

Life at the Limits: Capacities of Isolated and Cultured Lichen Symbionts to Resist Extreme Environmental Stresses

J.-P. de Vera · P. Rettberg · S. Ott

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Abstract Lichens are described as a symbiosis formed by a myco- and photobiont, capable of colonizing habitats where their separate symbionts would not be able to survive. Space simulation studies on the separated symbionts of the lichen *Xanthoria elegans* have been performed to test their capacity to resist the most extreme conditions. The isolated cultured symbiont cells were exposed to different doses of the UV spectrum, and to vacuum. Cultures of both symbionts were analysed by specific vitality tests (LIVE/DEAD-staining detected by Confocal Laser Scanning Microscopy). Growth capacity of symbiont cultures on different media was analysed after exposure to extreme environmental stresses. The data obtained support the hypothesis that the symbiotic state considerably enhances the ability of the respective symbionts to survive exposure to extreme conditions, including the conditions of space simulation. Species such as *X. elegans* may, therefore, be suitable for use as model organisms in exobiological studies.

Keywords Lichen symbiosis · Mycobiont · Photobiont · Viability · Space conditions · Culture experiments

Introduction

The progress of biological evolution fundamentally depends on the development of adaptation mechanisms. One key area in which this applies is in the development of resistance to extreme environmental conditions. Symbiosis is one of the most successful strategies for resistance and adaptation (Margulis 1993). At one level, endosymbiosis can be described as a dynamic enhancing machine of evolution. A different form of symbiosis is the association of two or more different eukaryotic and prokaryotic species, as seen in the

J.-P. de Vera (✉) · S. Ott
Institute of Botany, Heinrich-Heine-University, Universitätsstr.1, 40225 Düsseldorf, Germany
e-mail: devera@uni-duesseldorf.de

P. Rettberg
DLR German Aerospace Center, Institute of Aerospace Medicine, Radiation Biology, 51170 Köln,
Germany

development of biofilms (Belnap et al. 2001; Flemming and Wingender 2001) or in the lichen symbiosis (Hawksworth 1988; Honegger 1998). The distinctive symbiosis of lichens can be found in almost all biomes across the planet, and forms the dominate vegetation in some more extreme environments such as those of polar and high mountain regions. The association of two or more bionts (a green algae and/or cyanobacteria, and fungi) in lichens gives rise to the capacity to colonize habitats where each of the respective bionts would not be able to survive.

Multiple protective mechanisms are typical of lichens. Morphological and anatomical features including the structure of the cortex (de Vera et al. 2003) and the density of the gelatinous matrix (produced by mucilage which embeds the photo- and mycobiont cells), formed by extracellular polymeric substances (EPS) (Kappen 1988; Honegger 1993; Wierzchos and Ascaso 1996, 1998, de los Rios et al. 2002; Flemming and Wingender 2001; de los Rios et al. 2003) combine with the production of UV-screening secondary metabolites (Gauslaa and Ustvedt 2003; Wynn-Williams and Edwards 2002a; Wynn-Williams et al. 2002b; Cockell and Knowland 1999) to ensure to survival of the bionts in a lichen thallus in the face of extreme stresses such as total desiccation and high irradiation (Büdel and Wessel 1986; Fahselt 1995; Lud 2001; de Vera et al. 2003, 2004a). UV radiation, in particular, is known to have the capacity to damage eukaryotic organisms substantially. However, current knowledge on the influence of UV radiation on the lichen symbiosis and the respective symbionts is limited. In this study we utilise the extreme conditions of space simulation to address the questions of (i) which parameter(s) are lethal for the symbionts in both the symbiotic state and in isolation, and (ii) what is the resistance of the two symbionts to the biologically unrealistic challenges of simulated space conditions of UV radiation and vacuum.

Space simulation (UV- and vacuum conditions) experiments using the lichen *Fulgensia bracteata*, its photobionts and mycobiont (ascospores), and also on the lichen *Xanthoria elegans* and its mycobiont (ascospores) demonstrated that the viability of the entire lichen tissue was maintained during long times (up to 2 weeks) of exposure (de Vera et al. 2004a). Both the isolated photobiont cells of *F. bracteata* and the isolated hyaline ascospores of both lichens were more sensitive, losing viability after 8–16 h exposure time. Ascospores protected inside the fruiting body, where parietin acts as a UV-screening substance showed a higher resistance than the isolated spores and photobiont cells, and also maintained the capacity to germinate (de Vera et al. 2003, 2004a, b).

In the current study we document the resistance of isolated cultured symbionts of *X. elegans* to the extreme conditions of simulated space parameters. We hypothesize that the potential of the symbiotic state to resist the most extreme conditions is greater than that of the single isolated symbionts.

Materials and Methods

Biological Material

Samples of *Xanthoria elegans* (Link.) Th.Fr. were collected near the Sanetsch glacier (Wallis, Switzerland). *X. elegans* is a placoid crustose lichen widely distributed in alpine and polar regions, both of which typically experience high levels of insolation including UV-A and UV-B. For experimental use, clusters and single cells of the photobiont and ascospores of the mycobiont, of *X. elegans* were isolated and then cultured. After ascospore germination the mycelia developed were used in experiments.

Culture Conditions and Culture Properties

The mycobiont was cultured on a Malt Yeast (MY) medium (Esser 1976) and the photobiont on TOM agar medium (1%) (Ahmadjian 1967). All culture media were prepared to give a neutral pH (7). Cultures of mycobiont mycelia were incubated at 20° C. The photobiont was cultured at 16° C with light/dark-periodicity of 16 h light and 8 h dark. For each radiation experiment 8 replicates were used.

For the preparation of the photobiont culture on solid agar medium there are some essential particularities to be taken into account. Slightly homogenised liquid culture media with at the beginning more or less 500 (+60) cells per 500 µl were dispersed on the solid TOM 1% agar medium. The liquid medium consists of cells in clusters as well as single cells. Higher levels of homogenisation of the original liquid culture were not possible. The reasons: the use of higher spins causes cell death of the majority of the cell culture. In case of filtration the nutrient solution is disturbing this action either if used by dilution processes and no effective separation of clusters and single cells was achieved besides reaching a very undesirable low number of cells in the resulting liquid culture. Water and nutrient solution by density separation did also not get a satisfying result. Before the use of the culture in the exposing experiments a time of about 3 days incubation on solid medium has to be performed for adaptation and reaching the doubling rate on solid nutrient substrate for clusters as well as for single cells. Haphazardly some of the clusters started earlier cell growth and become denser during the adaptation phase. No rule for this early cluster growth can be distinguished, as the agar substrate was shaken and homogenized also before solidification. It can be supposed that some of the clusters of *Trebouxia* of the lichen *Xanthoria elegans* seem to be better adapted to the change from liquid to solid medium. The presence of clusters and single cells on the solid medium and the inhomogeneous cluster growth is very important for interpretation of the results we get after the experiments.

We have also decided not to exclude the data of samples with inhomogeneous cluster growth because it belongs to an essential behaviour of the entire photobiont culture on solid media and because the presence of dense clusters is more representing the morphologic structure of terrestrial alga assembled in the lichen community. By the expected data we would be able to see different behaviour on radiation effected single cells as well as dense cluster arrangements what is representing the potential of resistance of the entire photobiont culture with all its natural variations.

For space simulation (UV radiation combined with vacuum tests) the preparation of photobiont culture before exposure was slightly different. Agar media can not be placed in a vacuum chamber. Therefore the photobiont culture was placed on slices of MILLIPORE filters with a resulting starting number of 250 cells (clusters and single cells). The filter slices were placed on solid TOM (1%) agar medium for adaptation and reaching the doubling rate. The filters with these photobiont cells were striped off the initial solid culture medium and then exposed in the vacuum chamber to radiation and vacuum. After exposure the filter slices were replaced on the agar medium and checked for cell growth. 4 replicates were used for each of the exposure experiments.

Space Simulation Facility

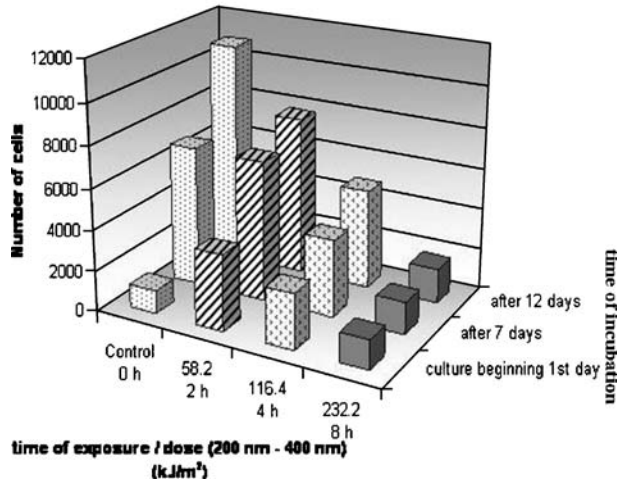
A detailed description of the simulated extreme space environment in one of the test beds of the German Aerospace Center (DLR, Cologne, Germany) is available in Horneck (1999) and de Vera et al. (2003, 2004a, b), and at the website www.dlr.de/Strahlenbiologie. The conditions applied during the experimental studies described here were: UV radiation covering a wavelength spectrum between 200 nm and 400 nm, with doses between 2.42

Table 1 Increase/decrease of cell amounts according to the applied radiation conditions

Parameters	0 h		2 h		4 h		8 h						
	time of exposure	dose (200–400 nm) kJ/m ²	1st day	after 7 days	1st day	after 7 days	1st day	after 12 days					
Control		58.2			116.4			232.2					
time of incubation	1st day	after 7 days	after 12 days	after 7 days	after 12 days	1st day	after 7 days	after 12 days					
Number of samples/number of cells	1	1,039	6,311	10,555	1,457	1,242	1,232	1,404	945	620	1,851	2,498	3,210
	2	<i>1,311</i>	<i>7,921</i>	<i>12,247</i>	1,301	852	410	1,484	1,208	961	1,007	871	524
	3	1,010	6,710	10,020	<i>5,817</i>	<i>11,391</i>	<i>14,729</i>	3,488	<i>6,617</i>	<i>8,921</i>	1,115	1,008	608
	4	1,050	6,751	10,198	<i>5,902</i>	<i>12,510</i>	<i>14,411</i>	3,828	<i>6,866</i>	<i>8,554</i>	1,003	811	497
	5	<i>1,407</i>	<i>7,277</i>	<i>12,576</i>	1,201	994	722	<i>3,931</i>	<i>5,822</i>	<i>8,897</i>	1,211	1,064	580
	6	1,001	6,310	10,361	<i>6,124</i>	<i>13,333</i>	<i>15,244</i>	1,498	1,302	1,211	1,174	826	552
	7	1,003	6,676	10,812	<i>5,887</i>	<i>12,821</i>	<i>14,560</i>	<i>3,963</i>	<i>5,666</i>	<i>8,124</i>	2,120	2,857	<i>3,911</i>
	8	1,004	6,436	10,212	1,662	1,060	824	1,841	1,473	1,253	2,052	2,678	3,812
Σ (number of cells)		8,825	54,392	86,981	29,351	54,203	62,132	21,437	29,899	38,541	11,533	12,613	13,694
Mean value		1,103.125	6,799	10,872.625	3,668.875	6,775.375	7,766.5	2,679.625	3,737.375	4,817.625	1,441.625	1,576.625	1,711.75

For each radiation test eight replicates have been used. Values in italics are Petri-dishes with higher amounts of densely formed clusters where early growth of some clusters during liquid/solid-adaptation phase has been observed

Fig. 1 Growth capacity of photobiont cells after exposure to UV radiation (UV, $200\text{ nm} \leq \lambda \leq 400\text{ nm}$). Eight replicates for each exposure experiment were analysed



and 465.6 kJ m^{-2} , and vacuum pressures between $2.8 \times 10^{-1}\text{ Pa}$ and $3.1 \times 10^{-5}\text{ Pa}$. Eight replicates were carried out for each exposure experiment.

Vitality Tests I: Growth Rate

Mycelia disks of about 1 mm in diameter were prepared from cultures and exposed to simulated space conditions. After exposure the disks of exposed mycelia were replaced on the original media and their growth was then measured after a further 1 month and 2 months of incubation. Control experiments have been applied. The growth rate of the photobiont cell culture was been measured after 7 and 12 days of incubation by recording the increase in biomass (cell quantities).

Vitality Tests II: Live/Dead Fluorescence Analysis by CLSM

Viability of the respective symbiont cells was assessed qualitatively by the use of Live/Dead staining kits (SYTOX-green and FUN I [Invitrogen, Molecular Probes, Karlsruhe]) after exposure to the simulated space parameters. FUN I indicates metabolically activity cells by colour—the cell becomes red if metabolically active. SYTOX-green stains the DNA of non-viable or damaged cells with an intense green colour. The fluorescence signals were identified using a confocal laser scanning microscope (CLSM) (Leica TCS NT). *In*

Table 2 Growth capacity of irradiated and non-irradiated (control) photobiont cells and clusters (UV, $200\text{ nm} \leq \lambda \leq 400\text{ nm}$) after incubation on TOM 1%-medium

Parameters	Time of exposure dose (200 nm–400 nm) (kJ/m ²)	0 h	2 h	4 h	8 h
Time of incubation		Mean values of number of cells (N)			
First day		1,103.1	3,668.9	2,679.6	1,441.6
After 7 days		6,799	6,775.4	3,737.4	1,576.6
After 12 days		10,872.6	7,766.5	4,817.6	1,711.75

For each exposure test eight replicates were used

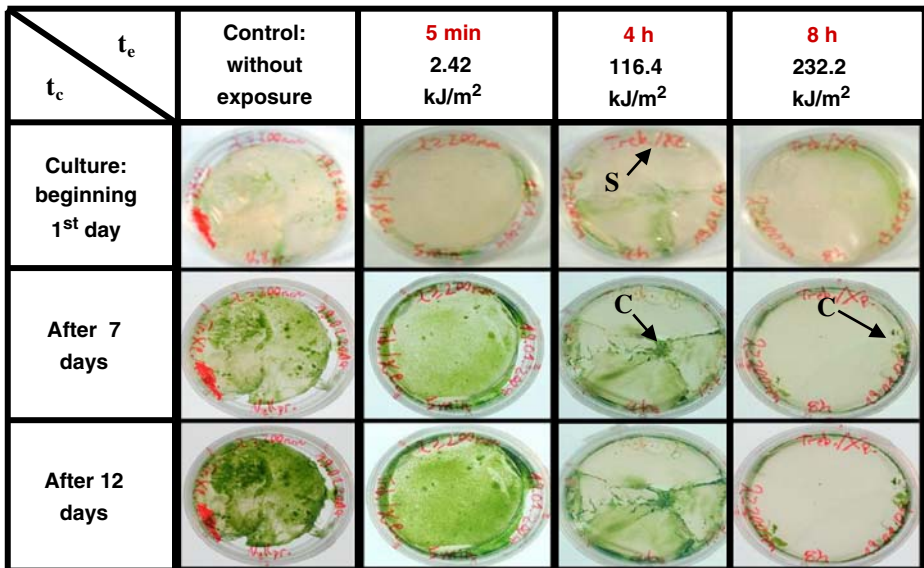


Fig. 2 Cell proliferation after the UV experiments. Single cells (*S*) show no progress in growth after an exposure time of 8 h (232.2 kJ/m^2) while clusters (*C*) of photobiont cells increase in growth

in vivo studies of single cells and clusters of the photobiont *Trebouxia* sp. (mol. ident.) and of the mycelia of the mycobiont of *X. elegans* were achieved by checking each of the laser scan-sections and fluorescent channels which are combined to form the overlay images (Fig. 6a–d). The CLSM channel and overlay-imaging technique in combination with the contour- and topographic imaging tools permitted precise location of viable and non-viable cells in clusters or mycelia. Detailed description of this methodology is given by de Vera et al. (2003). Image analysis by the cell counting program of Image Tool was applied for quantification of both bionts. When assessing viability of mycelia using these techniques, only the exterior part of the mycelia was examined, as the control mycelia indicated that the inner part was non-living.

Results

Growth Capacity I: Growth Capacity of Photobiont Cells

After exposure times between 2 and 8 h, corresponding to a total UV dose of $\sim 232.2 \text{ kJ m}^{-2}$, negative effects on growth were detectable. In the control samples the total cell number was

Table 3 Growth capacity of photobiont cells after applied simulated space conditions (UV irradiation $200 \text{ nm} \leq \lambda \leq 400 \text{ nm}$ up to doses of 232.2 kJ/m^2 and low pressures, up to $p \geq 3.6 \times 10^{-5} \text{ Pa}$)

Parameters	Time of exposure	0 min	5 min	2 h	4 h	8 h
	Dose (kJ/m^2)	Control	2.42	58.2	116.4	232.2
	Pressure (Pa)	1 atm	2.8×10^{-1}	4.9×10^{-5}	4.0×10^{-5}	3.6×10^{-5}
Time of incubation	Mean values of numbers of cells (<i>N</i>)					
First day		603.5	558	575.8	720.3	629.5
After 7 days		1,224.5	810.25	162.3	288	71.75

Four replicates were used for each space simulation test in this experiment

Table 4 Growth capacity of UV irradiated ($200\text{ nm} \leq \lambda \leq 400\text{ nm}$) and non-irradiated mycobiont mycelia (control) as well as after exposure to the combined vacuum pressures ($p \geq 3.6 \times 10^{-5}\text{ Pa}$)

Parameters	Time of exposure	0 h	2 h	4 h	8 h
	Dose (kJ/m^2)	Control	58.2	116.4	232.2
	Pressure (Pa)	1 atm	4.9×10^{-5}	4.0×10^{-5}	3.6×10^{-5}

Time of incubation	Mean values diameter (mm) of Mycobiont mycelia			
First day	0.9	1.2	1	1
After 1 month	3.5	2.1	1.9	2.1
After 2 months	4.1	4.3	3.3	3.3

For each space simulation experiment eight replicates were used

6 times higher after 7 days of incubation, and after 12 days nearly 10 times higher, than at the beginning of the experiment (Table 1 and Fig. 1). Growth (biomass) decreased with increasing length of UV exposure. After 2 h of UV exposure the total cell mass only doubled after 12 days of incubation, [control: doubling time approximately 1 day] and stopped its growth except of cultures with clusters after 8 h of UV exposure (Table 1, Table 2, Figs. 1, 2). If we have a closer look on the details it can clearly distinguished that the entire culture is only able to survive the applied radiation tests if dense cluster formation has occurred.

The combination of UV and vacuum exposure demonstrates the sensitivity of individual photobiont cells. After an exposure time of 5 min (dose of 2.42 kJ/m^2 and vacuum pressure $2.8 \times 10^{-1}\text{ Pa}$), clusters of photobiont cells doubled their biomass over the subsequent 7 day incubation. Individual cells showed no growth over the same interval. Higher UV doses and lower vacuum pressures (up to 232.8 kJ/m^2 and $3.2 \times 10^{-3}\text{ Pa}$) inhibited the growth of both single cells and cell clusters (Table 3).

Growth Capacity II: Growth Capacity of Mycobiont Cells

Exposure to high UV doses and low vacuum pressures had much less effect on the growth of mycelia. After an exposure time of 4 or 8 h (UV doses of 116.4 or 232.2 kJ/m^2) and low

Fig. 3 Growth capacity of mycobiont mycelia after exposure to space parameters. p , pressure; t_e , time of exposure; t_i , time of incubation. Eight replicates were used for each exposure experiment

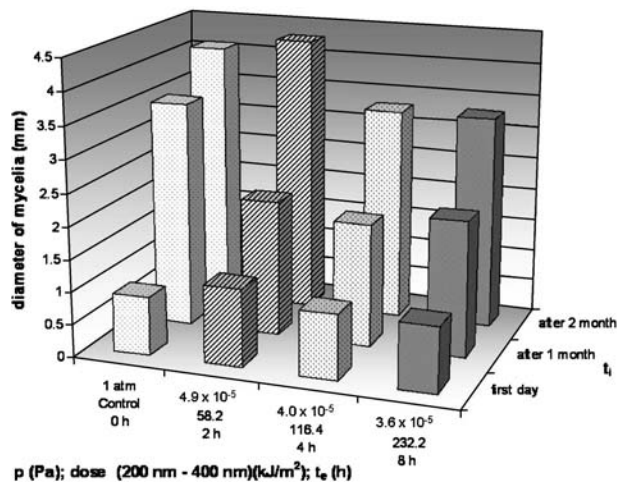
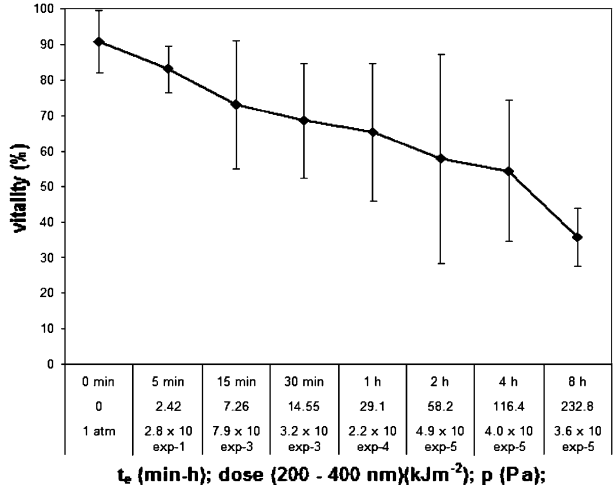


Fig. 4 Vitality of photobiont cells after exposure to polychromatic UV radiation (UV irradiation $200\text{ nm} \leq \lambda \leq 400\text{ nm}$ up to doses of 232.8 kJ/m^2) and vacuum pressure (up to $p=3.6 \times 10^{-5}\text{ Pa}$) quantified by analysis of the CLSM images (data represent mean values from eight replicates for each exposure time and the bars are indicating the range of CLSM measurements analysed by Image-Tool and ImageJ)

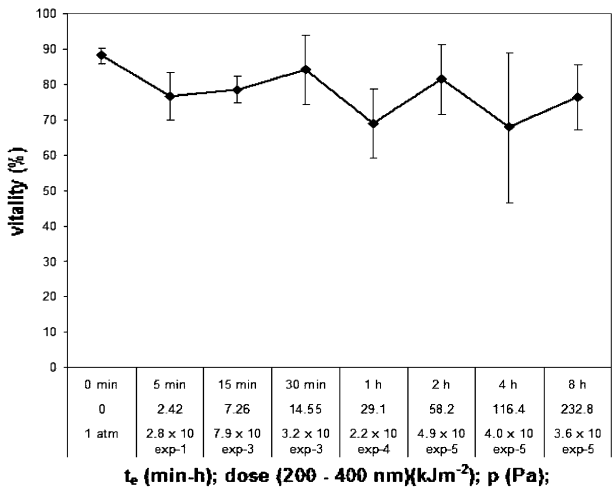


pressures approximately $4.0 \times 10^{-5}\text{ Pa}$ a small reduction in growth was detected (Table 4, Fig. 3). Control mycelia and those exposed for 2 h (dose of 58.2 kJ m^{-2} at $4.9 \times 10^{-5}\text{ Pa}$) both increased their biomass by a factor of four during the subsequent incubation, while those exposed for 4 or 8 h increased by a factor of 3.

Viability I: Viability of Photobiont Cells

As illustrated later on in Fig. 6, photobiont cells remained viable after exposure to a UV dose of 232.8 kJ/m^2 and a final vacuum pressure of $3.6 \times 10^{-5}\text{ Pa}$ when they were positioned in the centre of a cluster, receiving protection from the surrounding photobiont cells and the gelatinous matrix. Loose clusters and single cells were more affected. The presence of viable cells after 8 h exposure to simulated space conditions was only detected in clusters of the photobiont, with the proportion of viable cells decreasing from 82–95% (control) to 25–42% (Fig. 4).

Fig. 5 Vitality of mycobiont mycelia after exposure to polychromatic UV radiation (UV irradiation $200\text{ nm} \leq \lambda \leq 400\text{ nm}$ up to doses of 232.8 kJ/m^2) and vacuum pressure (up to $p=3.6 \times 10^{-5}\text{ Pa}$) quantified by analysis of the CLSM images (data represent mean values from eight replicates for each exposure time and the bars are indicating the range of CLSM measurements analysed by Image-Tool and ImageJ)



Viability II: Viability of Mycobiont Cells

The proportion of viable cells was 82–90% in controls, and 65–85% after 8 h exposure (Fig. 5), indicating only a minor decrease in viability. Unlike the results obtained after exposure of the photobiont, use of the dye SYTOX-green gave positive signals (indicating cell death) from only on a few cells. These cells were mostly located on the exterior of the mycelium. The proportion of dead cells was independent of the density of the structure of the mycelium (Fig. 6c,d).

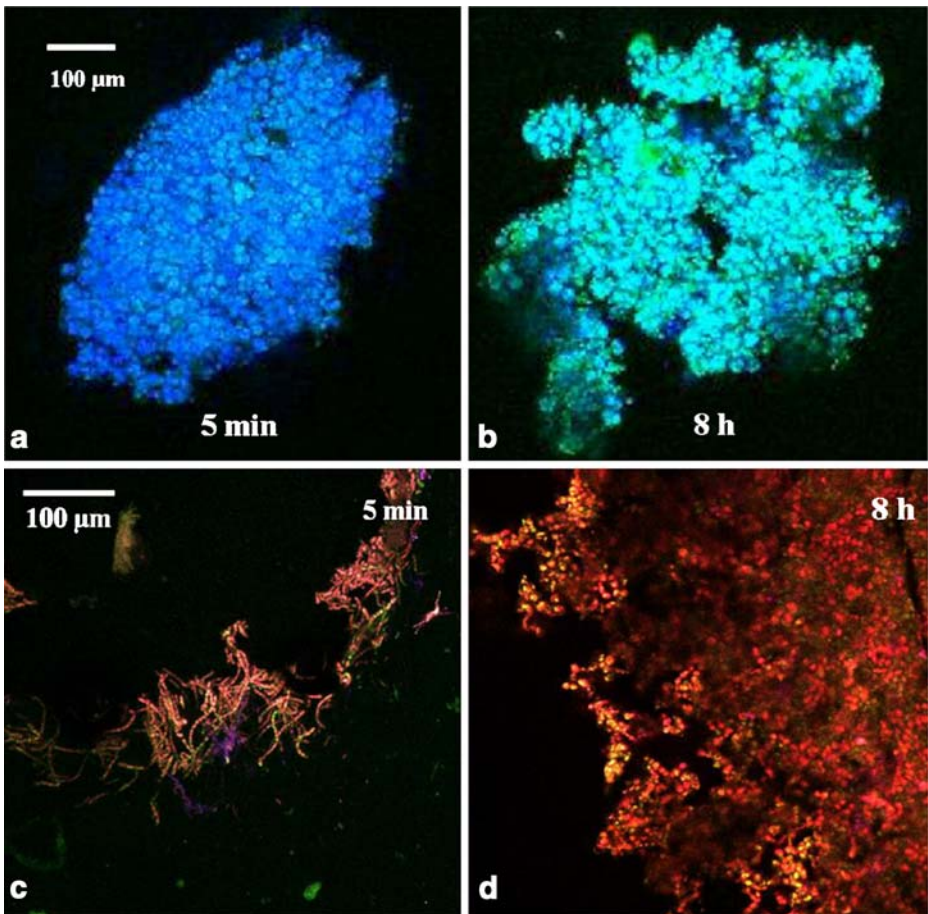


Fig. 6 Overlay images of photobiont cell clusters (**a, b**) and mycobiont mycelia (**c, d**) made by CLSM. After time of exposure to simulated space conditions of 5 min (2.42 kJ/m^2 ; $p=2.8 \times 10^{-5} \text{ Pa}$), the fluorescence dye SYTOX-green stains only three dead cells on the surface of a densely formed photobiont cluster (**a**) by a green colour whereas a loose photobiont cluster (**b**) has been more effected after $t_e=8 \text{ h}$ (232.2 kJ/m^2 ; $p=3.6 \times 10^{-5} \text{ Pa}$) and the majority of the cells is non-vital (green colour). No differences in the number of green or yellow stained dead cells occur between loose mycobiont mycelia and more compact structured. A change from pink to red coloured hyphae can be observed at the CLSM, probably due to UV induced parietin or carotene production

General Observations

Overlay images of CLSM (Fig. 6c–d) show that the auto-fluorescence of the lichen metabolite changes from pink to red directly after exposure to simulated space conditions. The early developmental stages of the mycelia of *X. elegans* are characterized by a white colour, showing no visible production of pigments. After exposure to simulated space parameters the colour changes into an intense pink over an incubation time of 2 months (Fig. 7). This probably indicates the production of parietin or carotene.

Discussion

Previous studies of *Xanthoria elegans* and *Rhizocarpon geographicum* have demonstrated a high capacity to resist the extreme stresses of simulated space exposure (de la Torre et al. 2004; de Vera et al. 2004a, b). These studies focused on viability and germination capacity of ascospores embedded in their natural fruiting bodies and in the isolated state. Before the current study there was very limited knowledge of the ability of each of the symbionts to resist these conditions.

The current study focused on the isolated photo- and mycobiont of *X. elegans*, a lichen that characteristically contains high concentrations of parietin (Honegger and Kutasi 1990; Solhaug et al. 2003), a very effective UV-screening secondary metabolite that causes the dark-orange colour of the thallus. UV-induced production of parietin in the lichen *Xanthoria parietina* has been demonstrated (Solhaug et al. 2003), and this response most likely underlies the colour change observed in the current study following UV exposure.

Photobiont

The very clear sensitivity to exposure to simulated space conditions demonstrated here in the photobiont of *X. elegans* mirrors that previously described in the lichen *Fulgensia*

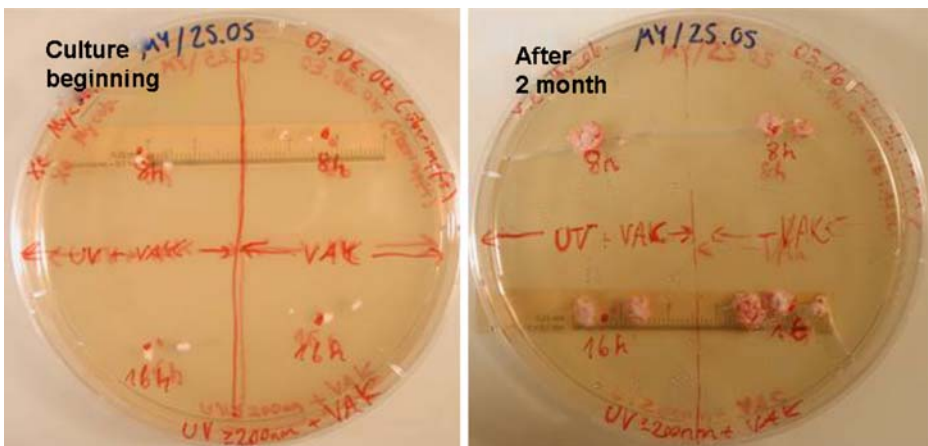


Fig. 7 Growth of mycobiont mycelia on malt yeast medium after exposure to the simulating space parameters (UV irradiation $200\text{ nm} \leq \lambda \leq 400\text{ nm}$ doses between 232.2 kJ/m^2 ($t_c=8\text{ h}$) and 465.6 kJ/m^2 ($t_c=16\text{ h}$) and low pressures, up to $p=3.1 \times 10^{-5}\text{ Pa}$). White or slightly pink coloured mycelia change to pink colour

bracteata (de Vera et al. 2004b). In *X. elegans* some measure of protection for up to 8 h exposure is provided within cell clusters. It is clear that, unlike the relatively high resistance of photobiont cells embedded in a lichen thallus (de Vera et al. 2003) single photobiont cells have much greater sensitivity, and would not be able to survive a hypothetical transfer of life between planets as postulated by the theory of Panspermia (Arrhenius 1903).

Mycobiont

Mycelia of *X. elegans* showed a much greater resistance to exposure to simulated space conditions than the photobiont. Both the presence of a well-developed mucilage layer, and UV screening secondary metabolites will contribute to this resistance. The role of the mucilage layer is comparable to that of bio crusts and biofilms (Belnap et al. 2001) where microorganisms are also protected against environmental stresses including UV radiation and desiccation by mucilage. The mucilage layer contains carbohydrates including the polyol mannitol and the disaccharide trehalose. These are compatible solutes and protective agents in lichens during total desiccation (Jennings and Lysek 1999), and may contribute to maintaining viability and growth capacity in the current study.

Conclusions

The current study clearly demonstrates surprisingly high resistance to exposure to the stresses of simulated space conditions in one of the isolated symbionts of the lichen *X. elegans*, although lower than when involved in the symbiotic association of the entire lichen thallus (cf. de Vera et al. 2003). The apparent resistance to these conditions of photobiont cells in a lichen thallus appears in reality to be due to the high resistance and protection provided by the mycobiont. The results obtained support the hypothesis that the symbiotic state of symbiont cells in lichens considerably enhances the ability of the respective symbionts to survive exposure to extreme conditions, including the conditions of space simulation.

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