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Standard Paper

Testing carbon and nitrogen sources for the *in vitro* growth of the model lichenized fungus *Endocarpon pusillum* Hedw.

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Abstract

To improve the efficiency of isolating and culturing lichen mycobionts, we performed a growth assay on an Australian strain of the soil-crust lichenized fungus *Endocarpon pusillum* Hedw. This assay determined the preferred nitrogen and carbon sources of the fungus by limiting the available nitrogen or carbon sources to single compounds found in soils, plants and lichen thalli. We found that the non-proteinaceous amino acid, GABA, produced the most growth of all nutrients when provided as the sole nitrogen source but was a poor carbon source. Fructose, glucose, cellobiose and sorbitol produced the most growth of all the carbon sources tested. Ammonium, nitrate and polyamines were poor nutrient sources. These findings correspond with reports of primary metabolite pools in other lichen species and may guide future studies involving growth of recalcitrant lichen mycobionts.

Key words: aminobutyric, GABA, growth, medium, nutrition, polyalcohol, polyol, rate

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Introduction

Lichens form when specific species of fungi contact a compatible photobiont, either a cyanobacterium or eukaryotic green alga (Green & Smith 1974; Almendras *et al.* 2018). The fungus encapsulates the algal cells and an unidentified inter-kingdom communication alters the algal cell morphology (Joneson & Lutzoni 2009). Lichen-forming fungi are known for their ability to produce an arsenal of compounds to protect the symbiosis from environmental stress, competition, pathogens and predation (Giez *et al.* 1994; Solhaug *et al.* 2003; Halama & Van Haluwin 2004). In return the algal cells fix carbon and, for cyanobacterial photobionts, nitrogen, which are then available to the fungus. Carbohydrates from the algal cells, such as hexoses and acyclic polyalcohols, are transferred to the fungus and assimilated (Richardson *et al.* 1968; Yoshino *et al.* 2020). A successful interaction between two compatible symbionts leads to changes in cell morphology and the formation of a stratified thallus structure (Athukorala *et al.* 2014). The growth of these thalli produces biomass in harsh or barren environments, allowing other species to enter that niche (Kumpula 2001; Asplund & Wardle 2017). Despite the abundance of lichen species, their importance in the environment, and their potential for some industries, there is still much to learn about their fundamental biology. This is largely due to the difficulties in obtaining axenic cultures of both symbionts, and the notoriously slow growth rate of lichen mycobionts.

Fungi are isolated using a variety of common media such as potato-dextrose (PD), yeast-sucrose, malt-glucose and Czapek-Dox agars (Crous *et al.* 2010). These media are often supplemented with nutrients that mimic the microbe's natural environment to encourage growth more reminiscent of that found in nature. For example, Campbell's™ V8 tomato juice is used to initiate sporulation in the wheat pathogen *Parastagonospora nodorum* (Abeysekara *et al.* 2009). However, relying on these media can lead to difficulties in cultivating uncharacterized fungi, or observing their fundamental biology. For example, mycotoxin production by *Fusarium* species is strictly controlled by specific nitrogen sources in the growth medium (Miller & Greenhalgh 1985; Maeda *et al.* 2017) and the wheat pathogen *Zymoseptoria tritici* grows in a filamentous form *in planta* but is yeast-like *in vitro* (Francisco *et al.* 2019).

Lichen mycobionts are often cultured using complex media such as potato-dextrose, Sabouraud-glucose, malt-yeast, or defined media such as Lilly and Barnett's medium or Czapek solution (Bando *et al.* 1997; Stocker-Wörgötter 2001; Stocker-Wörgötter *et al.* 2004; Brunauer *et al.* 2007). These media are often supplemented with extracts from the lichen's natural substratum, such as bark (Stocker-Wörgötter *et al.* 2004) or soil (Ahmadjian & Heikkilä 1970), or with polyalcohols (Brunauer *et al.* 2007). For example, Brunauer *et al.* (2007) grew *Xanthoria elegans* on Lilly and Barnett's medium supplemented with either ribitol or mannitol to examine differences in secondary metabolite production. Ribitol is produced by the algal cells associated with *Xanthoria*, while mannitol is the storage carbohydrate of the fungus. Interestingly, it was mannitol that induced the greatest production of secondary metabolites, rather than the algal carbohydrate. Co-culturing the mycobiont with its associated alga has proved useful. Wang *et al.* (2014) co-cultured the soil-crust lichen used in this study, *Endocarpon pusillum*, with its

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photobiont in a carbon-free medium to determine carbohydrate flux between symbionts, as well as changes in gene expression during the initial stages of symbiosis. Alternatively, cultivating thallus fragments has also proved useful for growth experiments. For example, Bando *et al.* (1997) grew thallus fragments of *Parmotrema tinctorum* in a range of media supplemented with various amino acids, vitamins and plant hormones to discover factors governing thallus growth. Interestingly, with the exception of asparagine and aspartate, all supplements tested reduced the growth of the thallus and, counterintuitively, diluting the media improved growth. Unfortunately, few amino acids and no polyalcohols were included in this study.

To discover media supplements that might aid isolating and culturing lichen mycobionts, we performed a six-month growth assay on a strain of the model soil-crust mycobiont, *Endocarpon pusillum*. This species has been the focus of resynthesis experiments in modern science for several decades (Ahmadjian & Heikkilä 1970). However, physiological observations and attempts at *in vitro* culture were published as early as 1878 (Zukal 1878). Its scientific history, cosmopolitan distribution and emerging genetic resources promote *E. pusillum* as an ideal model for studying lichen growth and resynthesis of lichens (Park *et al.* 2014; Wang *et al.* 2014, 2015; Mead & Gueidan 2020). Despite the advantages of this species, it is still slow growing compared with other model fungi such as *Aspergillus*. Accordingly, we undertook a growth assay to determine whether metabolites involved in plant microbe interactions might improve the growth of the mycobionts of an Australian strain of *E. pusillum*. The genome sequence of this Australian strain (EPUS1.4) has been published in a separate study (Mead & Gueidan 2020). The slow growth rate of the fungus made measuring biomass difficult. Therefore, we approximated relative growth by photographing flat black biomass against a white background and counting the proportion of black pixels. Ametrano *et al.* (2017) similarly measured the growth of rock-inhabiting fungi in co-culture with lichen photobionts, confirming that the method offers a more efficient alternative to weighing the biomass of slow-growing fungi. This assay limited the nitrogen or carbon sources available to the fungus to single compounds to determine metabolically preferred nutrients. These compounds were chosen on the basis of being commonly found in soils and plant apoplasts, and secreted by lichen photobionts (Richardson *et al.* 1968; Solomon & Oliver 2002; Mooshammer *et al.* 2014). Previous studies predominantly report growing *E. pusillum* on potato-dextrose, either on agar or in shaking liquid cultures (Wang *et al.* 2014; Li & Wei 2016; Yang *et al.* 2018). We assessed a range of carbon sources, ranging from two carbon molecules and essential primary metabolites such as acetate and citrate to more complex molecules such as polyalcohols, cellobiose and amino acids. We investigated the efficiency of inorganic and organic nitrogen compounds but focused on components of the γ-amino butyric acid (GABA) shunt and ornithine polyamine pathway. These compounds have been shown to play pivotal roles in growth, development and stress response in both plants and fungi (Chen *et al.* 2019; Rocha & Wilson 2019; Singh *et al.* 2020). Furthermore, these compounds have been reported to play varying roles in plant-fungal interactions. For example, the non-proteinaceous amino acid, GABA, was shown to be a highly abundant nutrient source in the apoplast of tomato leaves infected with the biotrophic fungus *Cladosporium fulvum* (Solomon & Oliver 2002).

The formulation of growth media is often crucial to successfully isolating and culturing fungi from the environment. To improve the success of isolating and growing lichen-forming fungi supplements are often added to traditional media. In this experiment we observed the growth response of the lichen mycobiont *E. pusillum* to various carbon and nitrogen sources with the aim of providing guidance for customizing growth media. This may facilitate the culture of lichen mycobionts and a further understanding of lichen biology.

Materials and Methods

Fungal strain and growth conditions

The specimen of *Endocarpon pusillum* (*C. Gueidan* 2364) used for isolation was collected from the CSIRO Black Mountain site in Canberra (35°16′S, 149°07′E), Australia in 2016. It was deposited in the CANB herbarium as accession CANB 913709. Ascospores were shot on Potato Dextrose Agar (PDA) plates and single ascospores were isolated and grown on liquid PD medium in an incubator with 20/18 °C 12-h day/night cycles. Stock cultures of the strain EPUS1.4 were grown at 22 °C in complete darkness in stationary liquid YSSG medium (yeast extract 5 g l^{-1}, sucrose 10 g l^{-1} sorbitol 10 g l^{-1}, GABA 1 g l^{-1}).

Growth assay cultures of *E. pusillum* were grown in a similar way to Li & Wei (2016) but in darkness in sterile multi-well plates. The plates were sealed with parafilm, maintaining constant humidity and inhibiting contamination. Each plate held 12 wells and each having a total volume of 3 ml (Nuncleon Delta coated, cat. 150628, ThermoFisher Scientific). Wells were filled with 2 ml of liquid minimal base medium (Table 1) then supplemented with a stock solution of the carbon or nitrogen source being tested. The nitrogen and carbon source treatments were randomly distributed among wells and the position of plates within the incubator was rotated weekly. The media were poured, inoculated and the plates sealed with micropore surgical tape (3M) under sterile conditions. Uninoculated YSSG medium was included to check for environmental contamination. Base minimal medium contained 10 mM sodium phosphate buffer and trace elements, and was adjusted to pH 6.5 with HCl. The base minimal medium was autoclaved then inoculated from a stock suspension of *E. pusillum* mycelia. The fungal biomass was washed three times with sterile distilled water before inoculation. The biomass was then finely blended in the base medium with a sterile Omni-Inc tissue homogenizer to a final concentration of 0.02 mg of fresh weight biomass per ml. The base medium for testing nitrogen sources was supplemented to a final concentration of 25 mM sucrose and the desired nitrogen source adjusted to 10 mM nitrogen. The base medium for testing carbon sources was supplemented with 10 mM mono-ammonium phosphate and the desired carbon source adjusted to 150 mM carbon (equivalent to 25 mM glucose). Carbon and nitrogen were supplemented from 1.5 M and 100 mM stock solutions, respectively, each adjusted to pH 6.5 with 1 M HCl or 1 M NaOH prior to sterilization. The components of each medium, at their final concentration, are described in Table 1. Note that GABA, ornithine and glutamine are both carbon and nitrogen sources. Including controls lacking any metabolizable carbon or nitrogen, this totalled 23 conditions. Four cultures in each condition were grown, totalling 92 samples, for 150 days before being quantified.

Colony surface area approximation

The colony surface area was used as an approximation of growth to overcome the need to weigh restrictively small biomasses of

Table 1. List of minimal media components. Media were derived from the base medium by the addition of carbon and nitrogen sources. Media used to test carbon sources contained 10 mM mono-ammonium phosphate as a nitrogen source. Conversely, media used to test nitrogen sources contained 25 mM sucrose for carbon. The base medium and stock solutions were adjusted to pH 6.5 with 1 M HCl or 1 M NaOH.

Media components	Final concentration (g l^{-1})
Base medium	
Di-sodium hydrogen phosphate	0.56
Sodium di-hydrogen phosphate	1.6
Trace element 100 × stock (ml l^{-1})	10
Carbon sources (equivalent to 25 mM glucose)	
Potassium acetate	7.4
Glycerol	4.6
Tri-sodium citrate	6.5
Xylose	4.5
Glucose	4.5
Fructose	4.5
Lactose	4.3
Sucrose	4.3
Cellobiose	4.3
Erythritol	4.6
Ribitol	4.6
Mannitol	4.6
Sorbitol	4.6
GABA	3.9
Glutamine	4.5
Ornithine hydrochloride	5.1
Nitrogen source (equivalent to 10 mM nitrate)	
Sodium nitrate	0.85
Mono-ammonium phosphate	1.15
GABA	1.03
Glutamine	0.745
Ornithine hydrochloride	0.843
Trace elements (100 × stock)	
Potassium chloride	50
Magnesium sulphate heptahydrate	50
Zinc sulphate heptahydrate	1
Iron sulphate heptahydrate	1
Copper sulphate heptahydrate	0.25

each sample. Each well was photographed on a white background with six technical replicates using a Leica M205C dissecting microscope at ×16 magnification (Fig. 1A). The images were then manually cropped using the software package GNU Image Manipulation Program (GIMP) to contain only the contents of the well (Fig. 1B). GIMP then converted all pixels to black or white and counted all black pixels as a proportion of total pixels

(GIMP 2019). To validate this method, images of exactly 25%, 50% and 75% black pixels were generated in Microsoft PowerPoint. These images underwent the same measurement procedure as the experimental well images and determined the measurement error rate of GIMP.

Growth measurements

The mean area covered in both the carbon and nitrogen test wells was normalised using the mean % area in the control wells containing no carbon and no nitrogen.

Biomass measurements

Media were gently removed from the wells so that the fungal biomass was not removed. The 12-well plates were dried at 65 °C for 4 days to remove all moisture. The plates were weighed before the biomass was removed. Biomass was detached from the plastic floor of the wells with a solution of 5 M HCl and 5% (v/v) hydrogen peroxide overnight at room temperature. The degraded fungal biomass was then gently washed away with hot water. The plates were subsequently wiped clean with paper towels, dried at 65 °C for 4 days and re-weighed. The difference in mass represents the total fungal biomass.

Statistical analyses

The black pixel count of each image was expressed as a proportion of total pixels for that image. The mean of the six technical replicates was taken for each well. The mean of the biological replicates for each of the control media (no carbon or no nitrogen) was determined, and each biological replicate was represented as a proportion of its respective control. A Shapiro-Wilks test using R (R Core Team 2017) determined all data followed a normal distribution, allowing a one-way ANOVA to determine variance between samples. Using R, a multiple comparisons test was performed using Tukey's Honest Significant Difference (HSD) test which determined which means were significantly different ($P < 0.05$). The R packages *ggpubr* (Kassambara 2019), *multcompView* (Graves *et al.* 2019), *dplyr* (Kassambara 2020), *rstatix* (Wickham *et al.* 2020) and *ggplot2* (Wickham 2016) were used to analyze and visualize the data.

Results

Technical error associated with measuring surface area covered by hyphal growth

There was < 1.5% deviation between the standardized images and the measured pixel count using GIMP (2019) (Fig. 2). This analysis did not describe the error associated with measuring biomass but indicated the technical error associated with pixel counts.

Growth of Endocarpon pusillum *on various carbon sources*

Endocarpon pusillum was grown on minimal media containing tri-ammonium phosphate as the sole nitrogen source and a selected sole carbon source (Fig. 3). The total biomass of the experiment, including all treatments and controls, reached 248.5 mg over six months and there was insufficient material to weigh the dry mass of individual samples. The fungus grew flat in minimal multicell layers against the bottom of the wells of

Fig. 1. Unmodified (A) and cropped black and white (B) images of *Endocarpon pusillum* biomass after six months of growth in minimal medium containing mannitol as the sole carbon source. Scale bar = 5 mm. In colour online.
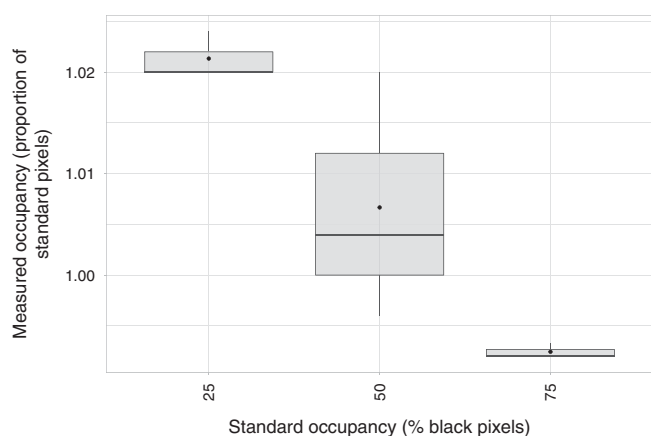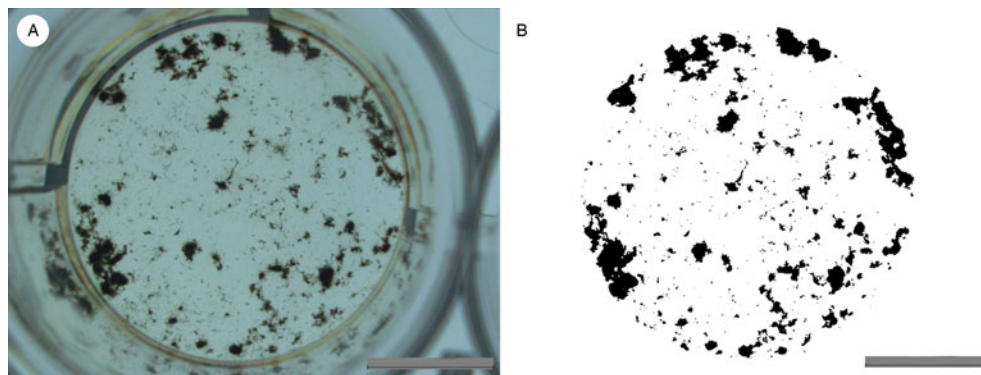

Fig. 2. Assessment of the error associated with estimating fungal growth. Standard images containing exactly 25, 50 and 75% black pixels were generated and the number of black pixels were measured using GIMP (2019). The results are expressed as the relative proportion of measured pixels compared to the predicted number. Black dots represent the mean, black lines within boxes represent the median, boxes represent the inner quartiles, and whiskers represent the minimum and maximum values.

12-well plates in liquid media with similar morphologies between replicates and between treatments, and low variability between replicates. The no carbon and no nitrogen controls produced some growth, equivalent to 27.0% (95% CI = 0.63) and 56.0% (95% CI = 3.42) respectively. These values were used to normalize the other test samples. The hexoses and hexose polymer, fructose, glucose, and cellobiose, produced the most growth of the sugar carbon sources and were comparable to the growth observed using the polyalcohol sugar, sorbitol. Interestingly, growth on sucrose was lower than on its monomer constituents, glucose and fructose. However, the growth on glucose was not significantly different from growth on sucrose. Similarly, growth on mannitol was lower than on its isomer, sorbitol, but not significantly (t-test: $P = 0.08$) (Fig. 3). Acetate, glycerol and citrate, as well as the amino acids, elicited poor growth whereby citrate and glutamine significantly reduced growth below that of the no carbon control.

Growth of Endocarpon pusillum on various nitrogen sources

Endocarpon pusillum was grown on minimal media containing sucrose as the sole carbon source and a selected sole nitrogen source. The non-proteinaceous amino acid, GABA, significantly elicited the most growth of all tested nitrogen sources (Fig. 4).

Interestingly, glutamine, a precursor to GABA synthesis, elicited significantly less growth than GABA or the no nitrogen control. Ornithine produced no significant difference to the no nitrogen control, whereas nitrate and ammonia produced significantly less growth.

Discussion and Conclusion

One of the major hindrances to studying lichens is the difficulty associated with isolating and culturing the mycobiont. In this study we tested several carbon and nitrogen sources that might improve the growth of the model lichenized fungal species *Endocarpon pusillum*. We found that the carbon sources glucose, fructose, cellobiose and sorbitol consistently led to significantly more growth than the no carbon control. This is not surprising since glucose is a key component of primary metabolism, whereas fructose, cellobiose and polyalcohols are plant-derived carbohydrates available to the fungus during its symbiotic association with a photobiont (Richardson *et al.* 1968). Cellobiose is a disaccharide of glucose monomers, which may explain the similar growth observed on these two carbon sources. The fungus may be assimilating cellobiose, when associated with its photobiont, by degrading the algal cell wall. This damage to the photobiont may represent a more parasitic interaction. The continuum between mutualism and parasitism in the lichen symbiosis is not a new concept (Richardson 1999; Hyvärinen *et al.* 2002; Ahmadjian & Jacobs 2011) and considering the multiple phylogenetic origins of lichenization (Gargas *et al.* 1995; Gueidan *et al.* 2008; Schoch *et al.* 2009) it is not unexpected that different lichen species may fall within this continuum. Alternatively, the propensity of *E. pusillum* towards cellobiose might be explained by the fungus having evolved to opportunistically recycle plant biomass in the soil or dead algal cells within the thallus.

Mannitol, sorbitol and fructose elicited similarly high levels of growth, with fructose providing the highest but most variable growth. Growth on these substrates correlates with known fungal metabolism whereby sorbitol can isomerize to mannitol which can then be oxidized to fructose for subsequent glycolysis (Solomon *et al.* 2007). Sucrose elicited less growth than its monomer constituents which might indicate that this species does not harbour an active invertase and cannot efficiently convert sucrose into monosaccharides. Future genomics and metabolic network modelling may provide some insight into this species' ability to assimilate disaccharides. Interestingly, the fungus can grow slowly on the common complex growth medium, potato-dextrose, but not when it is supplemented with Campbell's™ V8 tomato juice (results not shown), as is often required by plant pathogens
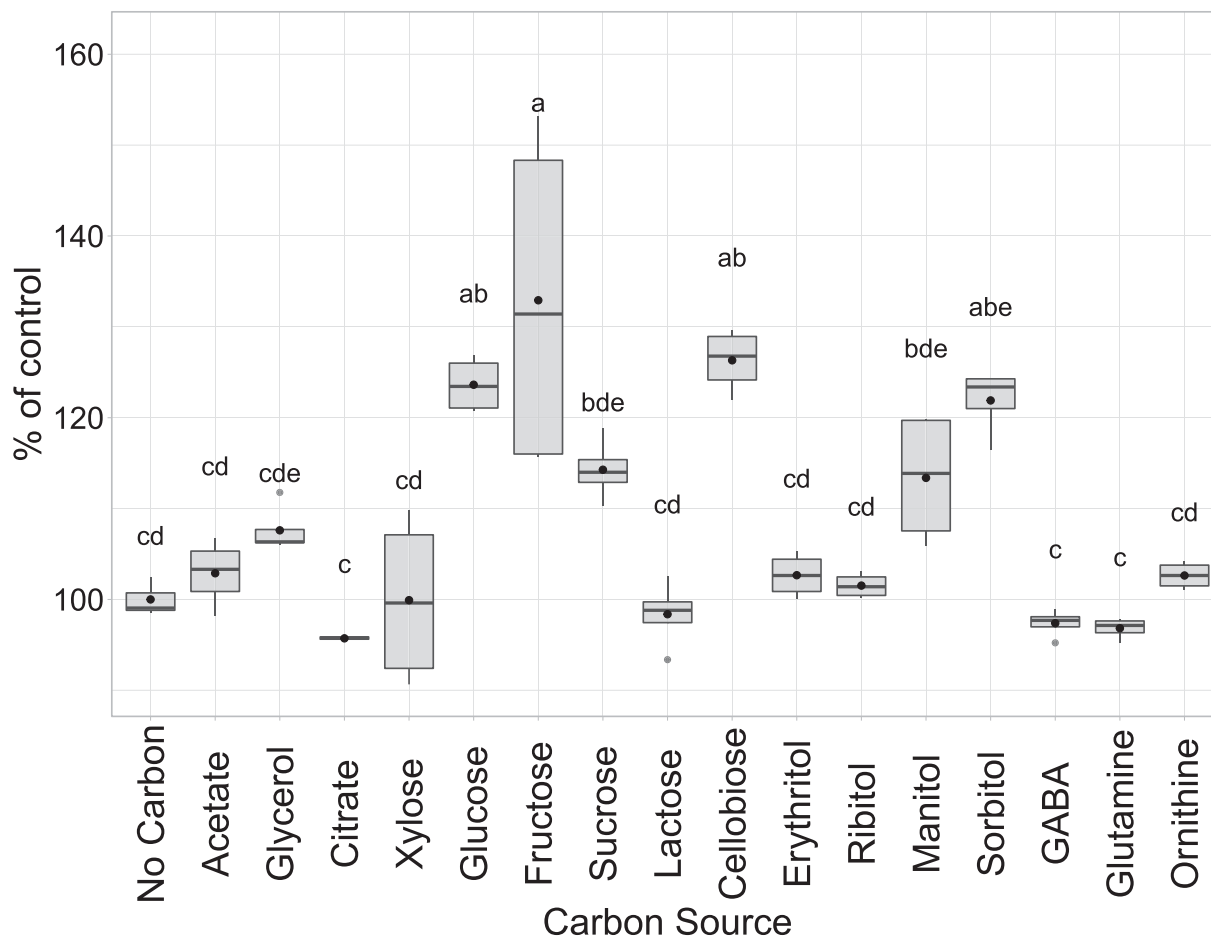
Fig. 3. Relative growth of *Endocarpon pusillum* on various carbon sources. Growth is relative to the control lacking any metabolizable carbon. Carbon source was adjusted to 25 mM glucose equivalent. Bars with the same letter are not significantly different based on Tukey's HSD test ($P < 0.05$). Black dots represent the mean, black lines within boxes represent the median, boxes represent the inner quartiles, and whiskers represent the minimum and maximum values. Grey dots represent outliers.

(Abeysekara *et al.* 2009). The other polyalcohols, glycerol, erythritol and ribitol, did not elicit the same level of growth as sorbitol, indicating that the mycobiont metabolism is selective. This correlates with other reports. Richardson *et al.* (1968) investigated the carbohydrates produced by photobionts of different lichen species and showed that there is a primary polyalcohol secreted in each case. Wang *et al.* (2014) found that growing *E. pusillum* in co-culture with its photobiont, *Diplosphaera chodatii*, led to the production of sorbitol (12.6 mg/100 ml), glucose (0.8 mg/100 ml) and sucrose (0.8 mg/100 ml) in the medium, but not fructose or mannitol. There were no carbohydrates found in media containing the symbionts in isolation, indicating that *E. pusillum* induced the release of predominantly sorbitol from the algal cells. Subsequent gene expression analysis of the fungus, when grown on several different carbohydrates, showed that sorbitol, mannitol, glucose and sucrose induced consistently high sugar transporter transcription, while other sugars did not (Wang *et al.* 2014). These reports show that identifying the dominant carbohydrate secreted by species of lichen photobionts may provide direction for which carbon source will improve the growth of desired mycobionts.

Amino acids were supplied as sole carbon or sole nitrogen sources. To test the ability of *E. pusillum* to grow on an amino acid as a nitrogen source, no other nitrogen sources were supplied in the minimal medium, and glucose was supplied as a carbon source. Likewise, to test the ability of the mycobiont to utilize specific amino acids as carbon sources, all other carbon was omitted from the medium and nitrogen was supplied as ammonium. All amino acids tested were poor carbon sources, despite pH buffering and equimolar carbon concentrations. This might be due to the carbon skeletons themselves proving difficult to metabolize but is more likely due to an imbalance of ammonium and carbon leading to ammonium toxicity. This hypothesis is reinforced by the detrimental effect of ammonia on the growth of *E. pusillum*. Exogenous nitrogen, including ammonia, has been reported to play a key role in lichen growth and development. The focus of these reports varied from the ability of nitrogen sources to induce metabolite production (Kinoshita *et al.* 2001), uptake (Dahlman *et al.* 2004; Hauck 2010), physiological effect (Gaio-Oliveira *et al.* 2005; Palmqvist & Dahlman 2006; Pirintsos *et al.* 2009) and in relation to nitrogenous air pollution (Pescott *et al.* 2015). The response to nitrogen source in these studies was predominantly species dependent; however, ammonia was generally more readily assimilated than nitrate. Most studies used lichens in the symbiotic thallus state, presumably due to the difficulties in cultivating the lichen mycobiont and to gain a more ecological understanding of the symbiosis. Unfortunately, including the photobiont precludes conclusions about the mycobiont's ability to use various nitrogen sources. However, the effect of these
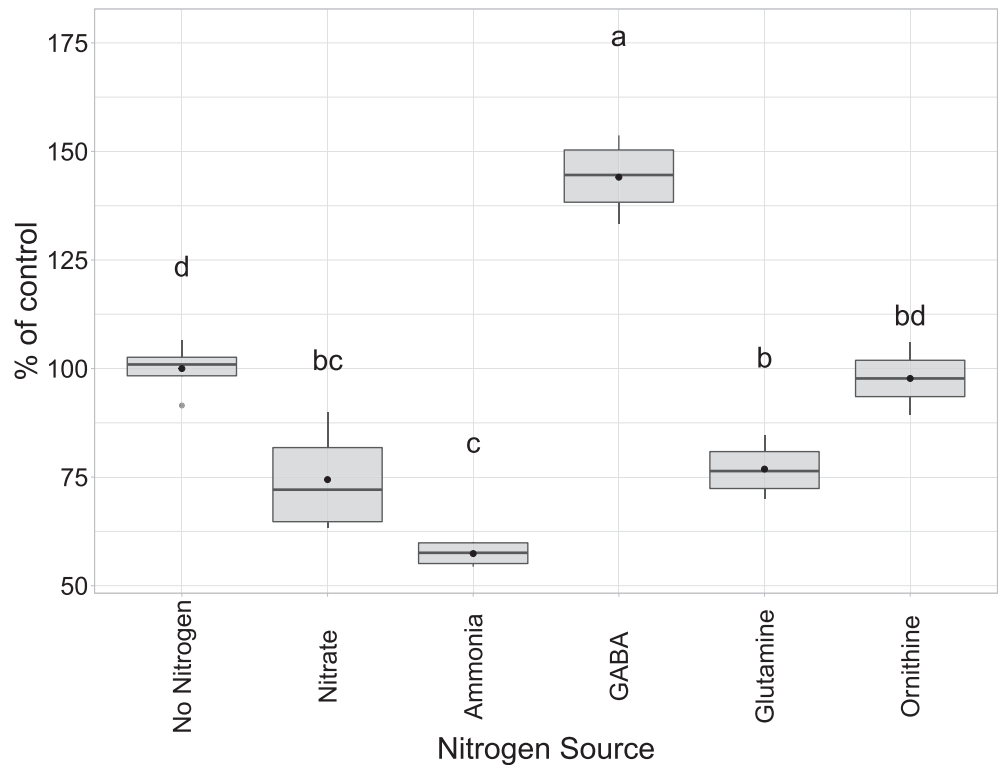
Fig. 4. Relative growth of *Endocarpon pusillum* on various nitrogen sources. Growth is relative to the control lacking any metabolizable nitrogen. Nitrogen source was adjusted to 10 mM nitrate equivalent. Bars with the same letter are not significantly different based on Tukey's HSD test ($P < 0.05$). Black dots represent the mean, black lines within boxes represent the median, boxes represent the inner quartiles, and whiskers represent the minimum and maximum values. Grey dots represent outliers.

compounds on lichen growth provides direction aimed at improving the growth of mycobionts in the future.

Only the amino acid GABA elicited more growth than the no nitrogen control when amino acids were supplied as the sole nitrogen sources. Glutamine and ornithine failed to elicit significantly more growth than the inorganic nitrogen sources, nitrate and ammonium. However, GABA produced 144% of the growth of the no nitrogen control, 194% and 251% of the growth elicited by nitrate and ammonium, respectively. GABA is a primary metabolite found in all eukaryotes. It is an important neurotransmitter in the vertebrate brain (Ge *et al.* 2006) and a key stress regulator in plants (Ramesh *et al.* 2015), yet the role of this small molecule is relatively unknown in fungi (Mead *et al.* 2013; Bönnighausen *et al.* 2015). GABA is produced from the tricarboxylic acid (TCA) cycle as a bypass from α-ketoglutarate to succinate, negating a key ATP producing step. The production of this molecule links the TCA cycle to several other key primary metabolic pathways, such as glutamine-ammonia dissimilation, alanine metabolism and the ornithine polyamine pathway. GABA and this polyamine pathway have been shown to play roles in fungal growth, development and pathogenicity (Bailey *et al.* 2000; Mead *et al.* 2013). Plant apoplastic GABA concentrations have been found to increase during pathogen infection leading to the hypothesis that fungal plant pathogens might induce GABA leakage to assimilate this nutrient (Solomon & Oliver 2002). Conversely, 100 μM GABA was able to trigger sporulation in *Parastagonospora nodorum* under normally prohibitive conditions (Mead *et al.* 2013). The GABA concentration was too low to be considered a nutrient source, suggesting the compound was being used as a signalling mechanism. In the lichen symbiosis, GABA may be acting as both a nutrient source and an indicator molecule, supplying a readily available nitrogen source to the fungus and signalling that a compatible alga has been found.

Mycelia grew in both the negative controls lacking carbon or nitrogen. This apparent growth does not represent an increase in biomass but the recycling of nutrients and metabolite reserves. Hyphae in a colony can fuse to share cytoplasm, known as a syncytium. Syncytia allow extending or exploratory hyphae to progressively move their cellular contents from one hypha to the next in search of nutrients (Plamann 2009; Roper *et al.* 2015). This creates a colony but is not indicative of an increase in biomass; instead, mass will be lost to respiration. Therefore, we normalized absolute measurements to their respective negative controls to account for this effect.

Citrate, nitrate, ammonia and glutamine showed decreased growth compared to their no carbon and no nitrogen negative controls. This is an artefact of fungal growth in low nutrient conditions and highlights the limitations of using a visual approximation for growth. The compounds that produced less growth than the negative controls could have had a toxic effect, such as ammonium toxicity, that inhibited growth. Alternatively, they could be poor nutrient sources that elicited minimal growth but did not trigger exploratory hyphae. Likewise, samples with equal growth to the negative controls, such as ornithine, are ambiguous. We cannot determine whether that particular nutrient, coincidentally, produced the same apparent growth as the negative control, or whether that nutrient is unavailable to the fungus and the apparent growth is due to exploratory hyphae. Despite these limitations, and the inherent phylogenetic variations in the mode of trophic assimilation, these experiments identified several metabolites most suited to growing *E. pusillum* and might be applicable to the culture of other slow-growing lichen-forming fungi.

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