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Growth chamber experiments on lichens: temperature and humidity regimes rapidly shape growth rates and carbohydrate contents

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Preface

This thesis is the final part of my master degree in General Ecology at the department of Ecology and Natural Resource Management (INA), Norwegian University of Life Sciences (NMBU). Finally, after one year of hard work, my master thesis has reached completion. I am satisfied with the whole process. It was very interesting to work on lichen, such an unique and important organism in the ecosystem.

First of all, I would like to express my deepest appreciation to my supervisors, Professor Knut Asbjørn Solhaug and Professor Yngvar Gauslaa, Norwegian University of Life sciences for their continuous guidance and cooperation during the whole process. It has been a pleasure to work with you. I was lucky to find both of you as my supervisors. I found you always available to ask for something. Thank you very much.

I would like to thank Massimo Bidussi, PhD student, Department of Ecology and Natural resource management for some discussion about the growth part of my thesis.

Special thanks to Professor Kristin Palmqvist, Umeå University, Sweden for her valuable comments specially on carbohydrate analysis part.

Last but not the least, I would like to thank my family: my parents and my brothers, for inspiring and supporting me spiritually all the time. I miss you lot.

This Master thesis is submitted to the Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences.

Thanks a lot.

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Abstract

This study assesses relative growth rates and carbohydrate contents of three lichen species under different temperature and humidity regimes in a short-term growth chamber experiment. Representatives from three functional groups: chlorolichens (*Parmelia sulcata*; green algal), cyanolichens *(Peltigera canina*; cyanobacterial) and cephalolichens (*Peltigera aphthosa*; green alga + cyanobacteria) were cultivated for 14 days (150 µmol photon m^{-2} s^{-1}; 12 h photoperiod) at four temperature regimes (28/23 °C, 20/15 °C, 13/8 °C, and 6/1 °C; day/night temperatures) and two hydration regimes (12 h day-time hydration; 12 h day-time + 12 h night-time hydration). These lichens showed much higher growth than earlier reported, particularly at 13/8 °C. A two-way ANOVA with temperature, humidity regimes as factors and specific thallus mass as a co-variate explained 57.8, 53.2 and 38.1 % of the variation in RGR for *P. aphthosa*, *P. canina* and *P. sulcata,* respectively. Significantly higher relative biomass (RGR) as well as thallus area growth rates (RT_AGR) were recorded when the thalli were hydrated day and night compared to hydration in day-time only in all species. Chronic photoinhibition was substantial in *P. apthosa* and *P. canina* when kept at lowest temperature regimes and also for the thalli kept dry at night, whereas *P. sulcata* was photoinhibited at the highest temperature for thalli kept dry at night. Strong, positive linear regressions occurred between RGR and maximal PSII efficiency (F_v/F_m) in all species. Metabolic activity at night improved recovery of photoinhibition and/or may enhance the conversion rate of photosynthates into thallus growth. Moreover, the carbohydrate pools in all the species were measured through HPLC. Unlike the dynamic growth patterns, carbohydrate concentrations varied little with temperature and humidity regimes. After 14 days cultivation, total carbohydrate pool decreased in *P. aphthosa* and *P. canina*, but slightly increased in *P. sulcata*. Mannitol occurred in all the species. Quantitatively, the largest carbohydrate pool was mannitol, glucose and arabitol for *P. aphthosa*, *P. canina* and *P. sulcata,* respectively. The RGR was significantly correlated with photobiont carbohydrate in all species.

Keywords: *Peltigera aphthosa*, *Peltigera canina*, *Parmelia sulcata*, Relative growth rate, Carbohydrates, Chlorophyll, Photoinhibition, Temperature, Humidity, Mannitol.

Abbreviations

RGR	Relative growth rate
RT_AGR	Relative thallus area growth rate
STM	Specific thallus mass
F_v/F_m	Maximal quantum yield of PSII
DM	Dry mass
A	Area
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
HPLC	High performance liquid chromatography
SE	South East

Contents

1. Introduction

Lichens are photosynthetically active symbiotic organism that can survive in almost all habitats over the world. Unlike plants, they do not use specialized organs such as root, shoot and leaves to cope with extreme environmental conditions. A lichen is an integrated thallus composed of mainly two partners (bipartite), the fungal partner and the photosynthetic partner. In addition, some lichen symbiosis consists three (tripartite) or more partners which is not widely known (Nash 2008). The fungal partner, the mycobiont, consists of lichenized fungi mainly (98 %) from ascomycetes (Honegger 1993). The photosynthetic partner, photobiont, is an alga and/or a cyanobacterium. These autotrophic organisms contribute by photosynthesis to provide the organism with fixed carbon. There are total 1600 genera of algae among which only 40 genera have been found to associate with the lichen-forming fungi (Friedl & Büdel 1996; Tschermak-Woess 1988). Most lichen photobionts are eukaryotic Chlorophyta (green algae) and some are from Xanthophyta (yellow-green algae). Green algae are photobionts in 90 % of all known lichens. *Trebouxia,* the most common genus, occurs in about 40 % of all lichens. Procaryotic photobionts occur only in about 8 % of the known lichens. The most common cyanobacterial photobiont genus is *Nostoc,* capable of both photosynthetic CO_2 fixation as well as N_2 fixation as reviewed by Palmqvist (2000). In lichens, the mycobiont takes up moisture leading to a mechanical change which allows more light to pass through the upper cortex (Gauslaa & Solhaug 2001) triggering algal photosynthesis and growth. During dry periods, the lichen becomes desiccated and does not grow. In terms of quantitative abundance and species diversity, lichens dominate almost 8 % of terrestrial ecosystem globally (Larson 1987). As they can withstand some extreme environmental condition, lichens form a dominated component of vegetation at higher latitudes (Longton 1988) under harsh environmental conditions. Lichens are important organisms in succession as pioneers in inhospitable environments such as tundra, exposed rock surfaces, asbestos, mortar and tropical leaf surfaces. Lichens are useful tools for monitoring air pollution in any areas (Skye 1979; Szczepaniak & Biziuk 2003). Although a lichen thallus is an important ecological entity as such, lichen-dominated communities are in danger all over the world. They are disappearing from many regions at a alarming rate (Elmendorf et al. 2012). Habitat destruction and fragmentation are main threats to lichens (Scheidegger & Werth 2009). Due to destruction of old growth forest all over the world, lichens are currently declining. Moreover, lichens are very sensitive to climate change affecting survival

and distribution (Ellis & Coppins 2007; Ellis et al. 2007). In addition, air pollution is major threat for poikilohydric organism like lichen in central Europe (Nimis et al. 2002). Thus, we need intensive investigation on lichen for the conservation of these unique organisms and to understand their susceptibility and decline.

The growth of an individual plant can be explained as the result of resource gain and subsequent biosynthesis of cellular compounds minus losses related to dispersal, fragmentation, grazing or necrosis (Palmqvist 2000). This is also true for the growth of lichens as the dominant part of both lichen and plant biomass is made of carbohydrate ((CH_2O)n) equivalents (Palmqvist & Sundberg 2000). The growth of lichen can be expressed as weight as well as thallus area gain. According to Gauslaa et al. (2009), lichen growth is often three-dimensional where the weight gain depends on photosynthetic carbon gain, whereas area gain depends on cell division and expansion (e.g., Palmqvist 2000). The formation of new lichen tissue requires the input of both carbon and mineral resources (Crittenden 1991). Moreover, lichens are considered as nutritionally specialized fungi which are capable of acquiring carbon (C) from algal or cyanobacterial photobionts (Honegger 1991; Richardson 1999). In this symbiotic organisms, only the photobionts (algae or cyanobacteria) synthesize carbohydrates (sugars or sugar alcohols) which are transferred to the mycobiont (fungus) (Armstrong & Smith 1996). Moreover, only the green algae produce acyclic sugar alcohols or polyols while the cyanobacteria produce glucose (Fahselt 1994; Hill & Smith 1972; Richardson & Smith 1966; Richardson & Smith 1968a). The type of sugar alcohols also vary with algal partner present in the lichens. Among eukaryotic photobionts, the most common green algae *Trebouxia,* as well as *Coccomyxa* and *Myrmecia,* export ribitol, whereas, *Trentepohlia* exports erythritol, and *Hyalococcus* exports sorbitol (Richardson 1985; Smith et al. 1969).

Lichens are considered to be slow-growing and long-living organisms needing long time in growth experiment, meaning that is should be difficult to observe effects of environmental factors on lichen growth within short time. But, recent studies on lichen growth (Bidussi et al. 2013; Denison 1988; Larsson et al. 2009; Pearson & Benson 1977) and synthesis of lichen compounds (Solhaug & Gauslaa 2004; Solhaug et al. 2003) under controlled laboratory condition suggest that growth can be measured within short period in growth chambers. The growth rate of lichen depends on different external and internal factors. Being poikilohydric

organism, lichen cannot maintain their water status (Green & Lange 1995). Therefore, growth is strongly correlated with external water availability (Armstrong 1992; Muir et al. 1997; Renhorn et al. 1996). Light is an important factor for lichen growth in the wet and metabolically active state (Palmqvist & Sundberg 2000). Though temperature is considered less important than humidity and light for lichen growth (Nash III 1996), it impact photosynthesis and respiration that affect lichen growth significantly. High temperature decreases carbon gain due to increased rate of respiration (Lange et al. 1994; Zotz et al. 1998). Moreover, the growth of lichen depends on the carbohydrates produced by photobiont and on the transfer of carbohydrates from a photobiont to a mycobiont. Long hydration periods without light may have negative impact on lichen growth because of excessive carbon loss by respiration. The future climate change as predicted by Stocker et al. (2013) will have negative impact on lichen communities because increased temperature and rainfall will likely affect lichen growth through negative carbon balance. As lichens grow slowly, very few studies have been conducted to observe the impact of environmental factors on lichen productivity. In addition, carbohydrate pools in lichen have rarely been quantified in functional experiments. Carbohydrate is the main substrate in respiration (Amthor 1995), and almost 50 % of the carbohydrates from the photosynthesis might be consumed in lichen respiration (Palmqvist 2000). Although the carbohydrate is the main requirement for energy and biosynthesis in growth and maintenance respiration, there is little information on how the carbohydrate production is affected by environmental factors and possible links between specific carbohydrates (photobiont or mycobiont) and lichen growth. Thereby, it is important to understand the carbohydrate pools and how they are influenced by external factors. In this study, I want to investigate the combined effects of moisture and temperature regimes on lichen growth and carbohydrate pools in short-time growth chamber experiments. This study includes three common and locally dominant lichens. One of them, the tripartite *Peltigera aphthosa* (cephalolichen) entails both green algal (*Coccomyxa*) and cyanobacterial photobionts (*Nostoc*). Next, the bipartite *Peltigera canina* (cyanolichen) has *Nostoc* as its only photobiont. Finally, the bipartite *Parmelia sulcata* (chlorolichen) has the green algal *Trebouxia* as its only photobiont. These species are selected to compare the observation with different photobionts and to observe the different carbohydrates produced by individual photobionts.

The objectives of this study are:

i. To study the growth of lichens as relative growth rate (RGR) and relative thallus area growth rate (RT_AGR) under different temperature and humidity regimes to assess the optimum growth conditions in growth chambers.
ii. To evaluate the use of growth chambers in lichen growth studies.
iii. To observe the effect of temperature and humidity on carbohydrate production in lichen.
iv. To assess the relationship between RGR and produced carbohydrates.

2. Materials and methods

2.1 Lichen materials

The *Peltigera* species *Peltigera aphthosa* (L.) Willd. and *Peltigera canina* (L.) Willd. were collected on 6th of September, 2013. *Peltigera aphthosa* was collected near Kollåsen, Ski, SE Norway (59. 753 °N, 10. 939 °E). The collection sites were fairly open, but partly shaded by the trees. The lichens were collected on shallow soils. *Peltigera aphthosa* (L.) Willd. is distributed in North America, Europe and Asia but mainly it is a circumpolar species in arctic, boreal, and temperate zones (Escudero 2003). Its lobes are broad, 2- 5 cm wide, dull grey-green when dry, bright green when moist. It is a cephalolichen containing two photobionts, green algae and cyanobacteria. The green algal photobiont (*Coccomyxa*) is found in the main thallus and the cyanobacterial photobiont *Nostoc* is located in superficial cephalodia (Rai et al. 1981). *Peltigera canina* (L.) Willd. was collected on soil close to a road crossing (59. 74114 °N, 10. 94065 °E) near Kollåsen in Ski. It is among the most widespread and common lichens in the world (Escudero 2003). Its lobes are wide and 5 - 10 cm in diameter. The color is dull brown, but become blackish when moist. The rounded lobes are soft when moist and papery when dry. It contains cyanobacterial photobiont *Nostoc* which assist in fixing atmospheric nitrogen. *Parmelia sulcata* Taylor. was collected on 7th of November, 2013 from the bark of trees located at Rustad (59. 66609 °N, 10. 81720 °E) in Ås, SE Norway. It is a widely distributed species and regarded as one of the most common taxa in temperate Europe. This species can grow in a wide range of environments. It mainly grows on bark or wood, but can also be found on siliceous rocks (Del Carmen Molina et al. 2011). It is foliose and the thalli are 4 - 20 cm in diameter. This lichen contains the most common green algal photobiont *Trebouxia*.

Fig. 1 A- *Peltigera aphthosa*, B- *Peltigera canina*, C- *Parmelia sulcata*

Photos by: Knut Asbjørn Solhaug

2.2 Growth experiment

The growth experiment was carried out following methods of Bidussi et al. (2013). All the collected thalli of each species were cleaned and stored in freezer for one month. The thalli were then air dried and transported to the laboratory. Firstly, eighty young and healthy thalli of each species with none or few reproductive organs were randomly selected. The selected thalli were then rinsed from debris. The unwanted mosses and green debris attached with lichens were cleaned. They were kept in the lab at 20 °C for 48 h before recording air dry mass (± 0.1 mg). Ten additional thalli of each species were selected for the purpose of measuring oven dry weight (DM) of all thalli. These were weighed and then put into the oven for 24 h at 70 °C. In the next day, these were reweighed (DM) until the weight became constant. The reduction factor in dry mass in the sacrificed thalli was used to calculate DM for all thalli. Afterwards, the samples were sprayed with de-ionized water and thallus area (A) was measured by a leaf area meter (LI3100 Licor, Lincoln, Nebraska) when the thallus was fully hydrated. Thalli of *P. aphthosa*, *P. canina* and *P. sulcata* had start DM of 184.1 ± 4.7 mg, 175.2 ± 5.4 mg and 240.5 ± 5.3 mg (mean ± 1 SE; n = 80) respectively, with corresponding thallus area of 13.3 ± 0.3, 14.4 ± 0.4 and 10.5 ± 0.2 cm^2 .

2.2.1 Experimental design

The growth experiment was carried out in two Sanyo MLR-351 growth chambers (Sanyo Electric, Japan). The thalli were cultivated for 14 days. Four diurnal temperature regimes (day/night): 28/23 °C, 20/15 °C, 13/8 °C, and 6/1 °C and two hydration treatment: 12 h dry + 12 h wet and 24 h wet were used. The daily photoperiod (150 µmol photons m^{-2} s^{-1}) was 12 hours for all days. The light condition was maintained by fluorescent lamps, Mitsubishi/Osram FL 40SS W/37. Twenty thalli of each species were grown in each temperature regime. In each treatment, thalli were cultivated in 20 open Petri-dishes (three thalli in each dish, one of each species) on top of 10 layers of filter paper. The hydration treatment was maintained by spraying deionized water. During cultivation, the lichens and filter papers were kept moist by spraying. The amount of water added in each treatment was adjusted for species and temperature. The water was added sufficiently to keep all the thallus equally hydrated until nearly the end of the light period. Pre-experiments were run to adjust the amount of added water to suitable levels. The lichens in 10 Petridishes were kept hydrated by spraying at the beginning and at the end of

the light period, whereas the remaining 10 Petridishes were sprayed in the beginning of the light period only. In this case, the former treatment kept the thalli hydrated during the light as well as the dark period, the latter treatment kept them moist during most of the day, but dry at night. Moreover, at the end of the photoperiod, these thalli were transferred to Petri-dishes with dry filter paper to accelerate drying and make sure that they remained dry during the entire dark period.

2.2.2 Growth rate measurements

Dry mass (DM) and Area (A) were quantified at the beginning and at the end of the experiment. Growth was measured as relative growth rate, $RGR = (\ln (DM_{end}/DM_{start}))*1{,}000/\Delta t$ (mg g^{-1} day^{-1}) and as relative thallus area growth rate, $RT_AGR = (\ln (A_{end}/A_{start}))*100/\Delta t$ (mm^2 cm^{-2} day^{-1}) , where Δt is the number of days between times start and end at which DM (g) and A (cm^2) were measured (Evans 1972), Δt = 14 days. Specific thallus mass, STM, was calculated at the beginning and at the end of the experiment as STM = DM/A. Changes in STM were calculated as $\Delta STM = 100*(STM_{end} - STM_{start})/STM_{start}$ and expressed as percentage change.

2.3 Measurement of photoinhibition

After the last dark period in growth experiment, all the thalli were taken out and measured the photoinhibition. For this purpose, the lichen was moistened and the thalli were kept in low light for 15 minutes. After that, the maximum photochemical efficiency of photosystem II (F_v/F_m) was measured with PAM 2000 fluorometer (Walz, Effeltrich, Germany).

2.4 Carbohydrate analysis

2.4.1 Extraction of carbohydrate

The amount of carbohydrates in the thalli were analyzed by following Gordy et al. (1978). After finishing the growth experiment, 100 mg dry weight of each thallus of each species were taken. The thallus was then ground to fine powder with a ball mill using small metal ball into an eppendorf tube. The soluble carbohydrates were extracted through heating the samples in 80 % ethanol with two changes of ethanol at 60 °C for 30 minutes for each change. The heating was carried out into a ultrasonic bath. In each changes, the extracts were centrifuged at 15000 rpm/min for 3 minutes. The supernatant from each changes were added together. The ethanol was removed from the supernatant at 60 °C by using a vacuum desiccator (Eppendorf AG 22331,

Hamburg, Germany). It is essential to remove the ethanol completely because it is eluted close to glucose on the HPLC and it is detected by the RID detector. Therefore, it can interfere with other carbohydrate peaks. After that, added 1.5 ml of water with the extract and heated at 60 °C for 30 minutes. The extract was then centrifuged at 15000 rpm/min for 3 minutes and the supernatant was collected. This supernatant was then filtered through a 0.45 µm GHP membrane filter (Millipore) before chromatography.

2.4.2 Separation of carbohydrates

Different techniques are used to separate and identify different carbohydrates. Among them the most common techniques are TLC (Thin Layer Chromatography), GC (Gas Chromatography) and HPLC (High Performance Liquid Chromatography). Nowadays, HPLC is widely used in this purpose as it is capable of rapid, specific, sensitive and precise measurements. In this experiment, HPLC technique was also used to separate and identify carbohydrates mainly sugar alcohols. During this experiment, Agilent 1200 series of HPLC (Agilent Technologies, Waldbronn, Germany) was used to analyze lichen extract. Carbohydrates mainly separated on the basis of their differential adsorption characteristics and analyzed by passing the solution through a column. Here, the column Agilent Hi-Plex Ca USP L19, 4,0 * 250 nm, 8 µm (p/n PL1570-5810) which is a specialized column for separating sugar alcohols was used and the sugar alcohols were detected by a Refractive Index Detector. For the mobile phase, 30 % acetonitrile and 70 % water were mixed together and used as solvent. The flow rate was 0.3 ml/min and the temperature of the column was 90 °C (Stephen Ball 2013).

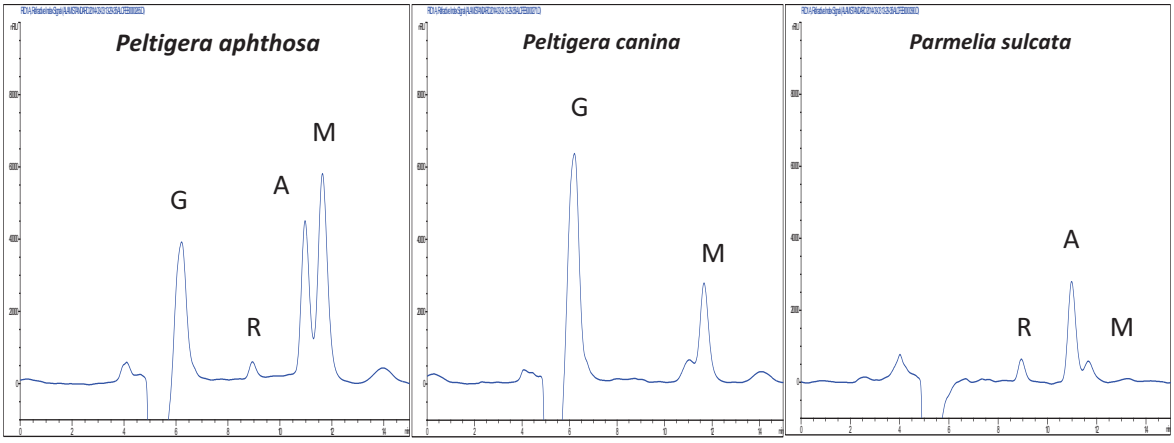

Fig. 2 The HPLC chromatogram trace showing the soluble carbohydrates peak. G = glucose, R = ribitol, A = arabitol, M = mannitol.

2.5 Chlorophyll analysis

2.5.1 Extraction of chlorophyll

The chlorophyll contents in the lichen samples were extracted followed by the procedure described in Palmqvist and Sundberg (2002). 10 - 12 mg of dry lichen samples from each species were ground to a fine powder on a ball mill in an Eppendorf tube. 1.5 ml of DMSO with $MgCO_3$ were added to each Eppendorf tube. The tubes were vortexed and incubated at 60 °C for 40 min using a water bath. They were vortexed several times during incubation. Afterwards, the extracts were centrifuged at 18000 rpm/min for 5 minutes and the absorbance of the supernatant was measured by a spectrophotometer.

2.5.2 Measurement of chlorophyll

The chlorophyll content was measured by using a Shimadzu UV2001 PC spectrophotometer. The absorbance for chlorophyll content was measured at 665 and 649 nm. The baseline absorbance was measured at 750 nm. After finding the absorbance at 649, 665 and 750 nm, chlorophyll *a* and chlorophyll *b* in mg g^{-1} was calculated according to equations from Wellburn (1994). The equations are stated below:

Chl *a* = $12.19*(A_{665} - A_{750}) - 3.45*(A_{649} - A_{750})$

Chl *b* = $21.99*(A_{649} - A_{750}) - 5.32*(A_{665} - A_{750})$

Peltigara canina is a cyanobacterial lichen that lacks Chl *b* and this equation is used for cyanobacterial lichen:

Chl *a* = $12.19*(A_{665} - A_{750})$.

2.6 Statistical analyses

All statistical analyses were run in Minitab 16 (Minitab Inc., State College, PA, USA). Two-way ANOVA was carried out using general linear model (GLM) to observe the effect of treatments on different parameters in three species. Temperature and humidity regimes were used as factors for both analysis. In growth analysis, STM at start was used as covariate and the parameters were RGR, RT_AGR, ΔSTM, Chl *a* and F_v/F_m. In carbohydrate analysis, STM at start and Chl *a* was used as covariate and the parameters were glucose, ribitol, arabitol, mannitol and total carbohydrate. When required, the variables were transformed to meet the requirements of the ANOVA. Correlation between individual carbohydrate and between RGR and different carbohydrates were also carried out. Means ± 1 standard error are given in text and figures.

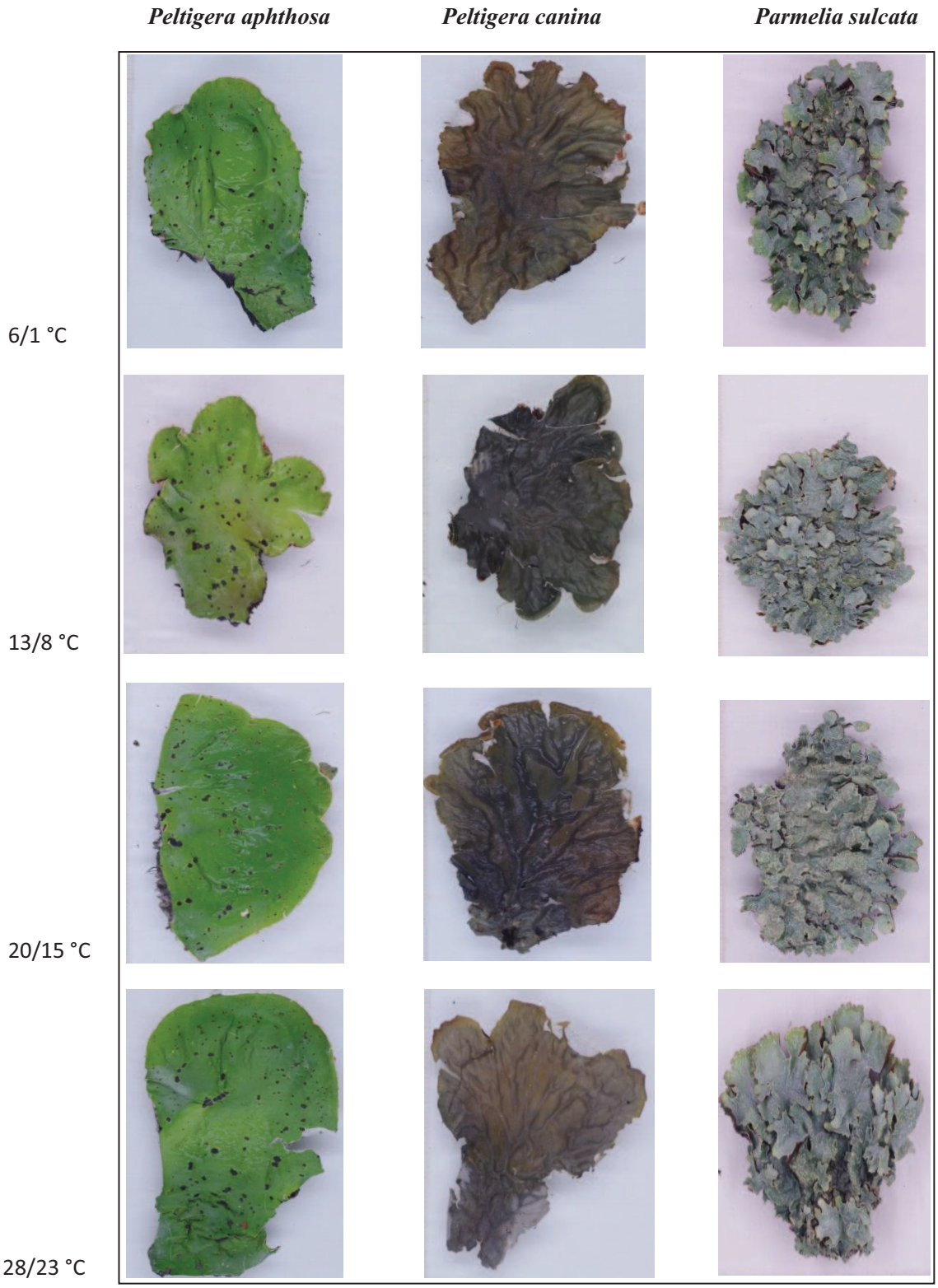

Fig. 3 Typical lichen specimen used in this study (Before the cultivation)

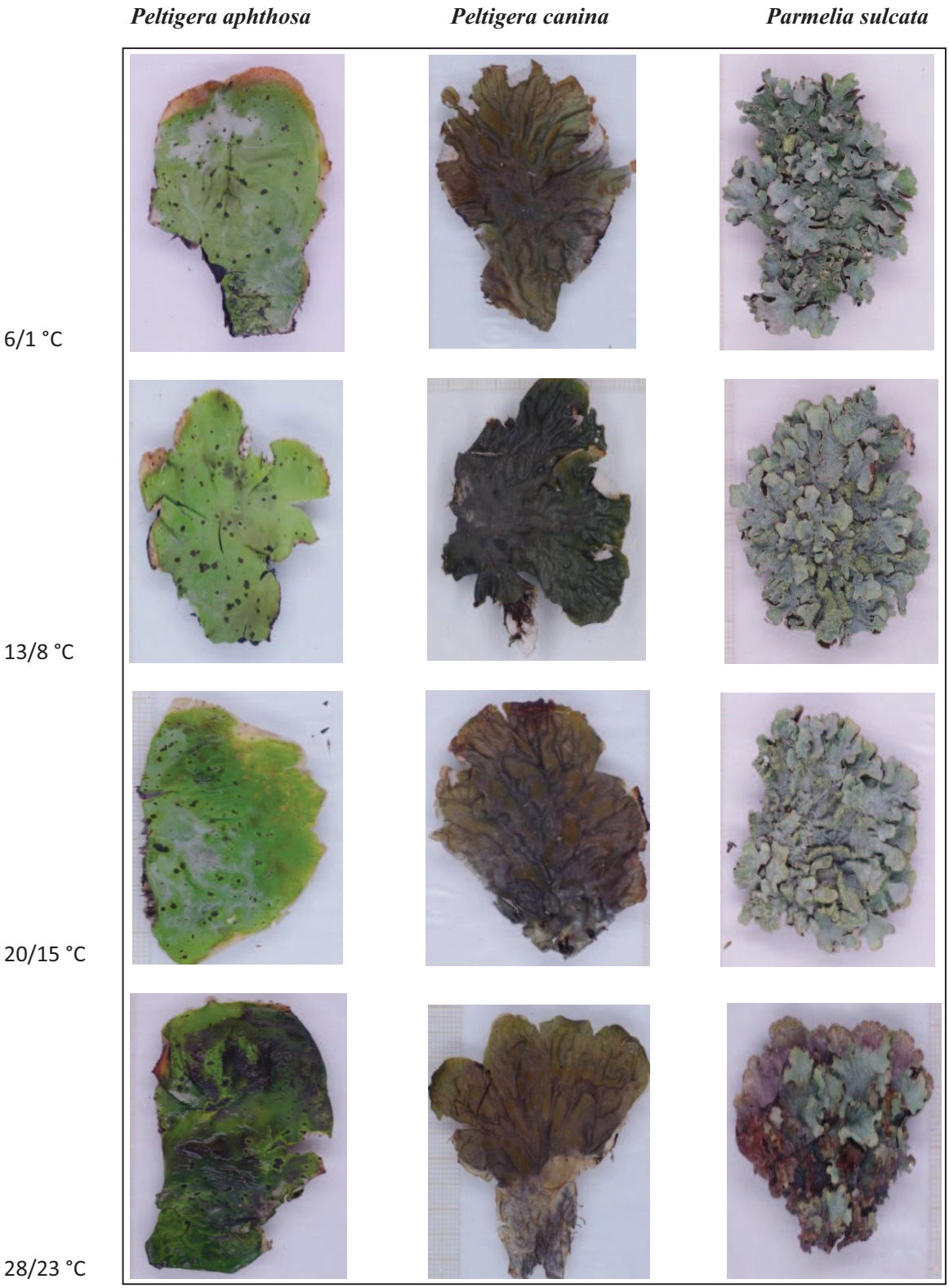

Fig. 4 Typical lichen specimen used in this study (After the cultivation)

3. Results

Photos of typical thalli before (Fig. 3) and after (Fig. 4) the growth experiment are shown. *Peltigera aphthosa* and *P. canina* were damaged at extreme temperature (6/1 and 28/23 °C), whereas *P. sulcata* showed strong, visible damage at the maximum temperature (Fig. 4).

3.1 Chlorophyll fluorescence

Maximal photosystem II activity (F_v/F_m) highly significantly differed between temperature and hydration regimes for all three species (Table 1). After the cultivation, the average F_v/F_m across all treatments was 0.506 ± 0.018, for *P. aphthosa*, whereas *P. sulcata* and *P. canina* had 0.647 ± 0.012 and 0.176 ± 0.017; (n = 80) respectively. In *P. aphthosa* and *P. canina*, the F_v/F_m increased with increasing temperature, whereas *P. sulcata* had almost the same values at all the temperature regimes (Fig. 5). Moreover, all species kept hydrated 24 h showed higher F_v/F_m values than those hydrated only 12 h. The thalli hydrated the whole day had fluorescence values means of 0.572 ± 0.021, 0.256 ± 0.025 and 0.677 ± 0.009 for *P. aphthosa*, *P. canina* and *P. sulcata* respectively, whereas those hydrated once in a day had the respective means 0.439 ± 0.025, 0.088 ± 0.013 and 0.617 ± 0.022 (n = 39 - 40). *Peltigera canina* showed almost three times higher F_v/F_m values in 24 hours hydrated thalli than those hydrated 12 hours a day. For *P. aphthosa* and *P. canina*, much photoinhibition occurred at lowest (6/1 °C) temperature and also in the thalli kept dry at night, whereas *P. sulcata* was photoinhibited at the maximum temperature (28/23 °C) for the thalli kept dry at night. F_v/F_m was a highly significant covariate in the ANOVA with RGR in all three species ($P < 0.05$, data not shown). At the end of the experiment, chlorophyll fluorescence value showed positive relationship with relative growth rate (RGR) in all species. Both *P. aphthosa* and *P. sulcata* showed almost similar regression curve (Fig. 6).

3.2 Chlorophyll *a* and *b*

The Chl *a* concentration in the thallus ranked from 0.60 to 2.37 mg g^{-1} in *P. aphthosa*, from 0.12 to 1.8 mg g^{-1} in *P. canina* and from 0.46 to 2.56 mg g^{-1} in *P. sulcata*. Chl *a* varied between the species, but not between the treatments (Table 1). The average Chl *a* content across all treatments was higher in *P. aphthosa* (1.10 ± 0.04 mg g^{-1}, n = 80) than *P. canina* (0.76 ± 0.04 mg g^{-1}, n = 80), whereas *P. sulcata* (1.44 ± 0.05 mg g^{-1}, n = 79) showed almost twice as high Chl *a*

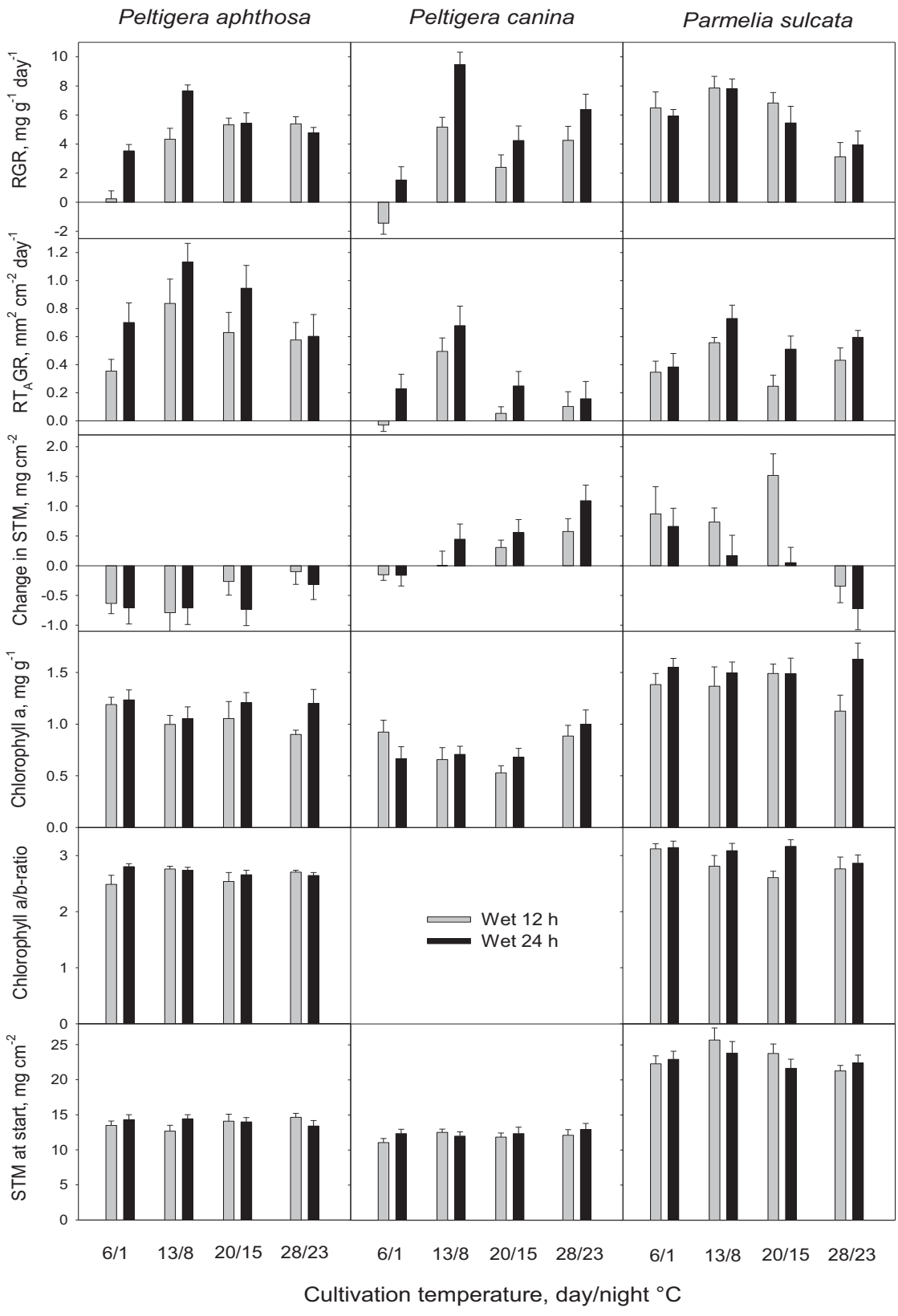
Peltigera aphthosa
Peltigera canina
Parmelia sulcata
RGR, mg g-1 day-1
RTAGR, mm2 cm-2 day-1
Change in STM, mg cm-2
Chlorophyll a, mg g-1
Chlorophyll a/b-ratio
STM at start, mg cm-2
Wet 12 h
Wet 24 h
6/1
13/8
20/15
28/23
Cultivation temperature, day/night °C

Fig. 5 Growth rates (RGR, RT_AGR), change in specific thallus mass during cultivation (ΔSTM), Chl *a* and maximal photosystem II activity (F_v/F_m) in *Peltigera aphthosa*, *Peltigera canina* and *Parmelia sulcata* cultivated for14 days at four temperature regimes (28/23, 20/15, 13/8, and 6/1 °C, day/night temperature) all with 12 h daily photoperiod (150 µmol photons m^{-2} s^{-1}) and two hydration treatments (wet 12 h, wet 24 h). Error bars indicate 1SE.

contents as in *P. canina*. Moreover, Chl *a:b* ratio was higher in *P. sulcata* (2.94) compared to *P. aphthosa* (2.67).

3.3 Effects of temperature and humidity on the Relative Growth Rate (RGR)

The average RGR across all treatments was 3.96 ± 0.46 mg g^{-1} day^{-1} for *P. canina* 4.58 ± 0.29 mg g^{-1} day^{-1} for *P. aphthosa*, and 5.92 ± 0.35 mg g^{-1} day^{-1} for *P. sulcata* (mean ± 1 SE; n = 78–80). Individual RGR-values ranked from -3.7 to 10.3 mg g^{-1} day^{-1} for *P. aphthosa,* -5.2 to 14.8 mg g^{-1} day^{-1} for *P. canina* and -2.9 to 12.6 mg g^{-1} day^{-1} for *P. sulcata*. Growth computed as percentage DM gain for the 14 days' period were 6.69 ± 0.42 %, 6.36 ± 0.76 % and 8.73 ± 0.52 % for three species, respectively. Both the temperature and humidity had strong impacts on lichen biomass growth among which temperature was the strongest (2-way ANOVA, Table 1). Interestingly, all three species showed the highest RGR at 13/8 °C (day/night). The highest mean RGR for one treatment was 7.66 ± 0.40 mg g^{-1} day^{-1} and 9.43 ± 0.88 mg g^{-1} day^{-1} for *P. aphthosa* and *P. canina* at the 13 °C and 24 h hydration treatment. In contrast, the highest mean RGR for *P. sulcata* (7.85 ± 0.81 mg g^{-1} day^{-1}) occurred at 13 °C and 12 h hydration treatment. At the highest temperature regime, the RGR of *P. sulcata* was much reduced (Fig. 5). Moreover, humidity had strong, significant effect on RGR for both *P. aphthosa* and *P. canina*, whereas RGR of *P. sulcata* did not respond to humidity regime (Table 1). The fastest RGRs consistently occurred in thalli hydrated continuously for 24 hours (5.34 ± 0.34 and 5.39 ± 0.67 mg g^{-1} day^{-1} for *P. aphthosa* and *P. canina*); the slowest RGRs were recorded for those hydrated only 12 h: 3.81 ± 0.43 and 2.56 ± 0.58 mg g^{-1} day^{-1} for the two species, respectively (means averaged across all four temperature regimes; n = 78 – 80). In *P. sulcata*, the average RGRs between two humidity regimes were not significantly different (Fig. 5, Table 1). *Peltigera aphthosa* showed larger differences in RGR between the two hydration treatments at the two lowest temperature regimes, but not at the higher temperature regimes. By contrast, RGR of *P. canina* responded more to temperature regimes than *P. aphthosa*, whereas RGR in *P. sulcata* declined with increasing temperature without any significant differences between the hydration treatments.

Table 1. Two way ANOVA for growth rates (RGR, RTAGR, ΔSTM), Chl *a* and F_v/F_m in *Peltigera aphthosa*, *Peltigera canina* and *Parmelia sulcata* cultivated for14 days at four temperature regimes (T) and two hydration treatments (H).

Parameter Source	*d.f*	**RGR**		**RT_AGR**		**ΔSTM**		**Chl *a***		**F_v/F_m**	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Peltigera aphthosa											
Temperature	3	22.98	0.000	4.28	0.008	2.11	0.106	1.52	0.216	38.05	0.000
Humidity	1	15.86	0.000	6.03	0.016	0.98	0.326	3.73	0.057	40.71	0.000
T*H	3	7.41	0.000	0.55	0.65	0.66	0.58	0.69	0.561	2.66	0.054
Error	72										
Total	79										
r^2_{adj}		**0.559**		**0.146**		**0.028**		**0.041**		**0.663**	
Peltigera canina											
Temperature	3	24.36	0.000	11.15	0.000	7.14	0.000	3.70	0.016	10.06	0.000
Humidity	1	18.54	0.000	4.24	0.043	2.51	0.118	0.24	0.269	52.47	0.000
T*H	3	0.61	0.610	0.58	0.629	0.52	0.628	1.20	0.316	2.70	0.052
Error	69										
Total	76										
r^2_{adj}		**0.537**		**0.305**		**0.190**		**0.096**		**0.521**	
Parmelia sulcata											
Temperature	3	7.86	0.000	5.08	0.003	6.77	0.000	0.31	0.819	10.00	0.000
Humidity	1	0.25	0.617	8.30	0.005	7.44	0.008	4.63	0.035	9.64	0.003
T*H	3	0.53	0.663	0.56	0.641	1.02	0.389	1.24	0.302	8.46	0.000
Error	71										
Total	78										
r^2_{adj}		**0.191**		**0.191**		**0.235**		**0.028**		**0.428**	

In addition, the interaction (Temperature * Humidity) highly significantly impacted the RGR of *P. aphthosa*, but neither for *P. canina* nor *P. sulcata* (Table 1). Moreover, RGR significantly declined with decreasing F_v/F_m for all the species ($P < 0.001$; $r^2_{adj} = 0.152$ to 0.444), especially for thalli cultivated at the lowest temperatures (6/1 °C) and kept dry at night (Fig. 5). There were no significant relationships between RGR and Chl *a* except for *P. sulcata* ($P < 0.05$, data not

shown). Moreover, STM_{start} was a highly significant covariate in the ANOVA with RGR ($P < 0.001$, data not shown) in *P. sulcata* but not significant for *P. aphthosa* and *P. canina*.

3.4 Effects of temperature and humidity on the Relative Thallus Area Growth Rate (RT_AGR)

The average relative thallus area growth rate (RT_AGR) across all the treatments was 0.72 ± 0.05 $mm^2\ cm^{-2}\ day^{-1}$ for *P. aphthosa*, 0.24 ± 0.04 $mm^2\ cm^{-2}\ day^{-1}$ for *P. canina* and 0.48 ± 0.03 $mm^2\ cm^{-2}\ day^{-1}$ for *P. sulcata* (mean ± 1 SE; n = 80). Moreover, area growth rates converted to percentage area gain for the 14 days' period were 10.9 ± 0.85 %, 3.6 ± 0.62 % and 6.9 ± 0.47 % for *P. aphthosa*, *P. canina* and *P. sulcata,* respectively. Interestingly, the overall RT_AGR of *P. aphthosa* was twice as high as *P. sulcata* and three times higher than in *P. canina*. Both treatments significantly influenced the RT_AGR in all species (2- way ANOVA; Table 1), with no significant interaction term. For *P. aphthosa* the highest mean RT_AGR was 1.13 ± 0.13 $mm^2\ cm^{-2}\ day^{-1}$, whereas *P. canina* and *P. sulcata* showed the highest mean RT_AGR 0.68 ± 0.14 and 0.73 ± 0.09 $mm^2\ cm^{-2}\ day^{-1}$ respectively (n = 10). For all species, these highest mean RT_AGR were recorded at 13/8 °C at 24 h hydration (Fig. 5). The area growth rate is higher at low than at high temperature regimes. At low temperatures (6/1 and 13/8 °C), the average RT_AGR was 0.76 ± 0.08, 0.34 ± 0.06 and 0.50 ± 0.05 $mm^2\ cm^{-2}\ day^{-1}$; n = 40, for *P. aphthosa*, *P. canina* and *P. sulcata,* respectively, and higher than that at the higher temperature regimes (0.69 ± 0.07, 0.14 ± 0.05 and 0.45 ± 0.04 $mm^2\ cm^{-2}\ day^{-1}$; n = 40, for the species, respectively). The contrast in RT_AGR between the two hydration regimes was also substantial. In all species, thalli hydrated 24 h showed higher RT_AGR than those hydrated only 12 h. *Peltigera aphthosa* hydrated the whole day had RT_AGR means of 0.84 ± 0.07 $mm^2\ cm^{-2}\ day^{-1}$ versus 0.59 ± 0.07 $mm^2\ cm^{-2}\ day^{-1}$ (n = 40) for those hydrated half of the day, whereas the respective means were 0.31± 0.06 and 0.15 ± 0.05 (n = 38 - 39) $mm^2\ cm^{-2}\ day^{-1}$ for *P. canina* and 0.56 ± 0.04 and 0.39 ± 0.04 (n = 39 - 40) $mm^2\ cm^{-2}\ day^{-1}$ for *P. sulcata*. The thalli of *P. canina* hydrated both day and night had twice as high RT_AGR as those hydrated only the day.

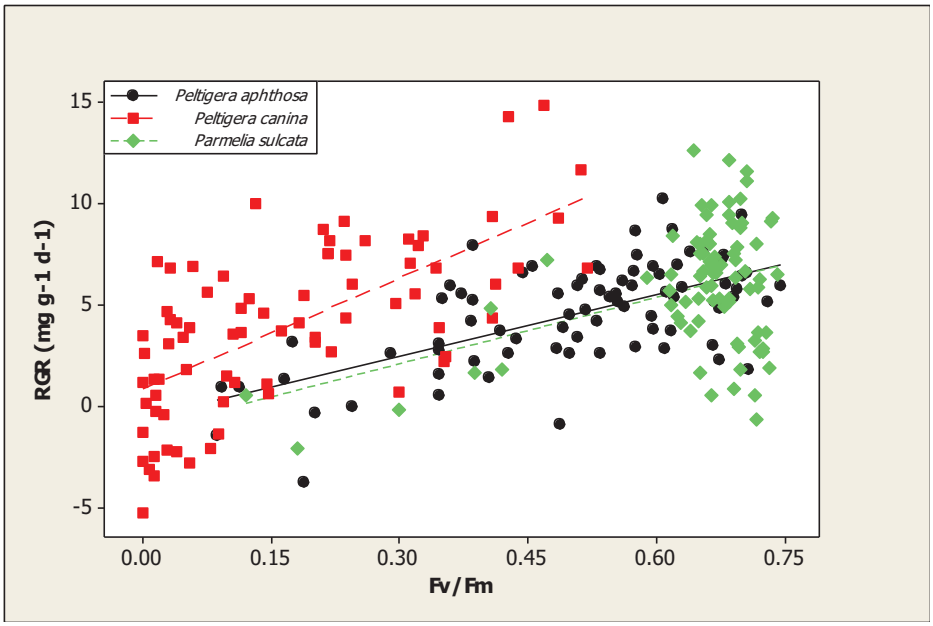

Fig. 6 The relationships between relative growth rate (RGR) and F_v/F_m measured at the end of the experiment in the thalli of *Peltigera aphthosa*, *Peltigera canina* and *Parmelia sulcata*. All species showed highly significant linear regressions ($P < 0.001$) between RGR and F_v/F_m: cephalolichen RGR = -0.54+10.11*(F_v/F_m); cyanolichen RGR = 0.87+18.22*(F_v/F_m); r^2_{adj} = 0.444 r^2_{adj} = 0.395; chlorolichen RGR = -1.18+10.97*(F_v/F_m); r^2_{adj} = 0.152.

3.5 Effects of temperature and humidity on the change in Specific Thallus Mass (ΔSTM)

At start, the specific thallus mass (STM) for three species were 13.8 ± 0.25, 12.1 ± 0.24 and 22.9 ± 0.46 mg cm^{-2} (mean ± 1 SE; n = 80) for *P. aphthosa*, *P. canina* and *P. sulcata* respectively. In *P. aphthosa*, the mean area growth exceeded biomass growth at all temperature and humidity regimes (Fig. 5), resulting in a net mean decrease in ΔSTM -0.53 ± 0.09 mg cm^{-2}; n = 80, (3.4 %). By contrast, *P. canina* and *P. sulcata* showed net mean increase in ΔSTM 0.28 ± 0.07 (2.5 %) and 0.37 ± 0.13 (1.8 %) mg cm^{-2} respectively, (n = 78 - 80). The variation in ΔSTM did not significantly differ with the treatments in *P. aphthosa*, whereas temperature was an important source of variation for ΔSTM in *P. canina* and *P. sulcata* (Table 1). Area growth was higher than biomass growth at lowest temperature (6/1 °C) in *P. canina* (-0.15 ± 0.09 mg cm^{-2}; n = 20) and at highest temperature (28/23 °C) in *P. sulcata* (-0.53 ± 0.22 mg cm^{-2}; n = 20). *Peltigera aphthosa* showed higher decrease in ΔSTM at low temperature than at high temperature for both hydration treatments (Fig. 5). By contrast, ΔSTM gradually increased with increasing temperature for both

humidity regimes in *P. canina,* but this trend was totally reversed in *P. sulcata.* In *P. canina* the thalli kept hydrated 24 h showed higher increase in ΔSTM than the thalli kept dry at night, whereas in *P. sulcata*, 24 h hydrated thalli showed higher decrease in ΔSTM than the thalli hydrated only 12 h (Fig. 5). *Parmelia sulcata* hydrated only during day time, showed the highest increase in ΔSTM at 20 °C (1.5 ± 0.36 mg cm^{-2}), whereas the thalli hydrated twice in a day experienced the highest ΔSTM at 6 °C (0.66 ± 0.30 mg cm^{-2}). In *P .canina*, the highest ΔSTM was recorded for both hydration regimes at 28 °C (0.57 ± 0.21 and 0.93 ± 0.22 mg cm^{-2} for both hydration regimes respectively, n = 9 - 10).

3.6 Effects of temperature and humidity on the soluble carbohydrates

The chromatogram trace showing the soluble carbohydrate peaks is shown in (Fig. 2). *Peltigera aphthosa* showed peaks of glucose, ribitol (photobiont carbohydrate) and arabitol, mannitol (fungal carbohydrate). Similarly, *P. canina* showed peaks of glucose and mannitol only, whereas *P. sulcata* had the peaks of ribitol, arabitol and mannitol. Before the experiment, the total carbohydrate concentration was 12.85 ± 1.5 %, 11.80 ± 0.83 % and 4.21 ± 0.55 %, (n = 5) for *P. aphthosa*, *P. canina* and *P. sulcata,* respectively. After 14 days' growth chamber cultivation at different temperature and humidity regimes, the carbohydrate concentration decreased in *P. aphthosa* (7.68 ± 0.18 %, n = 80) and *P. canina* (7.84 ± 0.19 %, n = 80), but increased slightly in *P. sulcata* (5.04 ± 0.14 %, n = 80) (Fig. 7). In *P. aphthosa*, temperature was a significant source of variation for ribitol, arabitol, mannitol and total carbohydrates except for glucose, whereas the humidity regimes showed significant effect for mannitol only (Table 2). Moreover, the interaction (Temperature*Humidity) showed some significant effect on all carbohydrates except mannitol (Table 2). STM at start was a highly significant covariate for glucose and ribitol (Table 2). In contrast, both temperature and humidity regimes significantly influenced all the carbohydrates in *P. canina* apart from mannitol that was not affected by humidity. Also, the interaction (Temperature*Humidity) showed no significant variation of the carbohydrates (Table 2). Chl *a* was a highly significant covariate for Glucose in *P. canina* (Table 2).

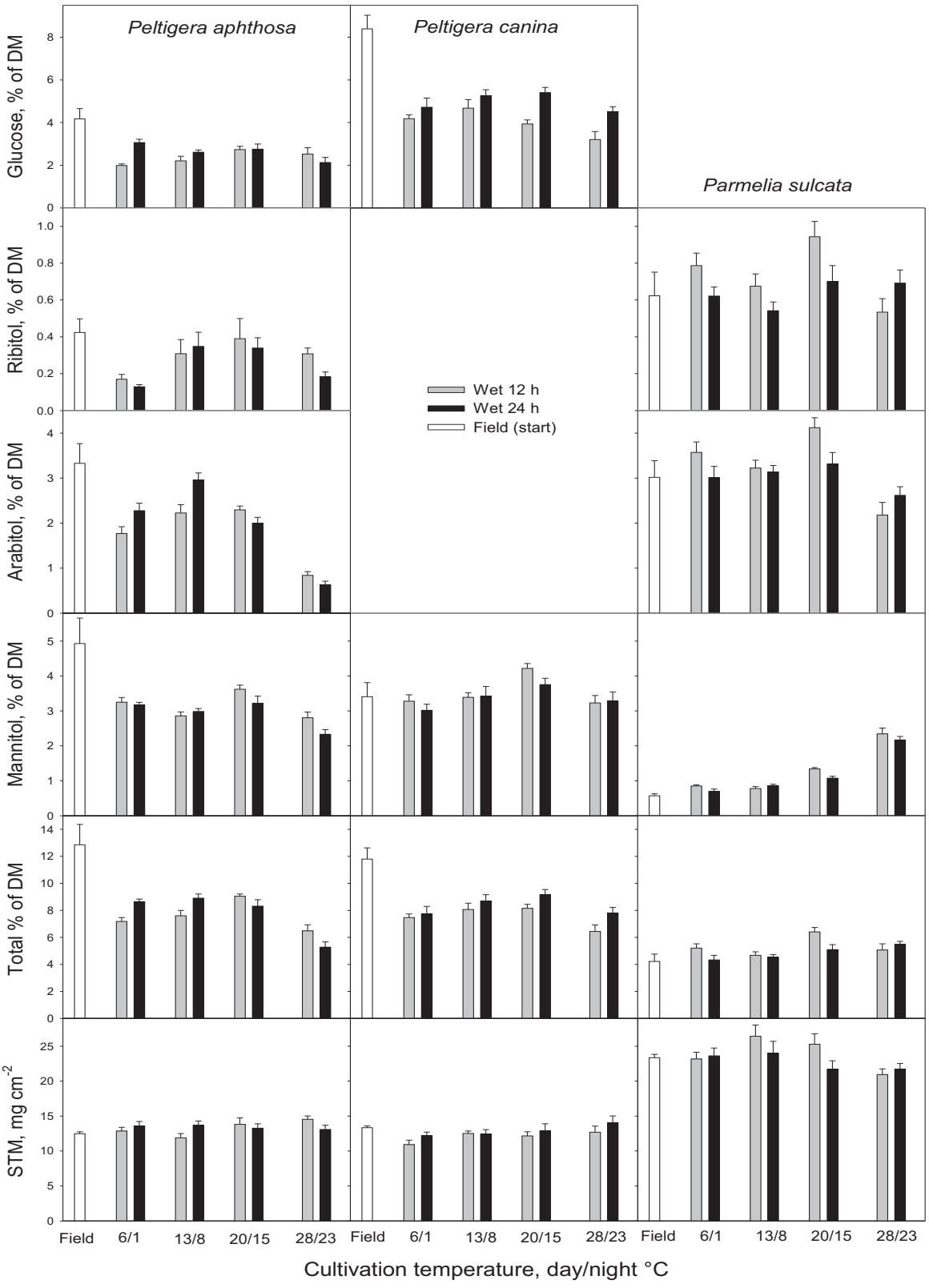
Peltigera aphthosa
Peltigera canina
Parmelia sulcata
Glucose, % of DM
Ribitol, % of DM
Arabitol, % of DM
Mannitol, % of DM
Total % of DM
STM, mg cm-2
Wet 12 h
Wet 24 h
Field (start)
Field
6/1
13/8
20/15
28/23
Cultivation temperature, day/night °C

Fig. 7 Concentration of carbohydrates (glucose, ribitol, arabitol, mannitol, total carbohydrates) and specific thallus mass (STM) at start in *Peltigera aphthosa*, *Peltigera canina Parmelia sulcata* cultivated for 14 days at four temperature regimes (28/23, 20/15, 13/8, and 6/1 °C, day/night temperature) all with 12 h daily photoperiod (150 µmol photons m^{-2} s^{-1}) and two hydration treatments (wet 12 h, wet 24 h).

For *P. sulcata*, the contrast in carbohydrates between temperatures and humidity were highly significant but the interaction (Temperature*Humidity) was not a significant source of variation (Table 2). Chl *a* was also a highly significant covariate for the variation of carbohydrates in *P. sulcata*. Mannitol was the only common carbohydrate pool found in all three species. The quantitatively most important carbohydrate in the cephalolichen was mannitol (3.03 ± 0.06 %), whereas glucose (4.49 ± 0.13 %) was the major carbohydrate for the cyanolichen and arabitol (3.15 ± 0.09 %) for the chlorolichen (n = 80). The average glucose concentration in *P. canina* (4.49 ± 0.13 %) was almost two times higher than *P. aphthosa* (2.5 ± 0.8 %, n = 80). Moreover, the amount of ribitol in *P. sulcata* (0.69 ± 0.03 %, n = 79) was twice as high as in *P. aphthosa* (0.27 ± 0.02 %, n = 80). In addition, mannitol % was almost the same in *P. aphthosa* (3.03 ± 0.06 %) and *P. canina* (3.41 ± 0.09 %) but much lower in *P. sulcata* (1.27 ± 0.07 %). The concentration of glucose was fairly similar in all treatments for *P. aphthosa,* but varied with temperature and humidity regimes in *P. canina* (Table 2). Highest concentration of glucose in *P. canina* (5.41 ± 0.24 %) was formed at 20/15 °C for thalli hydrated 24 h, and the lowest (3.20 ± 0.37 %) at 28/23 °C for thalli hydrated 12 h (n = 10). The concentration of glucose was consistently higher in the thalli hydrated 24 hours (4.9 ± 0.15 %, n = 40) than in those hydrated only 12 hours (3.9 ± 0.16 %, n = 38). Both in *P. aphthosa* and *P. sulcata*, the highest ribitol concentration occurred at 20/15 °C for the thalli hydrated in the morning only (0.39 ± 0.11 and 0.94 ± 0.08 %; n = 10, for *P. aphthosa* and *P. sulcata,* respectively) and the lowest was recorded at 6/1 °C for *P. aphthosa* and at 13/8 °C for *P. sulcata* (Fig. 7). *Parmelia sulcata* showed higher ribitol concentration in the thalli hydrated once (0.73 ± 0.04 %) than in thalli hydrated twice of the day (0.63 ± 0.03 %; n = 39 - 40). By contrast, arabitol percentage was the highest at 13/8 °C for the thalli hydrated 24 h in *P. aphthosa* (2.96 ± 0.15 %, n = 10), whereas *P. sulcata* showed the highest percentage of arabitol (4.12 ± 0.22 %, n = 10) at 20/15 °C for the thalli hydrated 12 h. For both species, the lowest percentage of arabitol was recorded at the highest temperature (Fig. 7). Moreover, average percentage of mannitol was much higher in *P. aphthosa* and *P. canina* than *P. sulcata* (Fig. 7).

Table 2. Two way ANOVA for Carbohydrates (glucose, ribitol, arabitol, mannitol and total carbohydrates) in *Peltigera aphthosa*, *Peltigera canina* and *Parmelia sulcata* cultivated for 14 days at four temperature regimes (T) and two hydration treatments (H) with STM_S (at start) and Chl *a* as covariate.

Parameter Source	*d.f*	**Glucose**		**Ribitol***		**Arabitol***		**Mannitol**		**Total(sugar)**	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Peltigera aphthosa											
STM_S	1	18.48	0.000	29.92	0.000	0.57	0.452	4.83	0.031	4.83	0.031
Chl *a*	1	0.31	0.581	1.59	0.212	0.50	0.484	0.20	0.656	0.00	0.982
Temperature	3	1.87	0.142	9.98	0.000	95.70	0.000	16.19	0.000	27.11	0.000
Humidity	1	3.45	0.067	0.48	0.490	0.12	0.729	5.64	0.020	0.52	0.475
T*H	3	4.20	0.009	4.84	0.004	5.66	0.002	1.42	0.245	6.31	0.001
Error	71										
Total	79										
r^2_{adj}		**0.335**		**0.435**		**0.790**		**0.422**		**0.564**	
Peltigera canina											
STM_S	1	1.74	0.191					7.83	0.007	0.56	0.459
Chl *a*	1	13.59	0.000					0.72	0.398	8.94	0.004
Temperature	3	8.68	0.000					7.44	0.000	8.48	0.000
Humidity	1	21.19	0.000					2.08	0.154	7.76	0.007
T*H	3	1.29	0.286					0.59	0.621	0.29	0.835
Error	67										
Total	76										
r^2_{adj}		**0.401**						**0.264**		**0.266**	
Parmelia sulcata											
STM_S	1			7.65	0.007	1.94	0.168	0.81	0.370	2.95	0.090
Chl *a*	1			23.11	0.000	17.87	0.000	1.08	0.301	18.93	0.000
Temperature	3			4.52	0.006	14.29	0.000	151.71	0.000	6.29	0.001
Humidity	1			14.20	0.000	9.64	0.003	7.49	0.008	13.71	0.000
T*H	3			2.98	0.037	2.69	0.053	2.79	0.047	2.63	0.057
Error	68										
Total	77										
r^2_{adj}				**0.457**		**0.503**		**0.863**		**0.418**	

*The ANOVA was run on log-transformed values.

For *P. aphthosa* and *P. canina*, the highest concentration of mannitol (3.62 ± 0.12 and 4.22 ± 0.14 %; n = 10, respectively) was recorded at 20/15 °C for the thalli kept dry at night. *Parmelia sulcata* showed increasing trend of mannitol percentage with increasing temperature for both humidity regimes (Fig. 7) and the highest percentage of mannitol (2.35 ± 0.16 %, n = 10) was recorded at 28/23 °C for the thalli kept dried at night.

Table 3. Pearson correlation coefficients between the concentration of carbohydrates in individual species. (G = glucose, R = ribitol, A = arabitol, M = mannitol; *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant, n = 80).

Species	**G vs. R**	**G vs. A**	**G vs. M**	**R vs. A**	**A vs. M**	**R vs. M**
Peltigera aphthosa	-0.287*	0.282*	0.586***	0.109 ns	0.465***	-0.076 ns
Peltigera canina			0.215 ns			
Parmelia sulcata				0.756***	-0.238*	0.012 ns

In addition, some carbohydrates were highly correlated with each other in individual species (Table 3). *Peltigera aphthosa* showed positive correlation between glucose and mannitol (r^2_{adj} = 0.586; P < 0.001), and between arabitol and mannitol (r^2_{adj} = 0.465; P < 0.001) but glucose and ribitol were negatively correlated (r^2_{adj} = - 0.287; P < 0.05). In *P. canina*, the carbohydrates were not correlated (Table 3). By contrast, positive correlation was found between ribitol and arabitol (r^2_{adj} = 0.756; P < 0.001) in *P. sulcata*, whereas arabitol and mannitol were negatively correlated (r^2_{adj} = - 0.238; P < 0.05). The overall ratio of fungal carbohydrate to photobiont carbohydrate is much higher in *P. sulcata* (6.98) than *P. aphthosa* (1.81) and *P. canina* (0.82). The RGR of three species were also highly correlated with the photobiont carbohydrates (Table 4). RGR of *P. aphthosa* and *P. canina* showed significant positive correlation with glucose (Table 4) whereas *P. sulcata* showed positive correlation with ribitol and arabitol (Table 4).

Table 4. Pearson correlation coefficients between RGR and carbohydrates in individual species (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = not significant; $n = 80$)

Species	**RGR vs. glucose**	**RGR vs. ribitol**	**RGR vs. Arabitol**	**RGR vs. mannitol**
Peltigera aphthosa	0.453***	0.143 ns	0.236*	0.063 ns
Peltigera canina	0.415***			0.108 ns
Parmelia sulcata		0.444***	0.600***	-0.336**

4. Discussion

4.1 Growth of lichens

Lichens growth is highly variable and depends on water availability, surrounding temperature, light received in metabolically active period, carbohydrate acquisition and nitrogen status. Due to slow growth rate, it may take long time to observe lichen performance under field conditions. By comparing field (Crittenden 2000; Gauslaa & Goward 2012; Larsson et al. 2012; Tømmervik et al. 2012) and growth chamber (Bidussi et al. 2013, this study) measurements of RGR and/or RT_AGR as growth measures, lichen can grow much faster in growth chamber than in nature. Assuming continuous exponential growth over time, the treatment giving the maximum mean RGR would have caused a doubling in DM after 90 days in *P. aphthosa*, 73 days in *P. canina* (13/8 °C, 24 h wet), and 87 days in *P. sulcata* (13/8 °C, 12 h wet) and annual RGR of 53.2, 58.5 and 53.8 g g^{-1} y^{-1}, respectively. With such high growth rates, effects of applied treatment can be detected after a short time span. These exceptionally higher growth rates in the lab can be explained by the poikilohydric character of lichens (Palmqvist 2000). In the field, lichens often become active at suboptimal temperatures and light (Green et al. 2008) because of rapid drying after cool mornings with dew, or cooler periods of rain (Lange & Green 2005). However, under favourable condition, e.g. kept hydrated most of the day at 150 µmol photons m^{-2} s^{-1}, they can grow much faster. Though lichen showed higher growth rate in the lab, it is not clear how long they can continue such high growth rate in this condition as they were not provided with any nutrients. The growth (RGR and/or RT_AGR) of lichen is a useful parameter to assess the

influence of different factors (Bidussi et al. 2013) as it integrates a number of responses affecting viability, reproduction and fitness (Larsson et al. 2012; Shriver et al. 2012). Growth parameters are important for understanding the function of lichen in ecological studies (Bidussi et al. 2013). All studied species increased their dry mass after 14 days cultivation in the growth chamber. Dry matter gain can be explained through net photosynthesis during the light period minus dark respiration during the night. Moreover, lichen biomass gain is primarily limited by the environmental factors that limit photosynthetic activity (Palmqvist 2000). The weight gain is achieved through CO_2 assimilation by the photobiont (Dahlman & Palmqvist 2003; Palmqvist 2000). Gas exchange measurements after spraying the thallus showed higher photosynthesis in *P. sulcata* than in *P. canina* (data not shown). Weight gain also depends on area gain as light is absorbed on an area basis and area expansion will increase the thallus capacity for additional resource acquisition (Dahlman & Palmqvist 2003). The overall weight gain in the experiment was higher in *P. sulcata* (8.73 ± 0.52 %), whereas *P. aphthosa* and *P. canina* showed a lower weight gain (6.69 ± 0.42 % and 6.36 ± 0.76 %, respectively). Higher RGR may result from higher Chl *a* concentration in *P. sulcata* (1.44 ± 0.05 mg g^{-1}) than in *P. aphthosa* (1.10 ± 0.04 mg g^{-1}) and *P. canina* (0.76 ± 0.04 mg g^{-1}). The photosynthetic capacity of lichen is strongly correlated with Chl *a* concentration (Palmqvist et al. 2002; Tretiach & Pecchiari 1995; Valladares et al. 1996); also the light use efficiency increases with increasing Chl concentration (Dahlman & Palmqvist 2003). The higher F_v/F_m in *P. sulcata* (0.647 ± 0.01) suggested that this species was less photoinhibited than *P. aphthosa* (0.506 ± 0.02) and *P. canina* (0.176 ± 0.02). Strong photoinhibition may reduce RGR. Moreover, after absorbing liquid water, chlorolichens with *Trebouxia* as their photobiont can induce photosynthetic electron transport and CO_2 fixation activity within shorter time (10 min) than lichens with *Coccomyxa* and *Nostoc* photobionts (Palmqvist 2000). This may contribute to comparatively higher RGR in *P. sulcata.* Although the thalli kept dry during nights could not have had dark respiration loss, their RGR was reduced significantly (Fig. 5, Table 1) compared to thalli kept wet all the time. The higher RGR in continuously hydrated thalli was also observed from Bidussi et al. (2013) in a similar growth chamber experiments with *Lobaria* species. The thalli kept wet 24 hours apparently repaired photoinhibition during the dark periods (Fig. 5, F_v/F_m), which may contribute to their higher RGR compared to thalli kept dry at night. Moreover, lack of active metabolism in dark periods may reduce growth in 12h hydrated thalli (Bidussi et al. 2013) as algae may respond to

photoperiod (Balzer & Hardeland 1991; Suzuki & Johnson 2001). The slightly higher maximum RGR in *P. canina* 9.43 ± 0.88 mg g^{-1} day^{-1} than *P. aphthosa* (7.66 ± 0.40 mg g^{-1} day^{-1}) and *P. sulcata* (7.85 ± 0.81 mg g^{-1} day^{-1}) can be explained by the advantage of utilizing liquid water for cyanolichen to restore photosynthesis after drying (Lange et al. 1986; Lange et al. 1993) than chloro- and cephalolichen. In fields, chloro- and cephalolichens are generally more active than cyanolichen due to their efficiency to utilize humid air or dew.

Area growth of lichen differs from mass growth. Photobionts contribute to mass growth by their carbon gain, whereas mycobionts contribute to area growth. By expanding thallus area, a lichen can increase its light harvesting area and occupy new space (Larsson et al. 2012). According to Jahns (1988), thallus area expansion is the result of marginal hypal growth including the photobiont cell division in the growing hyphal tips. Moreover, cell expansion growth in plants depends on cell wall properties and turgor pressure (Eqn 1).

Expansion growth = m (Ψp - Y) ..... Eqn 1

(m, the wall extensibility; Ψp, the turgor pressure; Y, the yield threshold which Ψp must exceed to allow growth (Nobel 1999). With no turgor pressure, area growth is hardly possible. Water availability is mainly responsible for turgor pressure in lichen which drives fungal hyphae expansion (Lew 2011; Wessels 1993) as well as thallus area growth (Gauslaa et al. 2009; Gauslaa & Goward 2012). The higher area growth (Fig. 5, RT_AGR) in *Peltigera* thalli hydrated both day and night compared to those hydrated only day-time can be explained by higher long lasting turgor pressure under continuous hydration. The continuously hydrated thalli experienced longer periods of high turgor pressure especially at night with low evaporative demands produced higher thallus area expansion than the thalli hydrated only once a day. Similar area expanding effects of moisture were found by Gauslaa et al. (2009) where the lichen sites with high water availability supported wider and thinner lobes than drier sites. Moreover, the increased ΔSTM in the thalli experienced nocturnal hydration in *P. canina* indicates that weight gain increased despite dark respiration loss, whereas dark respiration reduced weight gain in continuously hydrated *P. sulcata* thalli (Fig. 5). Dark respiration stimulates weight gain in *P. canina* and area gain in *P. aphthosa* and *P. sulcata.* According to a review of (Palmqvist 2000), dark respiration may provide energy required to translate photosynthates into new lichen tissues. Nevertheless, the relatively lower RGR and RT_AGR (Fig. 5) at maximum temperature was likely

the result of high respiration loss with increasing temperature as 10 °C increase in temperature can result in 2 - 3 times increase in respiration (Smith 1962).

F_v/F_m is often used as an indicator of viability in photosynthetic organisms (Nayaka et al. 2009). Normally, chloro- and cephalolichens have F_v/F_m values ranging from 0.6 to 0.76, whereas some cyanolichens have lower values such as 0.5 to 0.6 (Jensen & Kricke 2002). In this study, *P. sulcata* showed very little photoinhibition, whereas *P. aphthosa* and *P. canina* showed high photoinhibition especially for the thalli kept dry at night and the thalli at the lowest temperature (6/1 °C). The thalli kept dry at night suffered most from photoinhibition, as observed by Gauslaa and Solhaug (2004) and Bidussi et al. (2013). Metabolic activity at moist nights may repair the photinhibition (Bidussi et al. 2013). Lichens with lower light saturation point become photoinhibited strongly at low temperature as reported for shade adapted species like *Lobaria pulmonaria* by Pannewitz et al. (2002). Also, lichens with shade adapted nature become vulnerable to high light stress like photoinhibition (Coxson 1987; Demmig-Adams et al. 1990b; Manrique et al. 1993). Both *P. aphthosa* and *P. canina* are shade adapted, evidenced by strong photoinhibition when kept at 150 µmol photons m^{-2} s^{-1} in the lab. Again, at low temperature, when photosynthesis is less efficient, excess light makes the thalli susceptible to photoinhibition. The RGR of all species became strongly reduced with increasing reductions in F_v/F_m (Fig. 6), resulting in highly significant positive linear regressions between RGR and F_v/F_m at the end of the experiment (r^2_{adj} = 0.395; $P < 0.001$ for *P. aphthosa*; r^2_{adj} = 0.444; $P < 0.001$ for *P. canina* and r^2_{adj} = 0.152; $P < 0.001$ for *P. sulcata*). The stronger photoinhibition in *P. canina* than in *P. aphthosa* (Fig. 5), consistent with the data of Demmig-Adams et al. (1990a); (1990b) reporting hydrated cyanolichens have higher high light susceptibility than in hydrated chloro- and cephalolichens. Moreover, lichens with cyanobacteria lack the zeaxanthin-violaxanthin cycle (Demmig-Adams et al. 1990a; 1990b) and their PS II reaction-centre protein, D1, has an inherently lower resistance to photoinhibition (Clarke et al. 1993), which make them highly susceptible to photoinhibition. In general, during rehydration, antioxidants decrease and reactive oxygen species (ROS) is produced as shown in a chlorolichen (Weissman et al. 2005), which may cause reduced F_v/F_m (Bidussi et al. 2013).

4.2 Carbohydrates in lichens

Carbohydrate dynamics are rarely quantified in lichen studies. Very few studies (e.g., Armstrong 1975; Armstrong 1993; Armstrong & Smith 1994) have emphasized relationships between environmental conditions and carbohydrate allocation patterns. In this study, I have tried to quantify effects of external factors (temperature, humidity) on carbohydrate pools in a short term growth chamber experiment. The carbohydrates found in studied species were similar to those recorded previously in lichens with *Coccomyxa, Trebouxia* and *Nostoc* as photobionts (Armstrong & Smith 1994; Honegger et al. 1993; Lewis & Smith 1967; Richardson & Smith 1968b). Ribitol is the carbohydrate of the green algal photobionts *Coccomyxa* and *Trebouxia* transmitted to their mycobionts, whereas glucose is the photobiont carbohydrate in cyanobacterial lichen (Lewis & Smith 1967; Richardson 2002). A high concentration of glucose (2.5 % g^{-1} d. wt) was found in *P. aphthosa* (cephalolichen), which has not been recorded before. The glucose was likely produced by the secondary photobiont (*Nostoc*) present in cephalodia in *P. aphthosa*, whereas ribitol came from its primary algal photobiont. It is not clear if the glucose was transmitted to the mycobiont, or if it just accumulated in the extracellular sheath surrounding cyanobacterial cells (Honegger 1991) in the large cephalodia. Though lichens produce different carbohydrates, only the sugar alcohol (polyol) and/or glucose, depending on photobiont type, move to the fungus where they are utilized (Richardson 1985). Depending on species and season, the amount of sugar alcohol varies between 2 - 10 % of thallus dry weight as reviewed by Palmqvist (2000). The maximum polyol content (8.7, 3.4 and 4.2 % of thallus dry weight for *P. aphthosa*, *P. canina* and *P. sulcata* respectively) measured from this experiment suits this range. So far, the maximum polyol concentration was measured by Lewis and Smith (1967) in *Peltigera polydactyla* (10 % of thallus dry weight). 6 % polyol concentration from the same species was recorded by Drew and Smith (1966). Moreover, Pueyo (1959) measured polyol concentration of 1.7 - 4.9 % in eleven species and Honegger et al. (1993) measured 1 % dry weight or less polyol concentration in eleven cultured lichen fungi. Higher amounts of fungal carbohydrate (arabitol and mannitol) in all the species suggest that most soluble carbohydrates are located in the mycobiont and that the assimilated CO_2 by the photobiont as ribitol (chloro and cephalolichen) and glucose (cyanolichen) eventually are released to the fungus (Fahselt 1994).

Moreover, growth of lichens depends on photosynthesis rate of its photobiont and the subsequent allocation of carbohydrates (Armstrong 1993). The photobiont produces carbohydrates through photosynthesis that are further taken up by the fungus and used for growth and respiration. The lower carbohydrate pools in experimental thalli of *P. aphthosa* and *P. canina* than in control thalli suggest that these lichens in the growth chamber use their carbohydrate pool with a more rapid turn-over for growth and maintenance than they do in the field. *Parmelia sulcata* was different by allocating more carbon into carbohydrates. In *P. aphthosa*, the decreased ribitol concentration at extreme temperatures (6/1 and 28/23 °C) can be explained by high photoinhibition at low temperature (Fig. 5, F_v/F_m) and high respiration loss at high temperature. Comparatively lower glucose in the thalli hydrated once in *P. canina* consistent with the lower F_v/F_m values in these thalli (Fig. 5) and thus, likely lower photosynthesis than in those hydrated twice a day. The higher ratio between fungal to photobiont carbohydrate in *P. sulcata* suggests that a higher proportion of carbohydrates was allocated to the soluble fungal pool. For both *P. aphthosa* and *P. sulcata*, the arabitol pool decreased at maximum temperature (Fig. 7), whereas mannitol pool increased, suggesting that mannitol could be synthesized from arabitol. Under condition of stress, arabitol decreases and mannitol increases (Farrar 1973). The higher ribitol pool in *P. sulcata* than *P. aphthosa* (Fig. 7) is consistent with Richardson (2002) reviewing that lichens with *Trebouxia* have four times larger ribitol pool than in lichens containing other green algae. Higher Chl *a* concentration in *P. sulcata* (Fig. 5) may also contribute to higher ribitol production than in *P. aphthosa*. Large pool of ribitol in *Trebouxia* helps to protect against freezing temperature (Fontaniella et al. 2000). Arabitol concentration varied with temperature and humidity (Fig. 7, Table 2) which is consistent with the hypothesis that arabitol is a short term and readily mobilizable carbohydrate reserve (Armstrong 1993; Lewis & Smith 1967). Moreover, mannitol is the common and widespread sugar alcohol in lichens, found in all three studied lichens. Higher concentration of mannitol was recorded in *P. aphthosa* and *P. canina* than *P. sulcata* (Fig. 7). According to Richardson (2002), large pools of mannitol can support several days' respiration. Also, it works as a low molecular weight storage compounds (Sturgeon 1985). The strong correlation between RGR and glucose for *P. aphthosa* and *P. canina* (Table 4) suggest that glucose is a carbohydrate used for growth. In this case, the measured glucose in *P. aphthosa* is probably not mainly accumulating in cyanobacterial sheath in cephalodia. Alternatively, if glucose concentration is an indirect measure of cephalodial

biomass, the correlation between RGR and glucose may result from more cephalodia and thus an increased N_2 fixation in thalli and high in glucose. Moreover, ribitol in *P. aphthosa* may convert into fungal carbohydrate and serve other function (e.g., stress protection). In contrast, RGR in *P. sulcata* showed strong correlation with ribitol and arabitol.

5. Conclusion

Though lichens are generally considered to be slow-growing species, they can respond fast in growth chamber experiments. Therefore, short-term experiment can be important tool in functional lichen studies. All three studied lichens showed optimum growth at 13/8 °C. Also, the thalli of *Peltigera* species had higher growth when hydrated continuously day and night compared to those kept dry at night. Dark respiration can stimulate lichen growth by providing the energy to convert the photosynthates into new lichen tissue. Photoinhibition significantly decreased the RGR of the thalli cultivated at extreme temperatures. Moreover, carbohydrates are important for lichen growth and survival in extreme condition. The carbohydrate concentration measured in this experiment was quite high in all three studied lichens. RGR of lichen strongly depends on photobiont carbohydrate as it is the direct product from photosynthesis. The substantial reduction in carbohydrate pools in the two *Peltigera* species during the period with high growth is consistent with high turn-over rates and rapid metabolism in the growth chamber. Thereby, the carbohydrate pool is transferred to the mycobiont and converted to arabitol and mannitol with various functions.

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7. Appendix

Appendix 1. Table shows the values of RGR, RT_AGR, ΔSTM, F_v/F_m, Chl *a,* Chl *b* with treatments in *P. aphthosa*.

Treatment	Repl	RGR	RT_AGR	ΔSTM	F_v/F_m	Chl *a*	Chl *b*
6/1 DN	1	-3.72	0.43	-10.61	0.19	1.09	0.47
6/1 DN	2	-1.40	0.05	-2.58	0.09	1.41	0.51
6/1 DN	3	0.93	0.35	-3.52	0.11	1.01	0.78
6/1 DN	4	1.47	0.46	-4.32	0.41	1.27	0.47
6/1 DN	5	0.53	0.12	-0.89	0.35	0.82	0.37
6/1 DN	6	1.61	0.41	-3.46	0.35	1.62	0.59
6/1 DN	7	0.00	0.10	-1.42	0.24	1.24	0.43
6/1 DN	8	0.95	0.18	-1.23	0.09	1.18	0.57
6/1 DN	9	-0.33	0.51	-7.36	0.20	1.04	0.35
6/1 DN	10	2.24	0.93	-9.43	0.39	1.21	0.42
6/1 WN	1	1.32	0.16	-0.42	0.17	1.13	0.41
6/1 WN	2	2.65	0.50	-3.21	0.29	1.49	0.59
6/1 WN	3	2.78	0.56	-3.84	0.35	1.53	0.58
6/1 WN	4	3.36	0.70	-4.98	0.44	1.05	0.36
6/1 WN	5	3.09	1.38	-13.97	0.35	1.30	0.49
6/1 WN	6	5.54	0.39	2.34	0.37	1.70	0.56
6/1 WN	7	3.44	0.40	-0.71	0.51	0.96	0.32
6/1 WN	8	4.55	1.51	-13.67	0.50	1.44	0.53
6/1 WN	9	5.73	0.93	-4.87	0.54	1.06	0.36
6/1 WN	10	2.87	0.49	-2.80	0.48	0.71	0.25
13/8 DN	1	5.25	1.24	-9.51	0.39	0.96	0.33
13/8 DN	2	2.64	1.04	-10.29	0.43	0.83	0.31
13/8 DN	3	5.35	2.00	-18.55	0.35	0.79	0.29
13/8 DN	4	-0.84	0.16	-3.31	0.49	0.68	0.23
13/8 DN	5	4.23	1.07	-8.66	0.53	0.78	0.33
13/8 DN	6	4.19	0.76	-4.63	0.38	0.75	0.28
13/8 DN	7	6.26	0.67	-0.68	0.51	1.38	0.48
13/8 DN	8	5.93	0.74	-2.10	0.36	1.13	0.42
13/8 DN	9	2.60	0.14	1.75	0.54	1.31	0.47
13/8 DN	10	7.66	0.56	2.93	0.65	1.37	0.48
13/8 WN	1	7.62	0.88	-1.62	0.64	1.29	0.46
13/8 WN	2	8.64	1.19	-4.43	0.58	1.78	0.62
13/8 WN	3	6.89	1.23	-7.31	0.46	0.73	0.26
13/8 WN	4	5.59	0.30	3.68	0.49	1.08	0.46
13/8 WN	5	10.25	0.70	4.72	0.61	1.15	0.39
13/8 WN	6	7.49	1.55	-10.59	0.68	1.40	0.51

13/8 WN	7	7.03	1.45	-9.91	0.62	0.70	0.25
13/8 WN	8	8.73	1.29	-5.62	0.62	0.96	0.34
13/8 WN	9	6.90	1.69	-13.07	0.53	0.76	0.27
13/8 WN	10	7.48	1.07	-4.38	0.58	0.69	0.27
20/15 DN	1	6.72	0.67	-0.03	0.54	0.74	0.27
20/15 DN	2	3.71	-0.34	10.52	0.42	1.02	0.39
20/15 DN	3	6.00	1.09	-6.57	0.51	1.09	0.41
20/15 DN	4	4.94	0.82	-4.48	0.56	1.42	0.52
20/15 DN	5	3.84	0.92	-7.26	0.60	1.05	0.37
20/15 DN	6	7.93	0.51	4.03	0.39	2.37	1.97
20/15 DN	7	6.18	0.92	-4.10	0.56	0.81	0.30
20/15 DN	8	5.18	1.11	-7.95	0.56	0.71	0.26
20/15 DN	9	5.53	0.44	1.56	0.55	0.67	0.23
20/15 DN	10	3.17	0.15	2.36	0.18	0.66	0.30
20/15WN	1	2.29	0.08	2.06	0.67	1.83	0.68
20/15WN	2	6.61	1.06	-5.38	0.71	1.03	0.39
20/15WN	3	5.37	0.85	-4.25	0.69	1.04	0.47
20/15WN	4	7.30	0.69	0.54	0.68	1.32	0.55
20/15WN	5	3.74	0.45	-1.08	0.62	0.99	0.36
20/15WN	6	1.83	1.66	-18.71	0.71	1.63	0.69
20/15WN	7	5.96	0.70	-1.39	0.75	1.04	0.37
20/15WN	8	5.18	1.59	-13.99	0.73	1.34	0.46
20/15WN	9	6.41	0.87	-3.09	0.70	0.87	0.31
20/15WN	10	9.42	1.51	-7.63	0.70	1.00	0.34
28/23 DN	1	5.99	0.78	-2.49	0.57	0.95	0.36
28/23 DN	2	6.94	1.10	-5.49	0.60	0.87	0.31
28/23 DN	3	5.61	0.36	2.82	0.61	1.02	0.38
28/23 DN	4	6.54	0.18	6.89	0.60	0.88	0.31
28/23 DN	5	2.93	0.08	2.98	0.58	0.99	0.36
28/23 DN	6	6.63	0.98	-4.28	0.44	0.93	0.33
28/23 DN	7	5.42	0.30	3.46	0.62	0.88	0.34
28/23 DN	8	2.66	0.17	1.32	0.50	0.84	0.34
28/23 DN	9	6.71	1.01	-4.59	0.57	0.59	0.22
28/23 DN	10	4.46	0.82	-5.04	0.59	1.06	0.39
28/23WN	1	5.91	0.88	-3.93	0.63	1.17	0.42
28/23WN	2	2.83	-0.11	5.59	0.61	1.38	0.52
28/23WN	3	6.04	1.11	-6.77	0.68	0.90	0.34
28/23WN	4	5.20	0.51	0.13	0.67	0.95	0.40
28/23WN	5	4.81	0.57	-1.24	0.52	1.06	0.46
28/23WN	6	5.38	0.99	-6.08	0.55	1.04	0.40
28/23WN	7	3.00	0.07	3.33	0.67	1.67	0.63
28/23WN	8	3.89	0.32	1.03	0.49	0.74	0.27

28/23WN	9	5.81	0.26	4.54	0.69	2.15	0.77
28/23WN	10	4.82	1.44	-12.53	0.67	0.93	0.33

Appendix 2. Table shows the percentage of glucose, ribitol, arabitol, mannitol, and total carbohydrate with treatments in *P. aphthosa*.

Treatment	Repl	glucose %	ribitol %	arabitol %	mannitol %	Total %
6/1 DN	1	2.11	0.29	1.76	3.40	7.56
6/1 DN	2	1.90	0.21	1.90	3.34	7.35
6/1 DN	3	2.11	0.23	1.84	3.64	7.82
6/1 DN	4	1.86	0.08	2.39	3.05	7.39
6/1 DN	5	1.80	0.08	1.48	2.53	5.89
6/1 DN	6	1.93	0.16	2.13	3.49	7.71
6/1 DN	7	2.18	0.19	1.23	3.34	6.93
6/1 DN	8	2.33	0.11	2.50	3.60	8.53
6/1 DN	9	1.52	0.07	1.36	2.49	5.44
6/1 DN	10	2.14	0.28	1.10	3.62	7.14
6/1 WN	1	3.36	0.15	2.31	3.35	9.17
6/1 WN	2	2.73	0.14	3.32	3.44	9.63
6/1 WN	3	2.97	0.11	2.31	2.98	8.37
6/1 WN	4	3.13	0.14	1.76	2.85	7.88
6/1 WN	5	3.00	0.08	1.94	3.26	8.28
6/1 WN	6	3.93	0.17	1.49	3.06	8.65
6/1 WN	7	3.23	0.10	2.54	3.40	9.28
6/1 WN	8	3.53	0.11	2.01	3.21	8.86
6/1 WN	9	2.50	0.20	2.31	2.89	7.90
6/1 WN	10	2.23	0.09	2.77	3.33	8.42
13/8 DN	1	2.43	0.11	2.47	2.55	7.56
13/8 DN	2	1.39	0.38	2.55	2.57	6.89
13/8 DN	3	2.25	0.15	2.89	2.96	8.25
13/8 DN	4	1.57	0.37	1.74	2.69	6.37

13/8 DN	5	2.59	0.14	2.31	3.00	8.05
13/8 DN	6	2.74	0.08	1.88	2.56	7.27
13/8 DN	7	2.18	0.24	1.42	3.23	7.07
13/8 DN	8	1.93	0.32	2.27	3.22	7.74
13/8 DN	9	1.35	0.91	1.55	2.36	6.17
13/8 DN	10	3.61	0.38	3.17	3.44	10.60
13/8 WN	1	2.72	0.31	2.44	2.84	8.31
13/8 WN	2	3.09	0.28	3.54	3.10	10.01
13/8 WN	3	2.18	0.60	2.90	2.91	8.59
13/8 WN	4	2.17	0.23	1.96	2.62	6.98
13/8 WN	5	3.05	0.25	3.05	3.52	9.86
13/8 WN	6	2.34	0.94	3.49	2.91	9.67
13/8 WN	7	2.54	0.24	3.31	3.05	9.13
13/8 WN	8	2.66	0.15	3.06	2.85	8.72
13/8 WN	9	2.45	0.16	2.70	2.74	8.04
13/8 WN	10	2.92	0.34	3.15	3.32	9.74
20/15 DN	1	2.98	0.07	2.28	3.91	9.23
20/15 DN	2	1.88	1.12	2.23	3.57	8.79
20/15 DN	3	2.77	0.20	2.26	3.29	8.53
20/15 DN	4	2.57	0.49	2.95	3.23	9.24
20/15 DN	5	2.74	0.31	2.17	4.06	9.28
20/15 DN	6	2.50	0.87	2.15	3.21	8.73
20/15 DN	7	2.98	0.18	2.38	3.24	8.78
20/15 DN	8	2.40	0.33	2.44	3.67	8.84
20/15 DN	9	3.70	0.22	2.17	4.17	10.26
20/15 DN	10	2.84	0.11	1.91	3.87	8.73
20/15 WN	1	2.08	0.27	1.74	3.81	7.90
20/15 WN	2	1.74	0.67	2.09	2.22	6.71
20/15 WN	3	2.66	0.23	1.75	2.96	7.60
20/15 WN	4	3.68	0.20	2.42	3.76	10.06

20/15 WN	5	2.80	0.26	1.88	3.43	8.37
20/15 WN	6	1.64	0.66	1.19	2.45	5.94
20/15 WN	7	2.61	0.20	2.11	2.66	7.58
20/15 WN	8	3.30	0.25	2.29	3.14	8.98
20/15 WN	9	3.16	0.30	1.87	3.68	9.00
20/15 WN	10	3.87	0.33	2.65	4.10	10.95
28/23 DN	1	2.03	0.41	1.28	2.45	6.16
28/23 DN	2	3.20	0.37	0.99	3.57	8.13
28/23 DN	3	2.12	0.33	0.85	2.45	5.75
28/23 DN	4	3.76	0.29	0.98	3.37	8.40
28/23 DN	5	1.12	0.46	0.58	1.86	4.02
28/23 DN	6	2.01	0.22	0.73	2.86	5.82
28/23 DN	7	2.20	0.25	0.61	2.83	5.89
28/23 DN	8	2.49	0.32	1.21	2.82	6.84
28/23 DN	9	4.23	0.11	0.60	3.30	8.24
28/23 DN	10	2.11	0.32	0.57	2.52	5.52
28/23 WN	1	3.20	0.10	0.50	3.03	6.84
28/23 WN	2	1.48	0.11	0.38	1.82	3.79
28/23 WN	3	3.53	0.06	0.95	2.62	7.17
28/23 WN	4	2.40	0.18	0.87	2.28	5.72
28/23 WN	5	2.31	0.19	0.77	2.73	5.99
28/23 WN	6	1.72	0.14	0.46	2.32	4.65
28/23 WN	7	1.19	0.24	0.72	1.80	3.95
28/23 WN	8	2.25	0.25	0.92	2.47	5.88
28/23 WN	9	1.42	0.31	0.26	1.81	3.80
28/23 WN	10	1.75	0.25	0.48	2.47	4.96

Appendix 3. Table shows the values of RGR, RT_AGR, ΔSTM, F_v/F_m, Chl *a,* percentage of glucose, mannitol and total carbohydrate with treatments in *P. canina*.

Treat	Repl	RGR	RTAGR	ΔSTM	Chl *a*	F_v/F_m	glucose	mannitol	Total %
6/1 DN	1	-0.36	0.07	-1.43	1.09	0.02	4.96	2.68	7.65
6/1 DN	2	2.64	0.00	3.73	0.66	0.00	4.26	3.16	7.43
6/1 DN	3	-3.13	-0.21	-1.45	1.06	0.01	3.58	3.28	6.85
6/1 DN	4	-3.37	0.02	-4.88	0.68	0.01	3.65	3.50	7.15
6/1 DN	5	0.22	0.07	-0.62	1.09	0.10	5.22	4.36	9.58
6/1 DN	6	1.38	0.22	-1.21	0.38	0.02	4.38	2.71	7.09
6/1 DN	7	-5.23	-0.24	-3.90	1.10	0.00	4.19	3.73	7.93
6/1 DN	8	-2.77	-0.13	-2.05	1.43	0.05	4.04	3.12	7.16
6/1 DN	9	-2.48	0.02	-3.69	0.44	0.01	4.05	2.45	6.50
6/1 DN	10	-1.35	-0.13	-0.10	1.30	0.09	3.46	3.79	7.25
6/1 WN	1	1.18	0.40	-3.89	0.52	0.00	4.89	2.65	7.54
6/1 WN	2	-0.24	-0.14	1.68	0.86	0.02	6.37	3.65	10.01
6/1 WN	3	7.49	0.16	8.59	1.03	0.24	5.39	4.09	9.48
6/1 WN	4	4.64	0.54	-1.02	1.44	0.14	6.47	3.23	9.70
6/1 WN	5	0.66	0.61	-7.33	0.74	0.15	4.40	2.59	6.98
6/1 WN	6	-2.69	-0.22	-0.72	0.55	0.00	4.68	3.28	7.96
6/1 WN	7	2.71	0.37	-1.37	0.50	0.22	4.03	2.32	6.34
6/1 WN	8	1.49	0.24	-1.23	0.38	0.10	5.21	2.99	8.20
6/1 WN	9	-1.26	-0.25	1.74	0.12	0.00	1.71	2.64	4.36
6/1 WN	10	1.22	0.57	-6.13	0.49	0.11	4.06	2.75	6.81
13/8DN	1	3.13	0.72	-5.53	0.79	0.03	5.22	3.37	8.59
13/8DN	2	4.15	0.36	0.77	0.49	0.04	3.99	2.82	6.81
13/8DN	3	3.61	1.00	-8.59	0.49	0.11	5.21	3.82	9.03
13/8DN	4	3.48	0.38	-0.49	0.28	0.00			
13/8DN	5	9.99	0.55	6.43	0.48	0.13	4.73	3.71	8.44
13/8DN	6	6.46	0.25	5.62	1.52	0.10	4.72	2.73	7.45
13/8DN	7	4.84	0.34	2.09	0.79	0.12	3.99	3.17	7.16
13/8DN	8	3.74	0.85	-6.47	0.73	0.16	3.25	3.54	6.79
13/8DN	9	5.32	0.53	0.08	0.23	0.12	3.68	3.57	7.25
13/8DN	10	6.95	-0.04	10.77	0.75	0.06	7.30	3.75	11.05
13/8WN	1	7.97	0.40	5.67	0.85	0.32	6.22	4.54	10.76
13/8WN	2	7.55	1.02	-3.61	0.50	0.22	4.29	2.67	6.96
13/8WN	3	7.55	-0.09	12.49	0.64	0.22	3.91	2.72	6.63
13/8WN	4	9.12	0.71	2.92	0.59	0.24	4.27	2.89	7.16
13/8WN	5	9.31	1.12	-2.57	0.59	0.49	5.91	2.72	8.63
13/8WN	6	8.25	1.06	-3.19	0.46	0.31	5.90	3.05	8.95
13/8WN	7	7.06	0.07	9.34	0.64	0.31	6.31	4.21	10.52
13/8WN	8	8.41	0.93	-1.28	0.64	0.33	5.15	2.71	7.86
13/8WN	9	14.27	1.07	5.10	0.84	0.43	5.68	3.68	9.37

13/8WN	10	14.85	0.50	14.74	1.32	0.47	4.99	5.01	10.00
20/15DN	1	3.44	0.19	2.14	0.64	0.20	4.52	4.19	8.72
20/15DN	2	3.67	-0.08	6.39	0.13	0.12	3.88	3.59	7.46
20/15DN	3	4.16	-0.01	6.20	0.33	0.18	3.74	4.86	8.60
20/15DN	4	0.59	-0.01	1.00	0.47	0.02	3.16	3.51	6.68
20/15DN	5	3.15	0.28	0.54	0.46	0.20	3.70	3.95	7.65
20/15DN	6	4.35	0.20	3.35	0.41	0.24	4.61	4.70	9.31
20/15DN	7	-2.21	0.12	-4.70	0.55	0.04	2.97	4.18	7.15
20/15DN	8	5.53	0.04	7.46	0.77	0.32	4.50	4.54	9.05
20/15DN	9	-2.16	-0.23	0.16	0.89	0.03	3.68	4.09	7.77
20/15DN	10	3.44	0.03	4.47	0.63	0.05	4.59	4.54	9.13
20/15WN	1	-2.09	-0.27	0.90	0.49	0.08	4.93	3.89	8.82
20/15WN	2	6.07	0.60	0.14	0.68	0.41	4.84	3.77	8.61
20/15WN	3	4.28	0.42	0.06	0.66	0.03	5.73	3.94	9.68
20/15WN	4	0.70	0.16	-1.30	0.56	0.30	4.27	3.71	7.98
20/15WN	5	6.84	0.39	4.25	0.67	0.52	5.16	3.27	8.42
20/15WN	6	6.07	-0.24	12.53	1.05	0.25	5.23	3.26	8.49
20/15WN	7	6.83	0.65	0.40	0.58	0.44	5.89	3.46	9.35
20/15WN	8	5.06	0.22	4.11	0.48	0.30	6.15	3.51	9.66
20/15WN	9		0.51	17.25	1.25	0.38	6.85	5.24	12.08
20/15WN	10	4.39	0.05	5.61	0.37	0.41	5.04	3.51	8.56
28/23DN	1	3.90	0.69	-4.15	0.58	0.05	1.97	4.11	6.08
28/23DN	2	1.83	-0.34	7.55	0.55	0.05	2.11	2.81	4.92
28/23DN	3	8.75	-0.16	15.59	1.02	0.21	6.03	4.28	10.30
28/23DN	4	1.36	0.24	-1.47	0.87	0.01	2.24	3.06	5.30
28/23DN	5	4.67	0.49	-0.29	0.74	0.03	3.13	3.51	6.64
28/23DN	6	0.19	-0.26	3.99	0.63	0.01	2.65	3.45	6.10
28/23DN	7	8.18	0.24	8.48	0.88	0.26	3.06	3.00	6.06
28/23DN	8	1.15	-0.17	4.06	0.68	0.15	3.47	3.35	6.83
28/23DN	9	6.80	0.11	8.27	1.46	0.03	3.94	2.67	6.61
28/23DN	10	5.68	0.18	5.63	1.43	0.08	3.44	2.07	5.51
28/23WN	1	2.50	0.28	-0.47	0.72	0.35	4.08	2.87	6.95
28/23WN	2	6.84	0.54	2.00	0.88	0.34	3.62	2.39	6.01
28/23WN	3	5.45	0.31	3.32	1.49	0.19	4.81	2.21	7.02
28/23WN	4	7.16	-0.10	12.08	1.15	0.02	4.98	4.78	9.76
28/23WN	5	2.22	-0.23	6.54	0.44	0.35	3.62	3.29	6.91
28/23WN	6		0.69	13.87	1.80	0.54	4.66	4.31	8.97
28/23WN	7	9.39	-0.19	17.15	1.27	0.41	4.99	3.59	8.58
28/23WN	8	3.92	-0.16	8.08	0.47	0.35	3.97	2.81	6.78
28/23WN	9	8.19	-0.26	16.33	0.83	0.22	4.46	3.09	7.55
28/23WN	10	11.67	0.69	6.85	0.95	0.51	5.94	3.58	9.52

Appendix 4. Table shows the values of RGR, RT_AGR, ΔSTM, F_v/F_m, Chl *a,* percentage of ribitol, arabitol, mannitol and total carbohydrate with treatments in *P. sulcata*.

Treat	Repl	RGR	RT_AGR	ΔSTM	Fv/Fm	Chl *a*	Chl *b*	rib%	arab%	man%	Tot%
6/1 DN	1	0.55	0.48	-5.73	0.66	1.84	0.58	1.07	4.47	0.98	6.52
6/1 DN	2	12.18	0.04	17.93	0.68	0.99	0.34	0.50	3.04	0.68	4.22
6/1 DN	3	4.25	0.05	5.34	0.65	1.06	0.39	0.69	2.78	0.77	4.25
6/1 DN	4	7.03	0.27	6.25	0.66	1.48	0.43	1.03	3.40	0.92	5.34
6/1 DN	5	7.53	0.35	5.85	0.66	1.34	0.42	0.68	3.50	0.75	4.94
6/1 DN	6	8.09	0.42	5.54	0.65	1.52	0.45	0.97	4.70	0.90	6.57
6/1 DN	7	1.67	0.15	0.19	0.65	0.76	0.29	0.43	2.40	0.76	3.59
6/1 DN	8	7.89	0.37	6.06	0.69	1.64	0.48	0.83	3.73	0.89	5.44
6/1 DN	9	6.66	0.90	-3.21	0.65	1.65	0.56	0.82	4.27	0.97	6.06
6/1 DN	10	9.04	0.43	6.80	0.69	1.55	0.46	0.86	3.39	0.89	5.14
6/1 WN	1	5.22	0.05	6.83	0.67	1.82	0.55	0.37	1.75	0.38	2.50
6/1 WN	2	4.44	0.72	-3.83	0.62	1.54	0.55	0.84	2.84	0.72	4.40
6/1 WN	3	4.98	0.01	7.02	0.62	1.60	0.44	0.62	2.97	0.90	4.48
6/1 WN	4	5.14	0.00	7.40	0.69			0.82	3.93	0.76	5.50
6/1 WN	5	7.25	0.76	-0.54	0.69	1.45	0.45	0.69	4.06	0.94	5.68
6/1 WN	6	8.49	0.59	3.66	0.66	1.99	0.59	0.50	2.15	0.37	3.03
6/1 WN	7	5.30	0.69	-2.15	0.65	1.73	0.50	0.63	3.05	0.84	4.52
6/1 WN	8	7.57	0.30	6.61	0.65	1.34	0.44	0.62	3.24	0.81	4.67
6/1 WN	9	6.56	0.23	6.12	0.62	1.20	0.46				
6/1 WN	10	4.15	0.48	-0.89	0.63	1.29	0.46	0.50	3.15	0.51	4.16
13/8 DN	1	3.25	0.48	-2.10	0.72	0.80	0.34	0.38	3.10	0.76	4.24
13/8 DN	2	8.80	0.50	5.47	0.70	2.43	0.68	0.84	3.49	0.64	4.97
13/8 DN	3	8.02	0.69	1.64	0.66	2.00	0.59	0.63	2.66	0.74	4.03
13/8 DN	4	9.06	0.66	3.45	0.70	1.51	0.52	0.84	3.71	0.69	5.24
13/8 DN	5	5.85	0.38	2.97	0.66	0.69	0.36	0.42	2.71	0.54	3.67
13/8 DN	6	10.08	0.67	4.81	0.68	1.33	0.48	0.87	4.20	0.96	6.03
13/8 DN	7	6.99	0.39	4.41	0.68	0.75	0.37	0.75	2.83	0.97	4.56
13/8 DN	8	7.39	0.66	1.05	0.67	1.00	0.38	0.49	3.38	1.01	4.87
13/8 DN	9	12.63	0.54	10.67	0.64	1.91	0.53	0.98	3.65	0.91	5.55
13/8 DN	10	6.40	0.60	0.55	0.65	1.26	0.44	0.56	2.49	0.56	3.60
13/8WN	1	11.63	1.31	-2.05	0.71	1.48	0.48	0.58	2.57	0.81	3.96
13/8WN	2	5.33	0.82	-3.90	0.67	1.78	0.52	0.75	3.82	0.77	5.35
13/8WN	3	10.22	0.61	5.89	0.70	2.00	0.54	0.58	3.23	0.81	4.62
13/8WN	4	9.50	0.42	7.72	0.69	1.69	0.48	0.65	3.51	0.81	4.97
13/8WN	5	8.19	0.82	-0.04	0.66	1.05	0.41	0.43	3.12	0.90	4.46
13/8WN	6	6.33	0.89	-3.47	0.69	1.03	0.42	0.37	2.89	0.95	4.20

13/8WN	7	8.43	0.37	6.87	0.62	1.55	0.48	0.71	3.52	0.79	5.02
13/8WN	8	6.58	0.60	0.83	0.67	1.44	0.47	0.62	3.50	1.11	5.22
13/8WN	9	4.97	0.44	0.84	0.68	1.81	0.59	0.32	2.59	1.00	3.91
13/8WN	10	6.64	1.03	-5.00	0.67	1.10	0.41	0.41	2.61	0.65	3.67
20/15DN	1	5.71	0.38	2.68	0.62	1.06	0.36	0.62	2.98	1.22	4.83
20/15DN	2	7.53	-0.06	12.05	0.69	1.41	0.66	0.85	4.35	1.35	6.55
20/15DN	3	6.68	-0.22	13.28	0.70	1.42	0.57	1.22	4.17	1.44	6.83
20/15DN	4	9.12	0.31	8.87	0.73	1.58	0.72	1.29	4.60	1.38	7.27
20/15DN	5	8.07	0.53	3.96	0.65	1.57	0.49	0.82	4.08	1.36	6.26
20/15DN	6	5.34	0.44	1.32	0.69	1.05	0.46	0.92	3.80	1.38	6.10
20/15DN	7	8.00	0.37	6.25	0.72	1.72	0.58	1.05	5.20	1.54	7.78
20/15DN	8	1.84	0.00	2.66	0.70	2.02	0.79	0.47	3.01	1.12	4.59
20/15DN	9	5.98	0.41	2.67	0.66	1.44	0.53	1.05	4.49	1.41	6.95
20/15DN	10	9.91	0.32	9.87	0.66	1.63	0.64	1.16	4.50	1.22	6.87
20/15WN	1	0.85	0.12	-0.47	0.69	1.65	0.53	0.70	2.93	0.97	4.60
20/15WN	2	1.87	0.34	-2.17	0.73	0.98	0.36	0.37	2.35	0.81	3.54
20/15WN	3	3.64	0.68	-4.37	0.73	1.30	0.51	0.49	2.74	0.94	4.17
20/15WN	4	5.79	0.85	-3.73	0.71	2.02	0.57	0.84	3.51	1.05	5.41
20/15WN	5	2.91	0.28	0.22	0.70	1.01	0.35	0.51	2.46	1.25	4.22
20/15WN	6	6.25	0.61	0.28	0.72	1.47	0.50	0.86	3.61	1.09	5.56
20/15WN	7	9.33	0.32	9.03	0.74	0.80	0.23	0.74	3.36	1.13	5.23
20/15WN	8	2.74	0.23	0.61	0.72	1.75	0.53	0.54	3.06	0.94	4.54
20/15WN	9	9.97	1.00	-0.04	0.65	1.61	0.46	0.61	4.13	1.22	5.96
20/15WN	10	11.10	0.69	6.06	0.71	2.29	0.64	1.33	4.99	1.37	7.69
28/23DN	1	4.86	0.44	0.64	0.41	1.51	0.50	0.65	2.61	2.70	5.96
28/23DN	2	7.22	0.45	3.87	0.47	1.35	0.36	0.64	3.79	3.32	7.75
28/23DN	3	-0.19	0.22	-3.29	0.30	0.79	0.33	0.38	1.38	1.69	3.45
28/23DN	4	0.55	0.64	-7.87	0.12	0.46	0.16	0.25	1.03	2.04	3.32
28/23DN	5	1.64	0.21	-0.60	0.39	0.89	0.38	0.42	1.41	1.67	3.50
28/23DN	6	3.07	0.23	1.02	0.69	1.48	0.48	0.75	2.64	2.10	5.48
28/23DN	7	-2.08	0.20	-5.53	0.18	0.49	0.36	0.27	1.39	2.60	4.27
28/23DN	8	5.21	1.07	-7.46	0.63	1.47	0.59	0.96	2.45	2.18	5.59
28/23DN	9	7.17	0.58	1.90	0.66	1.93	0.54	0.66	3.07	2.79	6.51
28/23DN	10	3.71	0.27	1.36	0.64	0.88	0.32	0.36	2.01	2.39	4.76
28/23WN	1	3.64	0.52	-2.16	0.72	0.92	0.33	0.34	2.44	2.10	4.89
28/23WN	2	6.54	0.81	-2.14	0.74	2.57	1.11	0.76	3.44	2.14	6.33
28/23WN	3	5.90	0.40	2.69	0.72	1.96	0.62	0.79	2.89	2.03	5.71
28/23WN	4	2.89	0.60	-4.25	0.72	1.74	0.55	0.42	2.19	2.38	4.99
28/23WN	5	6.36	0.56	1.13	0.59	2.01	0.59	0.80	2.95	2.40	6.15
28/23WN	6	-0.67	0.61	-9.00	0.72	1.01	0.49	0.59	2.20	1.91	4.70

28/23WN	7	9.45	0.74	2.91	0.66	1.33	0.45	1.11	3.49	1.94	6.54
28/23WN	8	2.70	0.45	-2.49	0.72	1.85	0.55	0.85	2.84	1.90	5.59
28/23WN	9	1.86	0.45	-3.57	0.42	1.37	0.44	0.55	2.05	2.03	4.63
28/23WN	10	0.57	0.83	-10.26	0.72	1.52	0.63	0.70	1.67	2.90	5.28

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