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Root colonization with arbuscular mycorrhizal fungi and glomalin-related soil protein (GRSP) concentration in hypoxic soils from natural CO_2 springs

Irena Maček, Damijana Kastelec and Dominik Vodnik

University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Jamnikarjeva 101, SI-1001 Ljubljana, Slovenia

e-mail: irena.macek@bf.uni-lj.si

Changed ratios of soil gases that lead to hypoxia are most often present in waterlogged soils, but can also appear in soils not saturated with water. In natural CO_2 springs (mofettes), gases in soil air differ from those in typical soils. In this study, plant roots from the mofette area Stavešinci (Slovenia) were sampled in a spatial scale and investigated for AM fungal colonization. AM fungi were found in roots from areas with high geological CO_2 concentration, however mycorrhizal intensity was relatively low and no correlation between AM fungal colonization and soil pattern of CO_2/O_2 concentrations (up to 37% CO_2) was found. The relatively high abundance of arbuscules in root cortex indicated existence of functional symbiosis at much higher CO_2 concentrations than normally found in soils. In addition, concentration of two different glomalin-related soil protein fractions – EE-GRSP and TG-GRSP – was measured. No significant correlation between any of the fractions and soil gases was found, however the concentration of both fractions was significantly higher in the upper 0–5 cm, compared to the 5–10 cm layer of the soil.

Key words: arbuscular mycorrhizal fungi, Glomeromycota, root colonization, GRSP, glomalin, natural CO_2 springs, mofette, hypoxia, abiotic factors, extreme habitat, spatial distribution, soil fungi

Introduction

Arbuscular mycorrhizal (AM) fungi are obligatory biotrophic plant root endosymbionts, an ubiquitous functional group in soils, and are estimated to colonize around two thirds of plant species (Fitter and Moyersoen 1996). AM fungal symbiosis contributes significantly to global nutrient cycling and influences primary productivity in terrestrial ecosystems (Fitter 2005). AM fungi produce an extensive extra-radical mycelial network in soil and another AM fungal product, glomalin-related soil protein (GRSP), is transferred to soil via release from hyphae (Rillig et al. 2004, Driver et al. 2005). Glomalin has been reported to attribute a significant part, as much as 5%, of soil C (Rillig et al. 2001, Rillig et al. 2003), and to affect soil structure by increasing the stability of soil aggregates (Rillig and Mummey 2006).

Evidence is accumulating that AM fungi are subject to strong selection pressures from the abiotic soil environment, such as pH and temperature (Dumbrell et al. 2010, Dumbrell et al. 2011), and also soil hypoxia (Maček et al. 2011). Hypoxia is a stress factor that can determine the composition of native AM fungal communities and affect the functioning of symbiosis (Maček et al. 2011). Normally, soil CO_2 concentrations are about 50-times higher (up to 2% CO_2) than ambient atmospheric CO_2 concentration, and often fluctuate due to soil compaction, waterlogging, and/or vegetation (Bouma and Bryla 2000, Pfanz et al. 2004). Natural CO_2 springs (mofettes) are extreme ecosystems where soil CO_2 concentration can reach values above 80% CO_2 in the upper 10–20 cm of soil in the most extreme sites, representing a consistent environmental impact of the displacement of the soil atmosphere by CO_2, leading to localized hypoxia

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(Vodnik et al. 2006). Most of the research at natural CO_2 springs in the past has been focused on above-ground responses of vegetation (Raschi et al. 1997, Badiani et al. 1999, Vodnik et al. 2002a, 2002b, Pfanz et al. 2004, Pfanz et al. 2007). Much less work has been done on the below ground responses of plants (Maček et al. 2005) or AM fungi (Rillig et al. 2000, Maček et al. 2011). Rillig et al. (2000) reported a linear correlation between atmospheric CO_2 concentration and AM fungal root colonization, soil hyphal length, and glomalin concentration in a mofette, but discussed results mainly with respect to global atmospheric CO_2 increase and did not consider the effects of gas composition in the mofette soil. The latter has been proved as a crucial factor of plant (Vodnik et al. 2002a, 2002b, Pfanz et al. 2004, 2007) and microbial (Videmšek et al. 2009, Maček et al. 2011) performance. Despite limitations with experimentation on mofettes it has been recognized that they can be very efficiently used to study the effects of unique soil gaseous conditions on soil and plant processes. In this respect they can also serve as a model system to investigate the response of AM fungi to soil hypoxia.

The objectives of the present study were: (i) to quantify AM fungal colonization in plant roots in response to exposure to a gradient of soil CO_2/O_2 concentrations in the extreme environment of the Stavešinci mofette in a spatial pattern; and (ii) to analyze different fractions of soil GRSP when exposed to extreme soil CO_2 concentrations within this ecosystem.

Materials and Methods

Soil gas measurements and sampling

The study was conducted in 2003 in Stavešinci mofette area, NE Slovenia (for detailed description of the site see Vodnik et al. 2006, 2009b). The site is a flat post-agricultural area where geological CO_2 of ambient temperature, without traces of sulphur compounds, CH_4 or CO, is released into atmosphere via several vents (Vodnik et al. 2002a). The existing vegetation at the study site consists of C_3 and C_4 grasses, dominated by *Poa pratensis* L., *Dactylis glomerata* L., *Holcus lanatus* L., *Phleum pratense* L., *Setaria pumila* (Pior.) Roem & Schult, *Echinochloa crus-galli* (L.) PB, common grassland plants and some ruderals (Vodnik et al. 2002a, Pfanz et al. 2007). Because atmospheric CO_2 concentrations vary, depending on weather and wind conditions, from ambient to at least 1% at 0.5 m above-ground level (Vodnik et al. 2006), soil CO_2 concentration was found to be a better estimate of plant exposure to geological CO_2. Therefore, soil CO_2 and O_2 concentrations were measured by a portable gas analyzer GA2000 (Ansyco, Germany, method described in Vodnik et al. 2006) in two mofette locations, Plot 1 and Plot 2 (Fig. 1). For Plot 1 soil and root samples were taken in September 2003 in a regular sampling grid of 58 sampling points (1 m resolution) using a soil probe (Ø = 10 cm, 10 cm depth) in an area around a CO_2 vent (mofette) with soil CO_2 concentrations ranging from high CO_2 (max 37% CO_2) to ambient (control). Details on soil CO_2 and O_2 concentrations are shown in Fig. 2. Gas measurements within the Plot 1 were performed after soil sampling in December 2003 (Fig. 2). Since the soil CO_2 concentrations within Plot 1 did not reach the range of maximum concentrations that have already been measured in other studies within the Stavešinci mofette area (Vodnik et al. 2006), 29 additional soil samples were taken in a second, more extreme site (Plot 2). Samples were taken following soil air gas measurements for each sampling point in December 2003 by using a soil probe (Ø = 2 cm, in two soil depths 0–5cm and 5–10 cm) in a range of soil CO_2 concentrations from the control (< 3%) to the max (76%) (Fig.3). All samples were stored at 4 °C until further analyses. For both areas, the physical and chemical properties of the soil have been described before (Maček et al. 2009, 2011).

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Fig. 1. Natural CO_2 springs in Stavešinci mofette area. Sampling locations Plot 1 (back) and Plot 2 (front) are indicated by the oval lines.

AM fungal colonization

Roots were extracted from soil separately for each soil core within two weeks after sampling and stored in 70% EtOH. Roots were cleared with hot 10% KOH and acidified with 1N HCl. The fungal tissue inside roots was stained with 0.05% trypan blue in lactoglycerol. The AM fungal root colonization was assessed following Trouvelot et al. (1986) using an Olympus Provis AX70 microscope (n = 30 one cm root fragments from each soil core).

GRSP concentration

In each sample the soil was mixed and four replicates of 1 g subsamples were processed to extract easily extractable Bradford reactive soil protein (EE-GRSP, for terminology see Rillig 2004) and Bradford reactive soil protein after extensive extraction (TG-GRSP). The extraction method was described by Wright and Upadhyaya (1996) and Rillig et al. (2001). In short, replicate samples of sieved 1–2 mm soils were extracted with 2 ml of extractant. EE-GRSP was extracted with 20 mM citrate, pH 7.0 at 121 °C for 30 min. TG-GRSP was extracted with 50 mM citrate, pH 8.0 at 121 °C. Extraction of a TG-GRSP sample continued until the supernatant showed none of the red-brown color typical of glomalin (Wright and Upadhyaya 1996). Glomalin concentration was determined by the Bradford protein assay using 96-well plates as previously described for hyphae and soil extracts. Extracts were centrifuged at 10,000 RPM for 5 min to remove insoluble material.

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Bovine serum albumin standards were used in a range of 1.25 to 5.0 mg well^{-1}. The volume of extract tested was 50 ml, and blanks containing 50 ml of 20 mM citrate were used to correct for citrate in test samples.

Statistical analyses

Spatial distribution of soil CO_2 and O_2 concentration was presented (size of a dot in a figure corresponds to classes of measured variables). The Pearson or Spearman correlation coefficients were used as a measure of relationship between different variables. The averages of EE-GRSP and TG-GRSP concentrations in two soil layers (0–5 cm, 5–10 cm) were statistically compared using a paired t-test.

Results

Within the Plot 1 the maximum measured soil CO_2 concentration in the high CO_2 area was 36.9% (corresponding O_2 concentration 10.7%) and minimum CO_2 concentration in the control area 0.2% (corresponding O_2 concentration 17.0%) (Fig. 2). A wider range of CO_2/O_2 concentrations were measured within the second location (Plot 2), ranging from control (< 1% CO_2) up to maximum 76.1% CO_2 in soil air (Fig. 3). At both studied plots high correlation between soil CO_2 and O_2 concentration was found, as indicated by the Spearman's correlation coefficient -0.96 (Figs. 2 and 3).
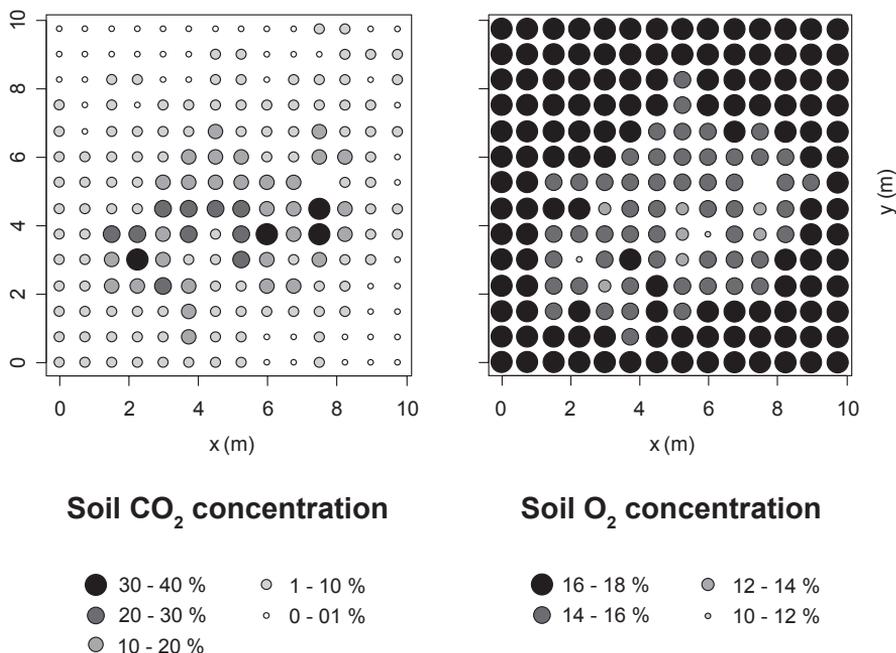

Fig. 2. The spatial pattern of soil CO_2 (left) and O_2 (right) concentrations in a 10 x 10 m area (x and y axes) in Stavešinci (Plot 1). The centre of a dot indicates the soil-gas sampling point. The sizes and the color intensity of the dots correspond to the class of soil CO_2/O_2 concentrations.
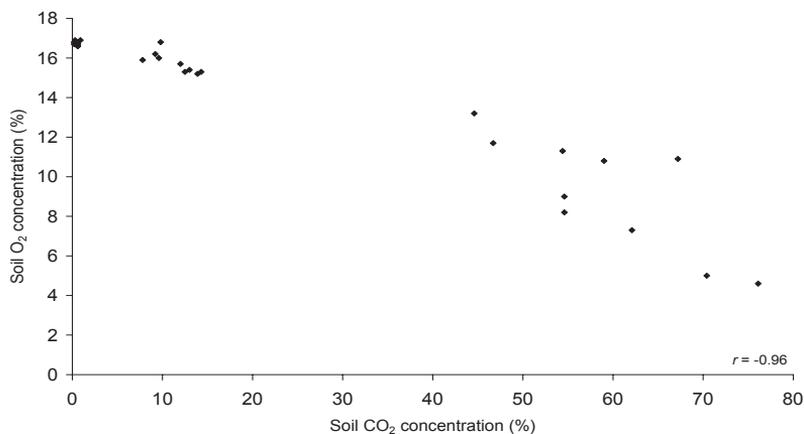
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Fig. 3. The correlation between soil CO_2 and O_2 concentrations within Stavešinci mofette Plot 2.

We found only 7% of root samples highly colonized with AM fungi (M – fungal intensity in roots > 45%) within Plot 1 (Fig. 4). M was significantly correlated with the arbuscule density (a) in the colonized root cortex (Spearman's correlation coefficient 0.53). The arbuscule density was relatively high and reached over 60% in 67% of the root samples (Fig. 4), which reflects physiological compatibility of the symbionts. No significant correlation between AM fungal colonization parameters and soil gases (CO_2 or O_2) was found.

Mycorrhizal intensity - M (%)

● 60 - 75 %	○ 15 - 30 %
● 45 - 60 %	∘ 0 - 15 %
● 30 - 45 %	

Arbuscule density - a (%)

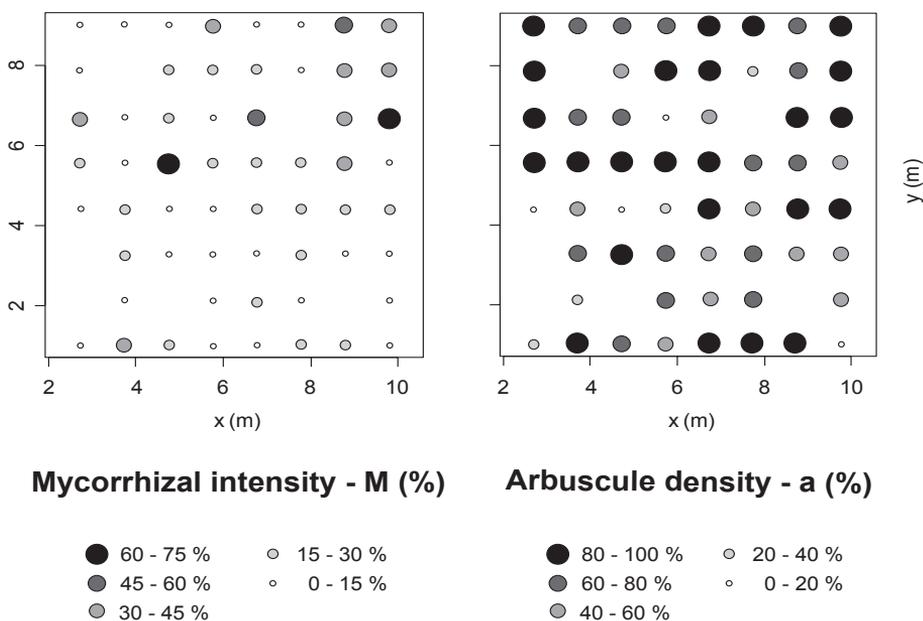| ● 80 - 100 % | ○ 20 - 40 % |
| ● 60 - 80 % | ∘ 0 - 20 % |
| ● 40 - 60 % | |

Fig. 4. Spatial presentation of AM fungal intensity (M) in roots (left), and the density of arbuscules (a) in the colonized root cortex (right) in an 8 x 8 m area (x and y axes) in Stavešinci mofette (subplot within Plot 1). The centre of a dot indicates soil core (Ø = 10 cm, 10 cm depth) sampling location. The sizes of the dots and the color intensity correspond to the class of the measured variable.

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Within the Plot 1 TG-GRSP concentrations ranged between 4.4–7.9 mg g^{-1} dry soil, and EE-GRSP concentrations between 1.1–2.1 mg g^{-1} dry soil (Fig. 5). Spearman's correlation coefficient 0.59 indicates significant correlation between both GRSP fractions, however no significant correlation between any of the GRSP fractions and soil gases (CO_2 or O_2) was found.

TG-GRSP (mg g^{-1} soil)   **EG-GRSP (mg g^{-1} soil)**

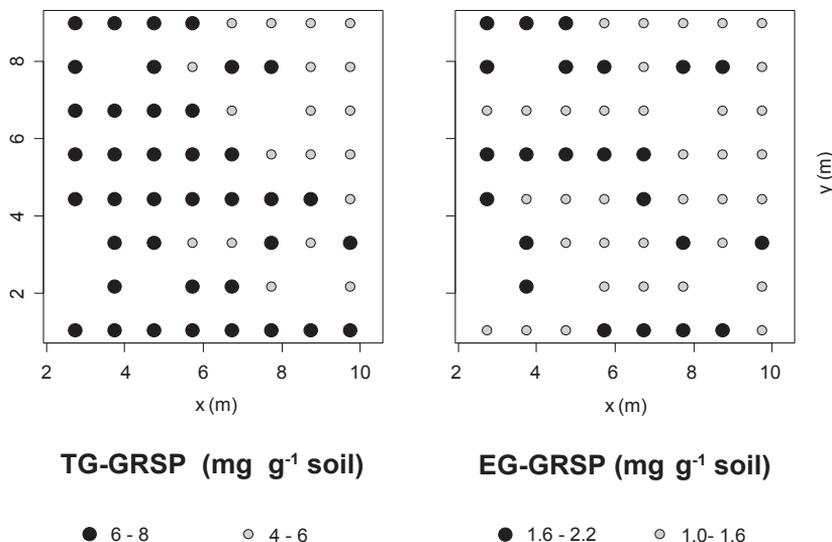- 6 - 8   ○ 4 - 6   ● 1.6 - 2.2   ○ 1.0- 1.6

Fig. 5. Spatial distribution of the soil's total TG-GRSP (left), and the easily extractable EE-GRSP (right) concentrations in an 8 x 8 m area (x and y axes) in Stavešinci mofette (subplot within Plot 1). The centre of a dot indicates soil core (Ø = 10 cm, 10 cm depth) sampling location. The sizes of the dots and the color intensity correspond to the class of the GRSP concentration.

The highest concentrations of TG-GRSP (10.6 mg g^{-1} dry soil) and EE-GRSP (4.6 mg g^{-1} dry soil) within the Stavešinci mofette area measured within this study coincided with the location (Plot 2) where the highest concentrations of geological gas were measured (76.1% CO_2 (Fig. 3)). The soil concentrations of both GRSP fractions were correlated with Pearson's correlation coefficients values 0.46 (0–5 cm soil layer) and 0.72 (5–10 cm soil layer), see Fig. 6. However, similar to Plot 1 (Figs. 2 and 5), no correlation between soil gases and any of the GRSP fractions was found.
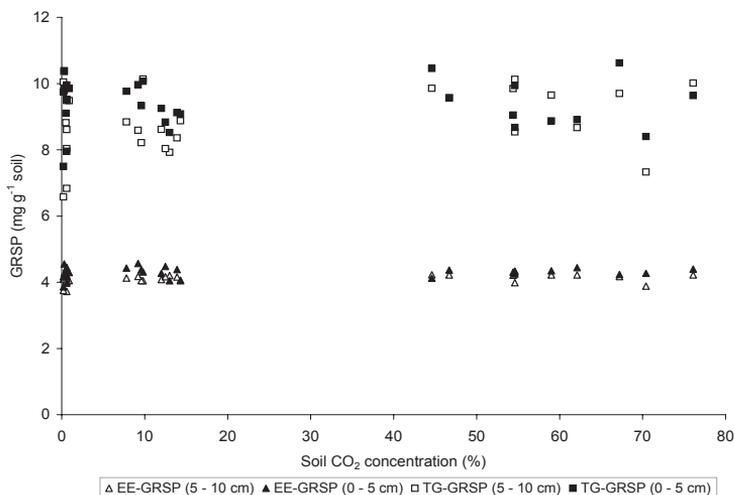

Fig. 6. The concentration of TG-GRSP and EE-GRSP in two soil depths (0–5 and 5–10 cm) and different soil CO_2/O_2 concentrations within Plot 2.

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Concentrations of EE-GRSP were significantly (p = 0.0000) higher in the upper, 0–5 cm, soil layer (4.3 ± 0.03 mg g^{-1} dry soil) compared to (4.1 ± 0.03 mg g^{-1} dry soil) in the lower 5–10 cm soil layer. In addition, significant differences (p = 0.0012) in TG-GRSP concentrations were found for both soil depths, with the average of 9.4 ± 0.14 mg g^{-1} dry soil in the 0–5 cm layer and 8.9 ± 0.19 mg g^{-1} dry soil in the 5–10 cm layer.

Discussion

This study highlights the response of AM fungal root colonization and soil concentrations of AM fungal product glomalin-related soil protein (GRSP) to a gradient of concentrations of soil gases (CO_2 and O_2) in mofette environment (natural CO_2 springs).

Soils can be often depleted in O_2 due to waterlogging and flooding. A similar situation occurs at natural CO_2 springs, where displacement of the soil atmosphere by CO_2 leads to localized hypoxia or even anoxia (Vodnik et al. 2006). In arbuscular mycorrhizas a part of the fungus (intraradical mycelium) lives in the primary root cortex, where the exchange between both partners in the symbiosis takes place, a second part extends into soil (extraradical mycelium). Root environment can be enriched in CO_2 and depleted (or enriched) in O_2, mostly due to root metabolism (Le Tacon et al. 1983) and other plant derived processes (aerenhymal tissues) (Vodnik et al. 2009a) and can differ from the gaseous conditions found in the surrounding soil. In the most extreme mofette sites, AM fungi are most probably not being supported by mycelium in surrounding soil and the root environment may play an important role in providing sufficient amount of O_2 for aerobic respiration of symbiotic fungi, which would be an obvious explanation as to how these aerobic organisms might survive in hypoxic or anoxic soils. We showed, that despite the prolonged stress and the carbon cost of colonization, no correlation between geological gas emission (CO_2 concentrations up to 37%, Fig. 2) and AM fungal root colonization was found, however mycorrhizal intensity in roots was relatively low (Fig. 4). Le Tacon et al. (1983) report on the reduced spore germination rate in the AM fungal species *Glomus mossae* in hypoxic conditions, in addition, decreasing O_2 concentration from 21% to 3% reduced hyphal growth. The same authors suggested that reduced O_2 partial pressure in the root intracellular environment, resulting from the root metabolism, could be an important factor for endophyte growth and development. The optimum concentration of O_2 for the formation of arbuscules is suggested to be 8–12%. Saif (1981) reports on the lower rate of *Glomus macrocarpus* root colonization in *Eupatorium odoratum* L. only below 2% O_2 in the atmosphere. For our study, roots were sampled at location where concentration of O_2 in soil air was still relatively high (10.7%, Fig. 2) in the most exposed site to geological CO_2, thus most probably no direct effect of soil hypoxia was present.

Some of our previous work (Vodnik et al. 2002a,b; Pfanz et al. 2007) showed the negative effect of high geological CO_2 concentration on photosynthesis and growth in mofette plants. Thus, as a significant sink for assimilated carbon, AM fungi could also be affected by a reduced carbon supply and not only by a direct influence of the soil gaseous regime. Relatively high abundance of arbuscules (Fig. 4) in plant roots from Stavešinci mofette indicates the physiological compatibility and exchange of nutrients between the symbionts also in this specific environment. Interestingly, one of our recent studies showed that distant assemblages of AM fungi are found in locations with high soil CO_2 concentrations (high CO_2) and control sites within Stavešinci mofette area (Maček et al. 2011). This was confirmed in both, sites with moderately (Plot 1) and extremely elevated soil CO_2 (Plot 2), which were the same as those used for this study. The molecular data showed that, though CO_2 concentration in Plot 1 was not as extreme as in Plot 2 (Figs. 2 and 3), the most abundant phylotype of AM fungi found in both high CO_2 sites was the same. This same AM phylotype was exclusive to high CO_2 sites and was completely absent in control sites, indicating a high level of AM fungal community turnover between high CO_2 and control sites. Thus, though AM fungal root

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colonization, as measured in this study, indicates the overall presence of mycorrhizal fungi in plant roots regardless of the soil gas regime, only studies employing molecular tools, can give more information on AM fungal communities and their composition in a certain environment.

A second parameter, indicating long-term presence of AM fungi in mofette areas, is glomalin (GRSP – glomalin-related soil protein, see Rillig (2004), a glycoprotein produced by AM fungi (Wright and Upadhyaya 1996). Standing stocks of glomalin in soil are determined by its production and decomposition and environmental factors could affect the two fluxes independently (Rillig 2004). Using Bradford reactive soil protein measurement (Wright and Upadhyaya 1996) we found relatively high amounts of GRSP (TG-GRSP up to 7.9 mg g^{-1} dry soil) in Plot 1. The TG-GRSP concentrations found in Stavešinci mofette are in the range of those found by Rillig et al. (2000) in mofette sites in New Zealand and Vodnik et al. (2008) in a FACE experiment. However, no data on soil CO_2 or O_2 concentrations are presented in the study from the New Zealand mofette site. Interestingly, the highest concentrations of GRSP (TG-GRSP 10.6 mg g^{-1} and EE-GRSP 4.6 mg g^{-1} dry soil) within the Stavešinci mofette area coincided with the location where the highest concentrations of geological gas were measured (soil CO_2 concentration 76.1% within this study, see also Vodnik et al. 2006) and corresponding O_2 concentration 4.6%, indicating soil hypoxia. Though some plants are mycorrhizal also in the most extreme areas (for Plot 2 see Maček et al. 2011) and thus some glomalin must be produced, the plant (root) biomass in those areas is small. The factor affecting glomalin accumulation in the extremely high CO_2 concentrations in soil is most probably a change in microbial decomposition. Indeed, measurements of microbial respiration and microbial biomass in soils from the Stavešinci natural CO_2 spring clearly demonstrated higher microbial respiration and higher microbial biomass in control sites compared to high soil CO_2 sites (Maček et al. 2009).

This work presents one of the few studies on AM fungi in mofette areas. We showed that despite the prolonged stress, root colonization with AM fungi also exists in sites with extreme soil CO_2 concentrations that can lead to soil hypoxia. In addition, relatively high concentrations of AM fungal product GRSP can be found in the most extreme sites in Stavešinci mofette area. A possible explanation for that is slow decomposition of GRSP in hypoxic soil.

Acknowledgements

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