Xerotolerant mycobiota from high altitude Anapurna soil, Nepal

U. Petrovič a,*, N. Gunde-Cimerman b,c, P. Zalar b,c

a Institute of Biochemistry, Medical Faculty, University of Ljubljana, Vrazov trg 2, SI-1000 Ljubljana, Slovenia
b Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia
c National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia

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Abstract

Xerophilic and xerotolerant microfungi were isolated from soil samples collected in Anapurna Mountains, Nepal, at altitudes from 3000 to 5400 m. The total numbers and proportions of xerotolerant and psychrophilic strains in comparison with mesophilic mycobiota were determined by using different enumeration, selective media and four isolation methods. The most extreme xerophilic fungi were taxonomically identified as belonging to the genera Eurotium and Aspergillus. The low water activity of the soil due to dry climate and frequent binding of water in ice crystals favors a high proportion of xerotolerant fungal species. The correlation between xerotolerant and psychrophilic fungi was observed. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The Anapurna Mountains in the Himalaya region can be considered an extreme alpine environment because of the several stress factors present. Some of those to which microorganisms have adapted through natural selection include extremely low temperatures in winter, down to −40°C, which undulate up to +20°C, high UV radiation, low oxygen concentrations, short growing seasons for plants, extremely oligotrophic soil with various nutrient deficiencies, mainly of nitrogen, and the presence of wind, snow and ice [1–4].

In the area of the Torung Pass selected for this study there is an additional extreme-habitat-forming factor, namely the dry climate resulting from the geography of the region. Most of the time soils in these habitats have extremely low water activities, which are even lowered when free water is bound in ice crystals. Water availability imposes physiological limits and controls the rate of spore germination, growth, reproduction and also effects the outcome of competitive interactions amongst fungi [5].

Descriptions of xerophilic and xerotolerant fungi in the literature are mostly limited to those causing spoilage of food preserved with high quantities of sugar or salt [6]. Few investigations have been made regarding biodiversity and occurrence of xerophilic and xerotolerant fungi in natural habitats, with the exception of highly saline soils [7–9]. Alpine environments, especially the Himalaya region, have been investigated for the biodiversity of mesophilic and psychrophilic mycobiota, but are completely neglected for the presence of xerophilic and xerotolerant fungi [3,4].

Using different isolation methods and a range of low water activity enumeration and selective media [10], filamentous fungi from soil samples taken at the Torung Pass area at an altitude of 3000–5400 m were isolated. The aim of the present study was to determine the total numbers, proportions and species diversity of xerophilic and xerotolerant fungi, as well as to compare psychrophilic with mesophilic mycobiota at the same time.

2. Materials and methods

2.1. Localities

Soil samples were collected in the Anapurna Mountains in Nepal at seven different localities on both sides and at the top of the Torung Pass: 28°47’ northern latitude, 83°57’ eastern longitude, at altitudes from 3000 to 5400 m.
The eastern side of the pass: locality 1 (3000E): a meadow, 3150 m; locality 2 (4000E): sandy soil, 3950 m; locality 3 (5000E): stone gravel, 5100 m; locality 4 (5400): stone gravel at the top of the Torung Pass, 5416 m. Western windswept side: locality 5 (5000W): sandy soil, 5100 m; locality 6 (4000W): dry soil, 3900 m; locality 7 (3000W): sandy soil, 2850 m.

2.2. Samples

To exclude allochthonous mycobiota, the samples were collected at each locality at a depth of 10 cm. Approximately 10 g of soil was taken from each sampling site. Samples were collected into sterile 100-ml flasks with a pre-sterilized spatula.

2.3. Media

The following selective media were used for isolation: Dichloran rose bengal agar (DRBC), \(a_w 1.000\) [11]; dichloran 18% glycerol medium - DG18, \(a_w 0.946\) [12]; malt yeast 40% sucrose agar (M40Y), \(a_w 0.890\) [13]; 3 M NaCl malt extract agar (MEA+3 M NaCl), \(a_w 0.872\) [13]; MEA+5.2 M NaCl, \(a_w 0.782\) [13] and 70% glucose/fructose malt yeast agar (MY70GF) \(a_w 0.723\) [13]. To prevent bacterial growth 200 mg l\(^{-1}\) of chloramphenicol was added to all media. The water activities, \(a_w\), of the media were determined by a water activity system CX-1, Campbell Scientific Ltd.

2.4. Isolation methods

We used the following methods to isolate the fungi from the soil samples:

For spread plating, 1 g of soil was diluted with 5 ml of distilled water or 20% glucose solution. 0.5 ml of the suspension was spread on a plate. For pour plating, 0.5 g of soil was diluted with different media: DRBC, DG18, M40Y, MY70GF and MEA+5.2 M NaCl. In all cases serial dilutions of soil were made, 10\(^{-1}\), 10\(^{-2}\) and 10\(^{-3}\), and the plates were incubated in duplicate.

For soil washing, 0.2 g of soil was washed with 1000 ml of distilled water through black ribbon filters and filters were incubated on different media.

The plates were incubated at 4\(^{\circ}\)C for up to 120 days or at 23\(^{\circ}\)C up to 90 days.

2.5. Determination of number of colonies per plate

The grown colonies were counted daily, visually and by microscopic examination. At the end of the incubation the total numbers of colony forming units, CFU, for different isolation methods, for different localities, and for different selective media were calculated and expressed as the number of CFU per gram of soil.

2.6. Taxonomic identification

The taxonomic identification of xerophilic and xerotolerant strains was performed at the National Institute of Chemistry, Ljubljana, Slovenia and at the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The isolated strains were included in the Slovenian Fungal Culture Collection (MZKI).

3. Results and discussion

The specific conditions present in the Anapurna Mountains exert selective pressures on the microorganisms living there, mainly enabling growth of those adapted to low water activities of soil and low temperatures. The Torung Pass was selected as a model area because of its altitude and comparable alpine ecotone above the timberline on both sides of the pass. In this region the majority of the land surface has no vegetation and consists of pebbles, pulverized calcareous rock debris and patches of organic detritus.

The main goal of our study was to evaluate the impact of the stress factors present in quantitative and taxonomic terms on the mycobiota present. Many investigators commonly isolate fungi from alpine soils on mesophilic media. This creates a bias towards those species with optimum growth at \(a_w \approx 1\). Documentation of the species present does not indicate the active population. In our study, strains able to grow under \(a_w 0.9\) were considered xerotolerant and those able to grow under \(a_w 0.8\) xerophilic. Two xerophilic species: *Eurotium rubrum* and *Aspergillus sp.*, 10 different genera and 22 different xerotolerant fungal spe-

![Fig. 1](image-url)
cies were isolated at the seven localities examined and six species: Aspergillus fumigatus, Cladosporium sphaerospermum, Fusarium oxysporum, Geomyces pannorum, Humicula sp., Penicillium griseofulvum and Mycelia sterilia, had frequencies of more than 10% in at least one sample. The xerotolerant species isolated at 4°C were: Botrytis cinerea, Cladosporium sp., C. sphaerospermum, Epicoccum nigrum, F. oxysporum, F. merismoides, G. pannorum and Phoma sp. At 23°C the following were isolated: Alternaria sp., A. fumigatus, Aureobasidium sp., Cladosporium sp., Gliomastix sp., F. oxysporum, F. equiseti, F. tabacinum, Humicula sp., Penicillium polonicum, P. griseofulvum, P. chrysogenum, Phialophora sp., Phoma leveillei, Ulocladium sp. and Mycelia sterilia.

For the quantification of mycobiota at different altitudes three different enumeration media were used: DRBC medium as a control for the recovery of a wide range of mesophilic species, and DG18 and M40Y, originally developed for enumeration of xerophilic fungi from dried foodstuffs [6], for isolation of xerotolerant species. The results, interpreted as the total number of CFU, were obtained using dilution and spread plating with water or 20% glucose solution than with pour plating (Fig. 1), probably due in part to the thermal inactivation of psychrotrophic fungi [10]. Dilution with 20% glucose solution, a technique that favors isolation of xerotolerant fungi [10], gave the highest yields of isolated CFU on all media used (Fig. 1). Also the majority of psychrophiles were obtained using a dilution with 20% glucose solution spread plating (data not presented). Correlation between psychrophily and xerophily was also observed with fungi isolated from the Antarctic soils [14] and from alpine environments [4].

Dilution plating techniques are designed to determine populations of viable fungal propagules per unit weight while direct plating techniques such as washing are designed for the assessment of active mycobiota from individual lumps [15]. The total number of obtained CFU with soil washing was relatively low, while the proportion of xerotolerant CFU was equal to the highest, obtained with soil water dilutions and spread plating (data not presented).

All isolation methods and media used showed a higher total number of xerotolerant CFU on the western side of the pass where strong upstream winds and even drier climate cause more intense desiccation in comparison to the eastern side (Fig. 2). A distinct profile was observed also with the total number of all isolated CFU (Fig. 2) and this is probably due to increased stressful abiotic factors at higher altitudes. With the exception of locality 3000E, a non-arid grassy meadow, all sampling sites were above the timberline with scarce vegetation. For this reason the total number of CFU at this locality stands out from the general trend towards higher numbers of CFU at lower altitudes (Fig. 2). The diversity of xerophilic species at this locality was also lower than at the same altitude on the
western side (data not presented). The lower total number and diversity obtained from 3000E was probably due to the selectivity of the isolation medium. In contrast, the reason for an extremely low number of CFU obtained from locality 5000E was the solid rocky texture of the soil, and the results can be compared with CFU obtained on rocky gravel in Antarctica [14].

With the low average temperatures in the Himalayas at these altitudes, lowering with the increase in altitude, a relatively high proportion of psychrophic fungi that grow best at temperatures below 20°C [12] was expected. Contrary to our expectation, Fig. 3 shows that the proportion of enumerated psychrophiles at the highest localities, at and above 5000 m, was less than 20% while at lower localities it arose to over 60% of the total CFU. The most important extreme-habitat-forming factor affecting the physiological adaptations of the soil mycobiota present at the Torung Pass area is the frequent variation of temperature, from −40°C to +20°C, and as a consequence, variation of the water activity of the soil that increases rapidly when snow and ice melt. It seems that in such conditions species and strains capable of adapting to rapid changes in the osmotic pressure of their surrounding are favored. The major adaptation mechanisms involved are a wide range of psychrotolerance and osmotolerance, in contrast to areas such as the dry valleys of Antarctica with permanently low temperatures and low water activities, with highly specialized psychrophilic microorganisms [14,16].

In our opinion investigating microorganisms with respect to extremes in their habitats is the best way to gain insight into different ranges of sensitivity to stressful environmental conditions.

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