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Abstract

A wide range of cold environments exist, with an equally broad variety of fungi and yeasts that have adapted to such environments. These adaptations, which affect membranes, enzymes and other cellular components, such as radical scavenging molecules, display a great potential for exploitation in biotechnology. Alterations have been detected in membrane lipids, with an increase in fatty acid unsaturated bonds that enhance their fluidity. We report new data on the different phospholipid composition in membrane lipids in the same fungal species from both Antarctic and temperate regions. The decrease in temperature causes intracellular oxidative stress by inducing the generation of reactive oxygen species. We report the results of the first analysis of the non-enzymatic antioxidant response and phenolic compound production by an Antarctic strain of *Geomyces pannorum*. A survey on yeasts from the cryosphere is reported with a focus on their adaptation to a cold environment. Some studies have shown that the number of macrofungi in glacier forefronts rises as deglaciation increases. The survival success of many plants in such areas may be attributed to their mycorrhizal associations. We highlighted the macrofungal biodiversity of some Italian alpine habitats, in which we *Inocybe microfastigiata*, *Laccaria montana* and *Lactarius salicis-herbaceae* were recorded for the first time in Lombardy (Italy).

Introduction

The term psychrophilic was coined by Morita ([1975](#)) for microorganisms whose cardinal minimum, optimum and maximum growth temperatures are, respectively, at or below 0, 15 and 20°C, whereas microorganisms with a higher growth optimum and maximum were called psychrotrophic (Eddy [1960](#)). Currently the terms psychrophile and psychrotolerant are preferred, because it is the former, not the latter, that has truly adapted to grow exclusively at low temperatures (Russell [2008](#)). Psychrophiles are a family of extremophiles, i.e. organisms that survive under physicochemical conditions far removed from those that represent a suitable environment for human beings (Gerday [2002](#); Selbmann et al. [2012](#), this issue).

Among the coldest environments, the cryosphere represents one of the largest unexplored and extreme biosphere systems, and it includes the ecosystems characterized by the presence of ice in extensive masses (Benn & Evans [1998](#)), such as cold deserts, glacial habitats and permafrost (Margesin & Miteva [2011](#)). Because of its extremely harsh climatic conditions, Antarctica has been for many years the geographic region chosen by a worldwide range of microbiologists for exploring diversity of cold-adapted microorganisms (Onofri et al. [2007](#)). However, a number of more recent studies highlighted that psychrophilic microorganism populations can successfully colonize non-Antarctic cryosphere as well (Buzzini et al. [2012](#)). Arctic areas and glaciers of high-mountain complexes, such as Himalaya, Alps and Andes are glacial areas which also represent rich sources of psychrophiles and psychrotolerants (Thomas-Hall et al. [2010](#); Turchetti et al. [2011](#)).

The viability of microorganisms in cold environments has been the focus of interest for many years, and glacial ice is an excellent preservation matrix for microbial life (Newsham [2012](#)); the longevity of microorganisms entrapped in ice (including those from alpine environments) has consequently become a subject of study (Catranis & Starmer [1991](#); Abyzov [1993](#); Persiani et al. [2011](#); among others).

Physiological mechanisms conferring cold tolerance on fungi are complex and not yet fully understood; they include increases in unsaturated membrane lipids (Russell [2008](#)), the production of RNA chaperones to suppress the formation of undesired secondary RNA structures (Kwak et al. [2011](#)), the synthesis of antifreeze (García-Arribas et al. [2007](#)) and cold shock proteins (Horn et al. [2007](#)), as well as cold-active enzymes (Gatti-Lafranconi et al. [2010](#)). A combination of these mechanisms is necessary for the psychrotroph or psychrophile to function (Robinson [2001](#)).

The aim of this study was to highlight some of the adaptations displayed by fungi and yeasts to cold environments, and particularly to focus on the large ecosystem complex of cryosphere, the Antarctic continent and alpine glacier forefront.

Increase in unsaturated membrane lipids in fungi from the Antarctic

The Antarctic region is dominated by microorganisms with a high level of adaptation. The Antarctic mycobiota in particular has been investigated, among others, by Italian researchers from a qualitative, ecophysiological, molecular and phylogenetic point of view (Onofri et al. [2007](#)). Most of the filamentous fungi are cosmopolitan species, some of which are psychrophilic whereas many

more are psychrotolerant; responses of Antarctic fungi to different stresses appear to be similar to those found in temperate regions (Ruisi et al. 2007; Onofri et al. 2011).

Cold-adapted microorganisms have evolved mechanisms to deal with the thermodynamic constraints of low temperatures, whereas membrane composition can determine the ability of fungi to grow outside specific temperature ranges. Psychrophiles increase the degree of disorder within macromolecules to maintain fluidity or flexibility and, consequently, to maintain their function at low temperatures (Russell 1990; Robinson 2001; D'Amico et al. 2006). Cold-adapted fungi and yeasts respond by modulating the fluidity of their membrane (Weinstain et al. 2000; Russell 2009; Turk et al. 2011), which is mainly achieved by altering the fatty acid composition through an increase in the proportion of unsaturated fatty acids.

In order to investigate how phospholipid composition has changed in the cell membranes in fungi, the composition of seven species from the Antarctic [*Aspergillus versicolor* (Vuill.) Tirab, *Cadophora fastigiata* (Lagerb. & Melin) Conant, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Geomyces pannorum* (Link) Sigler & J.W. Carmich, *Mortierella alpina* Peyronel, *Mortierella antarctica* Linnem., *Scolecobasidium salinum* (G.K. Sutherl.) M.B. Ellis] and of five of the same species from temperate habitats [*A. versicolor*, *C. cladosporioides*, *G. pannorum*, *M. alpina*, *S. salinum*] was analysed after the species had been incubated at two different temperatures (8 and 25°C). The Antarctic samples from which the fungi were isolated were collected in different sites of the "Terra Nova Bay" during Italian Antarctic Expeditions.

Different culture conditions were designed to support both mesophilic and xerotolerant growth by using the following culture media: malt extract agar, Czapek agar, Czapek yeast extract agar, potato dextrose agar and 25% glycerol nitrate agar (G25N, a_w 0.955) (Pitt & Hocking 1985).

The isolated fungal strains were then inoculated on liquid media (potato dextrose broth and Czapek broth) by suspending mycelia in distilled H_2O+Triton $\times$ 100 (0.1%, w/v). The fatty acid composition analyses of the mycelial polar lipid fraction were carried out after 14 days of incubation by means of thin layer chromatography and gas chromatography according to Finotti et al. (1993).

Table I shows the comparison of concentrations in the polar lipid fraction between common fungal species in the Antarctic and those in temperate regions. Each value is the mean of three determinations. The *t*-test was used to assess the significance of differences in concentrations between the strains grown at 25°C and those grown at 8°C. The table shows that the palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids are present in all the fungal strains analysed, regardless of the habitat of origin. As regards the unsaturated fatty acids, oleic acid quantities were higher ($p < 0.05$–0.001) both in the strains of *C. cladosporioides* and *S. salinum* and in the Antarctic strain of *C. fastigiata* when these strains were grown at 8°C than when they were grown at 25°C. Linoleic acid also increased ($p < 0.001$) in the *A. versicolor* and *G. pannorum* strains, as well as in the *S. salinum* strain from Antarctica when grown at 8°C. This increase was also observed in the Antarctic strains of *Geomyces vinaceus* and *G. pannorum* by Finotti et al. (1993).

Table 1 Comparison of mean value concentrations of fatty acids, as a percentage of the total fatty acids concentration, in the same fungal species from Antarctic and temperate locations (each value is the mean of three determinations ± SE; *p*, significance of differences in the concentration at 25 and 8°C, carried out with *t*-test).

		Saturated fatty acids						Unsaturated fatty acids									Others
Species	T (°C)	14:0	p	16:0	p	18:0	p	18:1	p	18:2	p	18:3	p	20:4	p	20:5	%
A. versicolor (Vuill.) Tirab.	25	3.9 ± 0.19	< 0.05	40.9 ± 0.47	< 0.001	15.4 ± 0.21	< 0.001	9.3 ± 0.27	< 0.01	**21.2 ± 0.27**	< 0.001	**0.4 ± 0.06**	< 0.001	nd		nd	8.93
From grassland soils of Spain	8	1.8 ± 0.21		30.1 ± 0.19		8.2 ± 0.21		3.8 ± 0.20		**40.2 ± 0.36**		**6.4 ± 0.15**		nd		nd	9.57
A. versicolor [a]	25	1.4 ± 0.21		27.2 ± 0.21	< 0.01	12.0 ± 0.21	< 0.01	25.3 ± 0.27	< 0.001	**24.6 ± 0.27**	< 0.001	**0.3 ± 0.06**	< 0.001	nd		nd	9.20
From moss, Laplung island 75° 34′ S 162° 55′ E (Soil T 2°C)	8	1.6 ± 0.15		29.0 ± 0.20		7.7 ± 0.15		9.2 ± 0.15		**39.0 ± 0.25**		**4.2 ± 0.15**		nd		nd	9.30
Cadophora fastigiata [a] (Lagerb. & Melin) Conant	25	nd		24.2 ± 0.17	< 0.001	4.5 ± 0.12	< 0.01	**9.4 ± 0.15**	< 0.05	47.0 ± 0.01	< 0.001	**0.9 ± 0.03**	< 0.001	nd		nd	14.03
From *Tigriopus* sp. (Copepod)	8	nd		17.8 ± 0.15		3.3 ± 0.15		**10.6 ± 0.27**		33.6 ± 0.21		**25.4 ± 0.21**		nd		nd	9.27

		Saturated fatty acids					Unsaturated fatty acids									
		14:0		16:0		18:0		18:1		18:2		18:3		20:4		20:5	Others
Species	*T* (°C)		*p*		*p*		*p*		*p*		*p*		*p*		*p*		%
Base 164° 06′ S 74° 41′ E																	
C. cladosporioides (Fresen.) G.A. de Vries	25	1.5 ± 0.15		16.6 ± 0.15	< 0.001	9.3 ± 0.21	< 0.001	**29.8 ± 0.32**	< 0.001	33.5 ± 0.27	< 0.001	nd		nd		nd	9.30
From grassland soils of Spain	8	1.5 ± 0.10		20.3 ± 0.27		19.1 ± 0.27		**49.8 ± 0.36**		8.4 ± 0.21		nd		nd		nd	0.90
C. cladosporioides	25	1.3 ± 0.15	< 0.05	18.0 ± 0.21	< 0.01	9.1 ± 0.15	< 0.001	**13.7 ± 0.21**	< 0.001	46.3 ± 0.32	< 0.001	**1.5 ± 0.12**	< 0.05	nd		nd	10.10
From soil beneath moss, Carezza Lake 74° 43° S 164° 03′ E (Soil T 3°C)	8	2.0 ± 0.15		20.6 ± 0.27		13.7 ± 0.15		**46.9 ± 0.35**		7.6 ± 0.17		**2.3 ± 0.21**		nd		nd	6.87
G. pannorum (Link) Sigler & J.W. Carmich.	25	nd		16.5 ± 0.15	< 0.01	1.2 ± 0.15	< 0.001	41.0 ± 0.25	< 0.001	**34.6 ± 0.27**	< 0.001	nd		nd		nd	6.73

		Saturated fatty acids						Unsaturated fatty acids									Others
Species	T (°C)	14:0	p	16:0	p	18:0	p	18:1	p	18:2	p	18:3	p	20:4	p	20:5	%
From grassland soils of Spain	8	0.5 ± 0.06		14.7 ± 0.09		6.7 ± 0.20		21.4 ± 0.22		**41.8 ± 0.12**		1.2 ± 0.12		nd		nd	13.63
G. pannorum	25	nd		16.1 ± 0.15	< 0.001	1.1 ± 0.12	< 0.01	41.2 ± 0.21	< 0.001	**34.7 ± 0.27**	< 0.001	nd		nd		nd	6.90
From soil, Inexpressible Island 74° 56′ S 163° 45′ E (Soil T 2.7°C)	8	1.3 ± 0.06		18.3 ± 0.15		4.1 ± 0.15		11.8 ± 0.15		**47.3 ± 0.32**		8.0 ± 0.15		nd		nd	9.20
M. alpina Peyronel	25	1.0 ± 0.10	< 0.001	22.2 ± 0.15	< 0.001	8.9 ± 0.21	< 0.001	9.4 ± 0.15	< 0.01	10.0 ± 0.21	< 0.001	nd		34.8 ± 0.27	< 0.001	0.6 ± 0.12	13.13
From soil of Larderello (Tuscany)	8	8.5 ± 0.21		46.3 ± 0.35		22.3 ± 0.27		6.2 ± 0.21		3.7 ± 0.15		nd		3.6 ± 0.12		nd	9.37
M. alpina	25	1.2 ± 0.10	< 0.01	24.0 ± 0.15	< 0.001	8.6 ± 0.23	< 0.01	25.8 ± 0.42	< 0.01	13.8 ± 0.27	< 0.001	0.5 ± 0.12		**12.5 ± 0.21**	< 0.001	nd	13.60
From soil beneath moss Carezza Lake 74° 43° S 164° 03′ E	8	3.2 ± 0.15		37.1 ± 0.21		10.8 ± 0.15		21.8 ± 0.21		5.3 ± 0.17		nd		**19.8 ± 0.27**		nd	2.00

Species	T (°C)	Saturated fatty acids 14:0	p	16:0	p	18:0	p	Unsaturated fatty acids 18:1	p	18:2	p	18:3	20:4	p	20:5	Others %
Mortierella antarctica[a] Linnem (Soil T 3°C)	25	3.8 ± 0.15	< 0.001	25.8 ± 0.19	< 0.001	5.6 ± 0.12	< 0.001	30.4 ± 0.25	< 0.001	18.1 ± 0.15	< 0.001	nd	**2.7 ± 0.15**	< 0.001	nd	13.73
From soil, Cape Russel 75° 00′ S 163° 45′ E (Soil T 2°C)	8	6.1 ± 0.07		41.5 ± 0.12		9.5 ± 0.12		19.0 ± 0.15		3.0 ± 0.06		nd	**20.7 ± 0.12**		nd	0.30
S. salinum (G.K. Sutherl.) M.B. Ellis	25	nd		26.8 ± 0.15	< 0.001	6.7 ± 0.17		**48.0 ± 0.30**	< 0.01	4.6 ± 0.17		nd	nd		nd	13.93
From sediment of Vico lake (Latium)	8	2.1 ± 0.12		22.5 ± 0.17		6.5 ± 0.21		**54.3 ± 0.38**		5.2 ± 0.15		nd	nd		nd	9.40
S. salinum	25	nd		18.2 ± 0.19	< 0.001	16.5 ± 0.09	< 0.01	**43.3 ± 0.17**	< 0.001	**9.6 ± 0.12**	< 0.001	nd	nd		nd	12.30
From moss, Baker Rocks 74° 14′ S 164° 47′ E (Soil T − 1°C)	8	nd		10.3 ± 0.21		18.4 ± 0.27		**50.3 ± 0.44**		**11.7 ± 0.21**		nd	nd		nd	9.30

		Saturated fatty acids			Unsaturated fatty acids					Others
		14:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	
Species	T (°C)	p	p	p	p	p	p	p		%

As regards the strains we analysed, the two species of *A. versicolor* and the Antarctic strains of *C. fastigiata* and *C. cladosporioides* displayed a significant amount of linolenic acid (18:3), which was higher ($p < 0.05$–0.001) at 8°C. The Antarctic strains of *M. alpina* and *M. antarctica* revealed high amounts of arachidonic acid (20:4), which increased ($p < 0.001$) in strains cultivated at 8°C.

Jang et al. (2005) reported that the production of polyunsaturated acids depends on the source of carbon and nitrogen in culture media, but also increases as the temperature decreases. In addition, Weete & Gandhi (1999) claimed that members of the subgenera *Mortierella* and *Micromucor* could be identified according to the presence or absence, respectively, of arachidonic acid. Worthy of note is the production of eicosapentaenoic acid (20:5) exclusively by the strain of *M. alpina*, which originated in a temperate region, when grown at 25°C. This fatty acid has been attracting a great deal of attention on account of its beneficial effects on human health (Dewey et al. 2007, among others) and for a rapid identification of important pathogen bacterial species (Moss et al. 1980).

Table II shows the significance of differences (*t*-test) in fatty acid concentrations (expressed as percentages of the overall fatty acid concentration in the polar lipid fraction) in fungal species that are common in both the Antarctic and temperate regions when grown at 8°C. Each value is the mean of three replicates.

Table 2   Significance of difference (carried out with *t*-test) in the mean value concentrations of fatty acids (± SE), as a percentage of the total fatty acids concentration, in the same fungal species from Antarctic and temperate locations, at 8°C.

		Saturated fatty acids					Unsaturated fatty acids						
		14:0		16:0		18:0	18:1		18:2		18:3		20:4
Species	T (°C)		p		p		p		p		p		p
A. versicolor (Vuill.) Tirab.	8	1.8 ± 0.21		30.1 ± 0.19	< 0.05	8.2 ± 0.21	**3.8 ± 0.20**	< 0.001	40.2 ± 0.36		6.4 ± 0.15	< 0.001	nd
A. versicolor [a]	8	1.6 ± 0.15		29.0 ± 0.20		7.7 ± 0.15	**9.2 ± 0.15**		39.0 ± 0.25		4.2 ± 0.15		nd

Species	T (°C)	Saturated fatty acids						Unsaturated fatty acids						
		14:0	p	16:0	p	18:0	p	18:1	p	18:2	p	18:3	p	20:4	p
C. cladosporioides (Fresen.) G.A. de Vries	8	1.5 ± 0.10		20.3 ± 0.27		19.1 ± 0.27	<0.001	49.8 ± 0.36	<0.01	8.4 ± 0.21	<0.05	nd		nd	
C. cladosporioides [a]	8	2.0 ± 0.15		20.6 ± 0.27		13.7 ± 0.15		46.9 ± 0.35		7.6 ± 0.17		2.3 ± 0.21		nd	
G. pannorum (Link) Sigler & J.W. Carmich.	8	0.5 ± 0.06	<0.001	14.7 ± 0.09	<0.001	6.7 ± 0.20	<0.001	21.4 ± 0.22	<0.001	**41.8 ± 0.12**	<0.001	**1.2 ± 0.12**	<0.001	nd	
G. pannorum [a]	8	1.3 ± 0.06		18.3 ± 0.15		4.1 ± 0.15		11.8 ± 0.15		**47.3 ± 0.32**		**8.0 ± 0.15**		nd	
M. alpina Peyronel	8	8.5 ± 0.21	<0.001	46.3 ± 0.35	<0.001	22.3 ± 0.27	<0.001	**6.2 ± 0.21**	<0.001	**3.7 ± 0.15**	<0.001	nd		**3.6 ± 0.12**	<0.001
M. alpina [a]	8	3.2 ± 0.15		37.1 ± 0.21		10.8 ± 0.15		**21.8 ± 0.21**		**5.3 ± 0.17**		nd		**19.8 ± 0.27**	
S. salinum (G.K. Sutherl.) M.B. Ellis	8	2.1 ± 0.12		22.5 ± 0.17	<0.001	6.5 ± 0.21	<0.001	54.3 ± 0.38	<0.01	**5.2 ± 0.15**	<0.001	nd		nd	
S. salinum [a]	8	nd		10.3 ± 0.21		18.4 ± 0.27		50.3 ± 0.44		**11.7 ± 0.21**		nd		nd	

Note: Bold values indicate data on the unsaturated fatty acid concentration that are significantly different.

Worthy of note is the fact that differences for unsaturated fatty acids were almost always significant ($p = <0.05–0.001$). Oleic and linoleic acid levels increased, respectively, in the Antarctic strains of *A. versicolor* and *S. salinum*, whereas linoleic and linolenic acid levels increased in the Antarctic strain of *G. pannorum*. The Antarctic strain of *M. alpina* displayed an increase in oleic, linoleic and arachidonic acid levels.

These results taken as a whole clearly show that the effect of low temperature is associated with an increase in membrane unsaturated fatty acids in all the species considered. This adaptation process is particularly evident in 80% of the Antarctic species.

Antioxidant activity in fungi from the Antarctic

Cold-adapted microorganisms have developed special mechanisms to overcome the life-endangering influence of low temperature and to survive cold-induced oxidative stress. The decrease in temperature causes intracellular oxidative stress by inducing the generation of reactive oxygen species (ROS) (Fridovich 1998; Gocheva et al. 2009) that can damage cellular components such as DNA, proteins and lipids. To scavenge ROS, aerobic cells have developed a complex defense system consisting of both low-molecular mass scavengers and high-molecular mass antioxidants, in particular antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Angelova et al. 2005; Chattopadhyay et al. 2011; Krumova et al. 2012).

In order to gain further insight into the possible mechanisms underlying fungal survival in extremely cold conditions, cell response of Antarctic fungi to cold stress was investigated by Tosi et al. (2010) and Kostadinova et al. (2012). The results of these investigations showed that fungal cell responses to cold shock are dependent to a greater extent on species than on the degree of cold stress.

Antarctic microorganisms are assumed to possess lower rates of enzymatic and transport processes; this promotes a decrease in ATP demand and a subsequent accumulation of electrons in the respiratory chain, which in turn leads to a sudden increase in the production of the number of ROS (Chattopadhyay 2002). The importance of SOD and CAT for stress protection has been highlighted by Tosi et al. (2010) and Krumova et al. (2012), who demonstrated that Antarctic fungi are good producers of SOD and CAT. However, an Antarctic strain of *G. pannorum* (Link) Sigler & Carmichael was one of the less efficient producers of CAT and SOD (Tosi et al. 2010; Krumova et al. 2012). How then can *G. pannorum* overcome the climatic conditions found in the Antarctic? This paper provides the first report of the non-enzymatic antioxidant response and phenolic compound production displayed by this Antarctic strain.

The fungus was isolated from a soil sample collected in the austral summer of 2006–2007 near the Bulgarian base of St Kliment Ohridski (62°38′29″S 60°21′53″W) on Livingston Island in the Maritime Antarctic. *G. pannorum* (strain *B*) was analysed under optimal growth temperature and after cold and heat shock. Analyses were carried out on lyophilized mycelium from 10-day-old cultures grown at 20°C in 250 ml shaking flasks containing liquid Sabouraud culture medium. The results were compared with those recorded for a *G. pannorum* strain isolated in a Thracian tomb in Bulgaria (strain *T*), with environmentally controlled conditions in the interior. Data-sets were subjected to a *t*-test for independent samples at a 5% level of significance.

The diphenylpicrylhydrazyl radical (DPPH) scavenging activity was estimated according to Molyneux (2004), and by following the method reported by Cheung et al. (2003) with some modifications. An amount equal to 2 g of lyophilized fungus was left shaking overnight in absolute methanol. An aliquot of 1 ml DPPH radical (Sigma) (methanol solution, 0.1 mM) was added to a test tube with 33 µl of the fungus extract in methanol. Pure methanol was used as a blank. The reaction mixture was vortex-mixed and absorbance (Abs) was determined after 1 hr by measurement at 517 nm with a Jasco 7800 spectrophotometer (Jasco, Easton, MD, USA). Ascorbic acid standards were prepared in different concentrations, whereas DPPH radical scavenging activity was expressed as mM of ascorbic acid equivalent (mg^{-1} of sample dry weight).

Total phenolics were assayed using the Folin–Ciocalteau reagent (Singleton et al. 1999), following Waterhouse (2001) with some modifications. A mixture was prepared with 1 ml of water, 65 µl of the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 13 µl of sample. The mixture was shaken and allowed to stand for 8 min, before the addition of 195 µl of saturated Na_2CO_3 (20%, w/v). After incubation in the dark for 2 h, absorbance was measured at 765 nm

versus a blank. The total fungal phenolic concentration was expressed as mM of gallic acid equivalent (mg^{-1} of sample dry weight) with the help of a calibration curve prepared with gallic acid. All samples were analysed in triplicate. Non-enzymatic antioxidant activity and phenol production were measured after cold and heat shock. Following 10 days of growth, cultures were maintained at 4°C or 37°C for 72 h, and then analysed according to the method cited above.

The DPPH-radical scavenging activity of the *G. pannorum* strain *B* is presented in Figure 1 and is compared with that of *G. pannorum* strain *T*. Antioxidant production at the optimal temperature for growth (20°C) is plotted together with the production after cold (4°C) and heat (37°C) shock. Production of strain *B* was significantly different from that of strain *T* at 20°C and after cold shock. When the Antarctic strain was under cold shock, the response was threefold higher than at 20°C; no difference was recorded after heat shock. Strain *T* did not exhibit any significant change after cold or heat shock. The strong radical scavenging activity response of the *G. pannorum* strain *B* after cold shock was consistent with fungus phenolic production (Figure 2) expressed as gallic acid equivalent. After a 4°C cold shock, phenolic production was 2.4-fold higher than that at 20°C. A significant difference was also recorded after heat shock, though it was less marked.

Figure 1 DPPH radical scavenging activity of a *G. pannorum* strain isolated from Maritime Antarctica (B) and from a climatically controlled environment (Thracian tomb) (T). Activity is expressed as mM of Ascorbic acid equivalent per mg of dry weight at optimal temperature (20°C) (grey column), after 72 h shock at 4°C (white column) and 37°C (black column). Data are the mean of three replicates with standard deviation (bar).
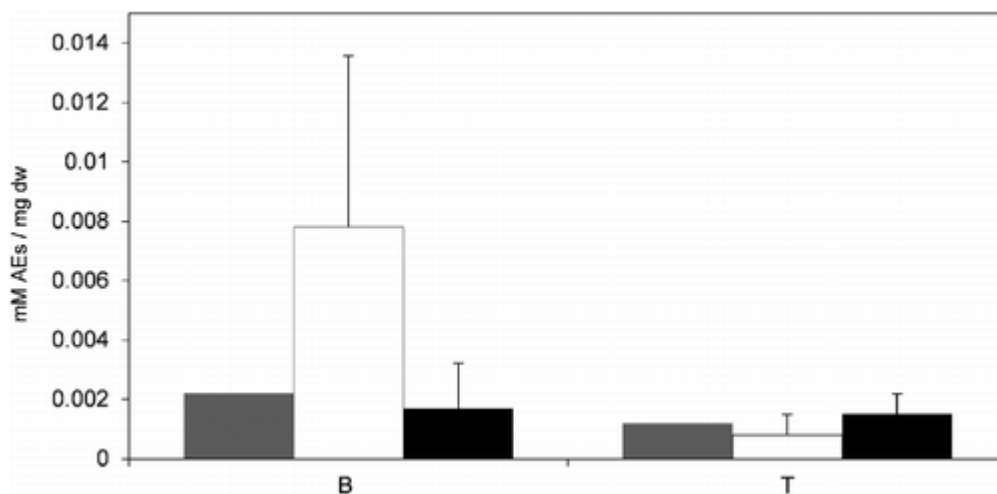
Figure 2 Total phenolic compound production of a *G. pannorum* strain isolated from Maritime Antarctica (B) and from a climatically controlled environment (Thracian tomb) (T). Activity is expressed as mM of Gallic acid equivalent per mg of dry weight at optimal temperature (20°C) (grey column), after 72 h shock at 4°C (white column) and 37°C (black column). Data are the mean of three replicates with standard deviation (bar).
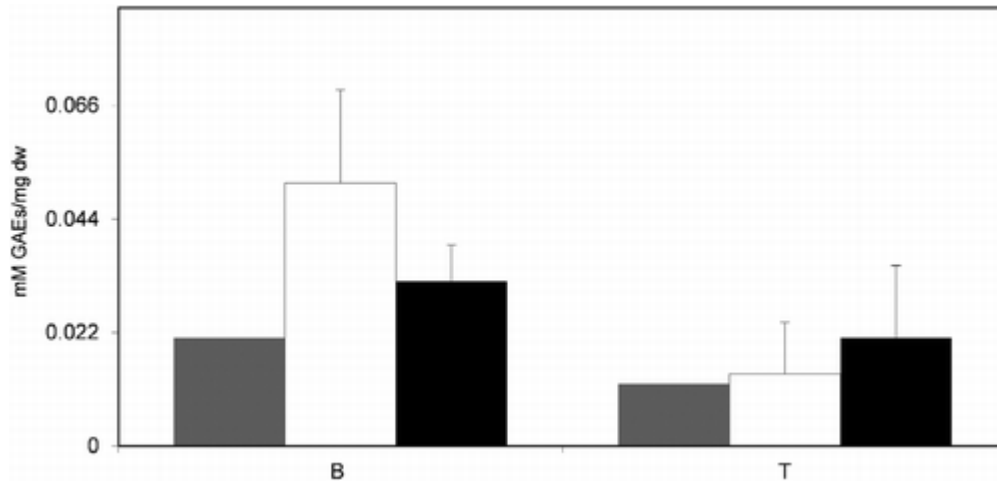
Non-enzymatic antioxidant activity is known to be partly due to the presence of phenolic compounds (Palacios et al. 2011). Our study clearly indicates that *G. pannorum* can overcome extreme cold conditions by means of a strong antioxidant response mainly through the production of phenolic compounds. *G. pannorum* is one of the most widespread fungi in Antarctica and its ability to buffer free radical damage with antioxidant compounds is partly responsible for its ability to survive in harsh environments.

Yeasts from the cryosphere

Yeasts can be defined as fungi whose asexual growth predominantly results from budding or fission, and which do not form their sexual states within or upon a fruiting body (Kurtzman et al. 2011). They include versatile species exhibiting heterogeneous nutritional profiles and a surprising aptitude for survival in a broad range of both natural and man-associated ecosystems (Hagler & Ahearn 1987; Starmer & Lachance 2011). Some authors have suggested that yeasts may be better adapted to low temperatures than bacteria (Margesin et al. 2003; Turkiewicz et al. 2003; Shivaji & Prasad 2009).

The cryosphere represents one of the largest unexplored and extreme biosphere systems, and it includes cold deserts, glacial habitats and permafrost (Margesin & Miteva 2011). As studies of Antarctic yeasts have been attracting a considerable amount of interest ever since the 1960s, hundreds of isolates have been characterized and several novel species described (Shivaji & Prasad 2009). Other glacial habitats are found in Arctic areas and on the glaciers of high-mountain chains, such as the Himalayas, Alps and Andes. Much like the cryosphere found in Antarctica, non-Antarctic cryosphere also harbours psychrophilic yeast life. Indeed, a partially habitat-dependent distribution has been observed in the non-Antarctic cryosphere (Buzzini et al. 2012), and some novel species have recently been described (de García et al. 2010a 2010b; Thomas-Hall et al. 2010; Turchetti et al. 2011; among others).

By comparing the list of yeast species isolated from the cryosphere, it is possible to observe that more than 120 different species have been found worldwide; approximately one third of these species belong to the genus *Cryptococcus* (Buzzini et al. 2012). As regards the ecology of psychrophilic yeasts that share such habitats, it remains to be seen whether some factors actually

affect the differential distribution of psychrophilic yeasts in the cryosphere. Moreover, the scientific literature documents the great diversity of isolation protocols used in over 60 years of investigations. As recently pointed out (Shivaji & Prasad 2009; Buzzini et al. 2012), most previous studies on yeast ecology in the Antarctic and non-Antarctic cryosphere have been conducted using markedly different isolation and incubation protocols. These differences may explain the apparently random distribution observed for most of the psychrophilic yeasts that inhabit cryosphere ecosystems (Vishniac 2006a, 2006b).

A considerable number of structural and functional mechanisms highlighting adaptation strategies adopted by psychrophilic yeasts to resist the severe conditions in the cryosphere have been reported since the 1960s. Moreover, some recent discoveries shed further light on the mechanisms governing both the survival and growth of psychrophilic yeasts. The adaptation strategies adopted by psychrophilic yeasts to overcome cold conditions in the cryosphere have recently been reviewed (Buzzini et al. 2012).

A number of studies have demonstrated that the ability to adjust membrane fluidity by regulating the synthesis of fatty acids, through mechanisms such as increased fatty acid unsaturation, decreased fatty acid average chain length and decreased sterol/phospholipid ratio, is crucial for adaptation to cold temperatures (Margesin & Miteva 2001). One of the mechanisms that has been studied most is that related to the unsaturation degree (Rossi et al. 2009).

Studies on the synthesis of antifreeze macromolecules have shown that trehalose biosynthesis pathways are found extensively in nature as a form of cell protection against osmotic stress. High cytoplasm concentrations of trehalose have been observed in *Mrakia frigida* and *Leucosporidium fellii* as a strategy to reduce the freezing point of intracellular fluid (Deegenaars & Watson 1997; Deegenaars & Watson 1998). More recently, Lee et al. (2010) reported the ability of an Arctic *Leucosporidium* sp. strain to secrete a glycosylated ice-binding protein.

The ability of psychrophilic yeasts to remain metabolically active in sub-zero temperatures was recently reported (Amato et al. 2009). Furthermore, recent comparisons of the dependence of growth kinetic parameters of psychrophilic and mesophilic yeasts within a wide range of temperatures revealed a clear dichotomy: the temperature at which microbial growth was fastest was generally different from the temperature that allowed the production of the highest amount of yeast biomass (Margesin 2009; Rossi et al. 2009).

The synthesis of psychrophilic enzymes is one of the psychrophilic yeast adaptation strategies which has been investigated most. The increased activity of psychro-enzymes at low temperatures is based on their improved structural flexibility in conjunction with a possible modification of the active site (Gerday et al. 1997; Feller & Gerday 2003). The enhanced plasticity of psychro-enzymes is frequently considered to be responsible for their weak thermal stability. This is due to their adaptation to cold conditions, which has led to structural changes that have increased sensitivity to various denaturing agents, thus pointing to a direct link between activity and stability of psychro-enzymes (Feller & Gerday 2003).

Macrofungal species from alpine ecosystems

Glacier forefronts are of particular importance because they represent a newly formed or exposed substrate that constitutes a unique habitat for primary succession and for ruderal organisms (Alfredsen & Høiland 2001). This habitat provides extreme examples of disturbance in which the primary production and amount of dead plant material are low above all because of slow nutrient turnover (Haselwandter & Read 1980). As a consequence, newly formed glacier forefronts are characterized by low nitrogen and organic matter levels (Jumpponen et al. 2012).

Fungi play a very important role in the initial colonization of these environments through a range of spore dispersal strategies that render different *taxa* more or less effective in inoculating these soils and hence in supporting the early establishment of mycorrhizal host plants (Cázares & Trappe 1994). Some studies have shown that the number of macrofungi in glacier forefronts rises as

deglaciation increases (Jumpponen et al. 1999), and the success of plant growth may be at least partly attributable to their mycorrhizal associations (Read & Haselwandter 1981).

Alpine tundra is another very important habitat, characterized by low thermal energy, temperature and nutrient supply, and high proportions of UV light, wind, snow and ice. Fungi have adapted to these life conditions just as other organisms have (Trappe 1988). Indeed, the short growth period favours short life cycles, with the suppression of the conidial or ascomatal state.

Physiological and biochemical adaptations to freezing temperatures, such as a change in the distribution of viable cytoplasm within *hyphae* in winter, might allow the mycelium to survive freezing events (Addy et al. 1994). Dwarfism, expressed in a reduced number of gills, and the size of the fruit bodies is another very common feature of alpine macrofungi.

Since macrofungi communities have rarely been studied in the alpine habitats of Italy, knowledge of the fungal flora in such environments is based on a limited number of sites (Jamoni 2008; Granito & Lunghini 2011; Venturella et al. 2011).

A recent contribution to our knowledge of fungal biodiversity in Italian high-mountain habitats has been provided by the PRIN 2008 project "Study and conservation of the fungal biodiversity in cold marginal habitats endangered from global change", which was designed to analyse the fungal communities in alpine environments characterized by the presence of glaciers found in the Apennines and Alps. These environments include the "Val Viola Bormina-Ghiacciaio Cima dei Piazzi" (IT 2040012) Site of Community Importance belonging to the EU Network "Natura 2000", which was investigated in 2010. The site, which lies at an altitude ranging from 1710 to 3441 m a.s.l. and located in the Central Alps, in Lombardy, is mostly covered by natural and semi-natural alpine and boreal grasslands (Parolo et al. 2008). Sporomata were collected during the summer and fungal *taxa* were identified. Fresh material was dried and voucher specimens were deposited in the Herbarium of the Department of Earth and Environmental Sciences, University of Pavia (Italy).

A total of 27 macrofungal species belonging to 17 genera (all members of phylum *Basidiomycota*) were identified. These include 14 Arctic-alpine species and 13 typical grassland *taxa* or ubiquitous species that occur in various habitats and frequently also appear in the alpine zone. The species collected contain some *taxa* that are remarkable on account of their rarity and geographical distribution. *Lactarius salicis-herbaceae* Kühner, one of the yellow alpine *Lactarius* species with violet staining milk, was collected from two alpine grassland plots (at 2290 and 2460 m a.s.l.) with snow bed vegetation including *Salix* shrubs, on calcareous soil. This is the first record of *L. salicis-herbaceae* in Lombardy. The fungus has previously been reported in the Italian Alps (Jamoni 2008) in localities with acidic soil. The white to cream lamellae and the relatively darker pileus (yellow to ochre), as well as the preference for acidic habitats, distinguish *L. salicis-herbaceae* from the similar *L. salicis-reticulatae* Kühner, which is characterized by deep yellow lamellae (almost peach coloured) that contrast with the pale (cream yellow, ivory, whitish towards margin) pileus, and grows on basic soil (Alfredsen & Høiland 2001; Gulden 2005). The finding of *L. salicis-herbaceae* for the first time on a calcareous bedrock widens both the ecological preferences and the geographic distribution of this species.

Inocybe microfastigiata Kühner was also collected near *L. salicis-herbaceae*. This small *Inocybe* species, which usually grows in association with dwarf willows, is found in the high-mountain belts of European mountains, having been reported in the Swiss, French and Italian Alps as well as in the Romanian southern Carpathians (Jamoni & Bon 1995; Ronikier 2008, among others). It represents a typical example of fungal alpine dwarfism as its basidiomes are much smaller (cap 1.5–2 cm in diameter, stipe $2–2.5 \times 0.3 \times 0.4$ cm) than those of the very similar species *I. rimosa* (Bull.) P. Kumm. (cap 7–10 cm in diameter, stipe $10 \times 0.3–1.2$ cm). The taxonomy of the *I. rimosa* group was somewhat confused for a long time and requires further revision since many species are described on the basis of small macro- and micro-morphological differences (Larsson et al. 2009). The exact ecology and geographical distribution of *I. microfastigiata* are not known. In this paper, *I. microfastigiata* is reported as the first record in Lombardy.

Another ectomycorrhizal basidiomycete observed was *Laccaria montana* Singer, which normally grows in the alpine zone (Mueller [1992](#); Kernaghan [2001](#)). Twenty sporocarps of this fungus were collected from one plot at 2460 m a.s.l. *L. montana* appears to be morphologically similar to *L. pumila* Fayod and *L. tortilis* (Bolton) Cooke, but differs insofar as the latter have two-spored basidia (Osmundson et al. [2005](#)). Despite being very similar, the small and striate forms of *L. laccata* (Scop.) Cooke var. *pallidifolia* (Peck) Peck differ insofar as *L. montana* has smaller, globose to subglobose basidiospores (Gulden [2005](#); Osmundson et al. [2005](#)). *L. montana* is restricted to Arctic, boreal and montane habitats and seems to prefer relatively disturbed soils present on ridges, open snow-beds, paths or open sandy areas (Alfredsen & Høiland [2001](#)). This species has been reported in the eastern and western Italian Alps (Bon [1987](#); Lo Bue et al. [1994](#); Jamoni [2008](#)), but has not previously been reported in Lombardy.

Another study was conducted in two permanent plots of alpine grassland (about 1000 m^2 each) situated along the path leading to Camoscere Lake (Elva, Cuneo, 2294 m) in southern Piedmont. The plots, which were visited during the summer months from 2004 to 2011, are dominated by the presence of *Helianthemum nummularium* (L.) Mill. subsp. *grandiflorum* (Scop.) Schinz & Thell plants. Although the vegetation is scarce, there is an abundance of ectomycorrhizal (ECM) fungi, which is indicative of these fungi's association with *Helianthemum* spp. Nine species were recorded, including a new *taxon*: *Amanita helianthemicola* Zotti, Vizzini & Traverso ([Vizzini et al. submitted](#)). The new species belongs to the *A. lividopallescens* complex in the section *Vaginatae* (Fr.) Quél. (Contu [2001](#)); it is very closely related to *A. lividopallescens* sensu Boudier (Boudier [1905](#)), and is characterized by clampless basidia and globose inamyloid basidiospores.

The other ECM species observed were *A. pantherina* (DC.) Krombh., *A. vaginata* (Bull.) Lam., *Boletus luridus* Schaeff., *Cortinarius anomalus* (Fr.) Fr. and *Russula pascua* (F.H. Møller & Jul. Schäff.) Kühner. It is noteworthy that a significant number of sporomata were observed during surveys, i.e. from 6 to 45 for each species. Besides the ECMs, three saprotrophic species were also collected: *Lepista irina* (Fr.) H.E. Bigelow var. *montana* Bon, *Agaricus campestris* L. and *Bovista plumbea* Pers.

Our data suggest that *Helianthemum nummularium* subsp. *grandiflorum* creates a suitable habitat for the growth of a wide range of ECM species, as it has previously been highlighted for *H. nummularium* subsp. *nummularium* by Barden ([2007](#)) in English grasslands. *Helianthemum* is known to form ectomycorrhizal associations, particularly with hypogeous ascomycetes in Mediterranenan areas (Turgeman et al. [2011](#)), whereas species strictly associated with *Helianthemum* have rarely been reported in alpine grasslands (Barden [2007](#)).

Acknowledgements

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