these compounds protect the lichen from lichenivorous snails (Asplund & Gauslaa, 2008), but the effect of individual compounds are not known.

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