Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach

F. Baptist,¹ L. Zinger,¹ J. C. Clement,¹ C. Gallet,²* R. Guillemin,¹ J. M. F. Martins,³ L. Sage,¹ B. Shahnavaz,¹ Ph. Choler¹,⁴ and R. Geremia¹

¹Laboratoire d’Ecologie Alpine, CNRS UMR 5553, Université de Grenoble, BP 53, F-38041 Grenoble Cedex 09, France.
²Laboratoire d’Ecologie Alpine, CNRS UMR 5553, Université de Savoie, F-73367 Bourget-du-Lac, France.
³Laboratoire d’études des Transferts en Hydrologie et Environnement, CNRS UMR 5564 Université de Grenoble, F-38041 Grenoble, France.
⁴Station Alpine J. Fourier, CNRS UMS 2925, Université de Grenoble, F-38041 Grenoble, France.

Summary

In alpine ecosystems, tannin-rich-litter decomposition occurs mainly under snow. With global change, variations in snowfall might affect soil temperature and microbial diversity with biogeochemical consequences on ecosystem processes. However, the relationships linking soil temperature and tannin degradation with soil microorganisms and nutrients fluxes remain poorly understood. Here, we combined biogeochemical and molecular profiling approaches to monitor tannin degradation, nutrient cycling and microbial communities (Bacteria, Crenarcheotes, Fungi) in undisturbed wintertime soil cores exposed to low temperature (0°C–6°C), amended or not with tannins, extracted from Dryas octopetala. No toxic effect of tannins on microbial populations was found, indicating that they withstand phenolics from alpine vegetation litter. Additionally at –6°C, higher carbon mineralization, higher protocatechuic acid concentration (intermediary metabolite of tannin catabolism), and changes in fungal phylogenetic composition showed that freezing temperatures may select fungi able to degrade D. octopetala’s tannins. In contrast, negative net nitrogen mineralization rates were observed at –6°C possibly due to a more efficient N immobilization by tannins than N production by microbial activities, and suggesting a decoupling between C and N mineralization. Our results confirmed tannins and soil temperatures as relevant controls of microbial catabolism which are crucial for alpine ecosystems functioning and carbon storage.

Introduction

In arctic and alpine ecosystems, seasonally snow-covered soils sequester a very large pool of organic carbon, which appears particularly vulnerable in the context of global warming (Hobbie et al., 2000). A positive feedback between increased soil respiration and rising atmospheric CO₂ has been put forward several times in global carbon balance models (Knorr et al., 2005). However, whether snow-covered ecosystems are carbon sources or sinks is still highly debated (Mack et al., 2004; Knorr et al., 2005). This is partly explained by the incomplete understanding we have of the processes involved in the wintertime heterotrophic respiration in relation to snow cover duration in cold ecosystems (McGuire et al., 2000; Monson et al., 2006). The variations of soil microbial activity in relation with the dynamic of the snow cover and litter inputs are poorly documented, though some seasonal changes in the structure and function of microbial communities in alpine soils have been described (Lipson et al., 2002; Schadt et al., 2003; Lipson and Schmidt, 2004). It has been shown that the cooler temperature at the end of the growing season triggers a marked shift to a psychrophilic microflora dominated by fungi (Lipson et al., 2002). Additionally, the microbial biomass increased sharply during wintertime. These microbial communities are able to decompose efficiently recalcitrant carbon sources, such as polyphenols, which are likely to be abundant in alpine plants tissues (Steltzer and Bowman, 2005). It is widely recognized that phenolics play a major role in nutrient cycling and litter decomposition through their multilevel interactions with mineralization processes (Cornelissen et al., 1999; Hättenschwiler and Vitousek, 2000). Aside from their toxicity towards some microorganisms, polyphenols, especially the tannin fraction, are expected to affect the availability of nitrogen to plants during their growing season, mainly through complexation.
of the organic nitrogen in soils (Kraus et al., 2003; Kaal et al., 2005; Nierop and Verstraten, 2006).

Fungus-dominated microbial communities are particularly abundant in the most constraining habitats of the alpine landscape such as exposed dry meadows (Nemergut et al., 2005). These ecosystems are dominated by slow-growing plant species — mainly Kobresia myosuroides, Dryas octopetala — and are characterized by a low net primary productivity, a high soil organic matter (SOM) content, and a limited supply of soil nutrients (Choler, 2005). Furthermore, D. octopetala produces high amounts of polyphenols, with proanthocyanins as the major tannin compounds. In dry meadows, the low and irregular snow pack leads to frequent periods where soils are frozen (< -5°C) between plant senescence and renewal. However, the impact of repeated low temperature events on both recalcitrant litter decomposition and soil functioning remains unknown. Additionally, these abiotic constraints are likely to be modified by climatic change. Recent climate scenarios for the Alps show changes in the seasonality and quantity of snow at high altitude, i.e. above 2000 m (Beniston, 2003; Keller et al., 2005). The predicted decrease in precipitation between autumn and early spring will most likely reduce the winter snow-covered period of alpine dry meadows, consequently, increasing the length of the soil freezing period. It is not known to what extent these changes will affect the wintertime decomposition of organic matter, recalcitrant compounds in particular.

In this study, we focused on the combined effect of low temperature (< 0°C) and the input of recalcitrant compounds on alpine soil functioning. We expected a shift in microbial communities as a consequence of changes in these two ecological factors during the late-fall critical period. We set up an incubation experiment with soil cores under laboratory conditions to disentangle the effects of temperature and tannin addition on the diversity of microbial communities and the carbon and nitrogen cycles. More specifically, we addressed the following questions: how does tannin input affect (i) carbon and nitrogen mineralization and (ii) overall soil bacterial and fungal phylogenetic structures? (iii) how are these functional and phylogenetic responses are modulated by a prior treatment at freezing temperature (−6°C)?

We simulated a late-fall litter flux by adding tannins extracted from D. octopetala leaves to soil cores collected in dry meadows during the fall, and we mimicked the snow-pack reduction by a freezing treatment. We monitored the C and N soil dynamics (including tannin evolution) and assessed the microbial soil diversity through rRNA genes (16S rRNA gene for prokaryotes, ITS for fungi) using molecular profiling [single-strand conformation polymorphism (SSCP)] in addition to classical microbial techniques.

Results

Impact of tannin on structure and metabolism of microbial populations were addressed in an incubation experiment with soil cores under laboratory conditions. Four treatments were applied (n = 3). In W/S and T/S treatments, soil cores were amended, respectively, with water and tannins extracted from D. octopetala leaves, and they were all maintained at 0°C during 45 days. In W/F and T/F, soil cores were also amended with water and tannin solution respectively, but then were stored at −6°C during 15 days (day 15) and kept at 0°C for four more weeks.

Phenolic metabolism

At day 15, more than 10% of the added tannins were recovered from the soil samples, 12% for S/T (Stable/Tannin treatment) and 17% for F/T (Freezing/Tannin treatment) (non-significant difference, U = 2.5, P = 0.376). After 45 days, both temperature treatments had a recovery fraction of around 5%. In treatment W, no tannins were detected in the soils at days 15 and 45, while they were present in low but detectable amounts at day 0. When comparing the phenolic acids, significantly higher levels of protocatechuic acid (last aromatic degradation metabolite before ring fission) were observed in the treatment T, than in the treatment W, irrespective of temperature and sampling times (Fig. 1). The accumulation of this degradation product was higher in the F/T treatment than in the S/T treatment for both dates, indicating a better metabolizing of the tannins in soils submitted to the freezing treatment. Similar patterns were observed for other phenolic acids: vanillic and p-hydroxybenzoic acids (data not shown).

Changes in microbial biomass and diversity

Microbial and fungal biomasses and bacterial counts were not significantly affected by temperature or tannin amendment (Table 1). However, these treatments affected microbial diversity differently. The F/T and S/W treatments had the strongest impact on the bacterial communities (Fig. 2A). Moreover marked differences between day 15 and day 45 are supported for all the treatments except for F/W.

For crenarchaeotes, we observed the formation of new SSCP peaks for all the treatments (data not shown). The F/T treatment had a contrasted effect on the structure (peak distribution) of the crenarchaeote communities compared with other conditions (data not shown) as there were less peaks, which suggests the dominance of few phyotypes.

Diversity of fungal communities was significantly affected for all conditions and especially for S/T and F/W. The SSCP profile of day 45 (F/T) was an outlier because
the data file containing the migration value for statistical analysis was corrupted. The longest distance corresponded to the F/W cores, which showed fewer peaks than the other treatments (Fig. 3D). The day 0 and S/W profiles (Fig. 3A and B) had more than 10 peaks. The S/T profiles (Fig. 3C) presented a low signal, but as many peaks as S/W and day 0. A broad analysis of the raw data for S/T revealed that the baseline increased, which may indicate the co-migration of numerous fungal phylotypes (Loisel et al., 2006). For all profiles, the predominant phylotypes became relatively more abundant between days 15 and 45. Moreover, the F/T profiles presented more peaks than the S/T ones (Fig. 3E). These results suggest that tannins prevented the loss of fungal phylotypes due to freezing.

**Impact on carbon mineralization**

Between days 0 and 15, the total CO₂ efflux measured with the F treatment was significantly lower (approximately two- to fourfold) than with the S treatment (Table 2). Although marginally significant, the tannin treatment (F/T) led to an increase (approximately twofold) in CO₂ efflux between days 0 and 15 compared with the F/W treatment. However, the total CO₂ efflux at 0°C was not affected by the presence of tannins.

On day 15, when the temperature shifted from -6°C to 0°C, the CO₂ efflux doubled from soils of F/T treatment and increased fourfold in the F/W treatment. Between days 15 and 45, no additional differences were detected between the treatments with and without tannins (Table 2). These results indicated that tannins enhanced the CO₂ efflux only with the freezing treatment between days 0 and 15.

**Impact on nitrogen cycling**

The nitrogen dissolved in the soil extracts was mainly in organic forms [-529.6–1416.7 μg N g⁻¹ dry weight (dw), 90.4–99.6% of total dissolved nitrogen (TDN)], while ammonia (-3.2–67.1 μg N g⁻¹ dw, 0.3–8.7% of TDN) and nitrate/nitrite (0.1–10.2 μg N g⁻¹ dw, 0.0–1.3% of TDN) made up smaller proportions of the TDN. Total dissolved nitrogen and dissolved organic nitrogen (DON) soil contents were changed neither by the temperature (F versus S treatments, data not shown) nor by the addition of tannins (T versus W treatments). Within S treatment, net N mineralization rates between days 15 and 45 were not influenced by the presence of tannins, whereas they were significantly reduced in soils previously stored at -6°C (F treatment, Fig. 4A). This effect was even stronger in soils in the F/T treatment, for which we measured net N immobilization values suggesting that the production of inorganic N was not sufficient to compensate for its disappearance.

Net mineralization potentials (NMP) measured on soil subsamples at days 0, 15 and 45 were 10–400 times higher in the F/W treatment than in the S/W treatment (Table 1). The NMP at day 15 was significantly lower in the F/W treatment than in the S/W treatment, and it increased significantly between days 15 and 45 in the F/W treatment but not in the S/W treatment (Table 1). Differences between day 15 and day 45 were tested by Wilcoxon signed rank test (upper case, P < 0.05) and differences between treatment (temperature or tannin amendment) by Mann–Whitney rank sum test (lower case, P < 0.05). n = 3 ± standard error of the mean.
higher than the net N mineralization measured between days 15 and 45 (Fig. 4B and C). Yet, NMP increased during the incubation in the case of the F/W treatment but not in the F/T treatment. On days 15 and 45, NMP from soils amended with tannins were significantly lower than for the unamended ones (Fig. 4B). In treatment S, soils incubated with tannins (S/T) had also lower NMP than on soils amended with water (S/W) but only on day 15 (Fig. 4C). Net mineralization potentials were therefore strongly reduced in F/T treatment compared with the others.

Discussion
In our study, the role of tannins was evaluated through the combination of biogeochemical analyses with molecular profiling approach. The few other studies which examined the impact of polyphenols through purified tannin addition focused on forested ecosystems, characterized by faster nutrient cycling and higher productivity (Bradley et al., 2000; Fierer et al., 2001). Furthermore, unlike those studies, we used undisturbed soil cores instead of composite and homogenized samples to maintain the vertical stratification and its associated physical and microbiological properties.

Impact on carbon and nitrogen cycles
The organic and inorganic nitrogen soil concentrations measured in the alpine soils, as well as the dominance of organic N forms, were in accordance with the literature (Tosca and Labroue, 1986; Lipson et al., 1999; Zeller et al., 2000). Similarly, CO₂ efflux measurements were within the range of previous observations (Leifeld and Fuhrer, 2005; Schimel and Mikan, 2005). After 1 month, only a minor fraction of the added tannins was recovered from the amended soils. The disappearance of tannins could be explained either by degradation or by insolubilization, due to complexation with proteins or adsorption on organo-mineral soil fractions (Kaal et al., 2005; Nierop and Verstraten, 2006).

In stable treatment, tannin addition had limited effects on C and N mineralization as well as on microbial biomass indicating (i) that tannins were not used as a significant extra C source and (ii) that they did not inhibit microbial communities. The slight increase in protocatechuic acid 802

Table 2. Soil water content, daily mean CO₂ efflux over the days 0–15 and days 15–45.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Soil water content (%)</th>
<th>CO₂ efflux (µg C g⁻¹ C day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0-T15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/W</td>
<td>33.4 (1.0)</td>
<td>318.8 (70.4)</td>
</tr>
<tr>
<td>F/W</td>
<td>34.8 (3.8)</td>
<td>76.7 (7.8)</td>
</tr>
<tr>
<td>S/T</td>
<td>37.6 (4.1)</td>
<td>267.9 (71.9)</td>
</tr>
<tr>
<td>F/T</td>
<td>30.9 (4.5)</td>
<td>128.6 (19.2)</td>
</tr>
<tr>
<td>T15-T45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/W</td>
<td>33.2 (1.2)</td>
<td>281.3 (131.0)</td>
</tr>
<tr>
<td>F/W</td>
<td>34.9 (3.1)</td>
<td>269.4 (51.5)</td>
</tr>
<tr>
<td>S/T</td>
<td>39.1 (4.2)</td>
<td>210.7 (23.7)</td>
</tr>
<tr>
<td>F/T</td>
<td>34.0 (4.7)</td>
<td>273.8 (94.5)</td>
</tr>
</tbody>
</table>

The differences between days 15 and 45 within each treatment (upper case, P < 0.05) were tested by Wilcoxon signed rank test and the differences between treatment (temperature or tannin addition) within each period (lower case, P < 0.05) were tested by Mann–Whitney rank sum test. n = 3 ± standard error of the mean.
Fig. 3. Capillary electrophoresis-SSCP profiles of fungal communities for each treatment. A = day 0; B = S/W; C = S/T; D = F/W; E = F/T. Black lines: day 15; grey lines: day 45. All profiles are displayed for an arbitrary fluorescence intensity interval of 4000.

Fig. 4. A. Net N mineralization rates between days 15 and 45 in soils for F and S treatments amended with sterilized water (white bars) or a tannin solution (black bars). B and C. Net N mineralization potentials in soils after 0, 15 and 45 days of incubation in the F treatment (B) and the S treatment (C), amended with sterilized water (white bars) or a tannin solution (black bars). Mann–Whitney rank sum tests were performed to test for differences between treatment (temperature or tannin addition) within each period (lower case, \( P < 0.05 \)). \( n = 3 \pm \) standard error of the mean.
indicated only weak tannin degradation. This suggests that tannins were preferentially adsorbed on organo-mineral soils or complexed with decomposing organic compounds (Fierer et al., 2001). But this hypothesis, largely mentioned in the literature, is hypothetical as efficient methods to quantify such insoluble compounds are missing (Lorenz et al., 2000; Kanerva et al., 2006). Also, we did not assess the impact of a prolonged exposure to tannins as it occurs in natural habitats, but rather try to mimic a pulse of tannins corresponding to the litter fall period. Thus, it should not be considered as a long-term effect.

The incubation of alpine soils at –6°C led to a decrease in C mineralization between day 0 and day 15. Lower temperatures strongly affect soil processes by lowering microbial enzymatic activities (Schimel et al., 2004) but do not affect microbial biomass as already shown by others authors (Lipson et al., 2000; Groffman et al., 2001; Grogan et al., 2004). Surprisingly, higher CO₂ effluxes were detected in F/T than in F/W treatments and higher concentrations of protocatechuic acid in F/T than in F/W treatments and higher concentrations of protocatechuic acid in F/T than in S/T treatments in the first step of the experiment. These results indicate a higher tannin degradation at –6°C. Added to limited microbial growth, this suggests that some microorganisms may be able to use this C source, unlike the populations present at 0°C (S/T). However, the lack of data on N mineralization between day 0 and day 15 prevents us from drawing further conclusions.

From day 15 to day 45, the only significantly affected process was net N mineralization, which decreased in F/W-treated alpine soils. This confirms that a prolonged pre-period of frost slowed down N cycling in alpine soils (Schimel et al., 2004). The reduction of metabolic activities in the soil microbial community may be responsible for this effect. The addition of tannins in our alpine soils amplified the freezing effect on net N mineralization described previously and even led to apparent N immobilization (F/T, Fig. 4A). Possibly, the complexing capacity of tannins may have been detected only when microorganisms were less active due to freezing, suggesting that the kinetics of N mineralization by living microorganisms was faster than that of abiotic N immobilization by tannins. Both processes occurred in the stable treatment, but N microbial mineralization was dominant and microorganisms transformed N₉org into NH₄⁺ and NO₃⁻ in much larger quantities than could be complicated by tannins. As a result, we measured no tannin effect on net N mineralization in soils for the S treatment (measured as the amount of NH₄⁺ and NO₃⁻ produced).

In F treatment, the reduction of microbial activities reduced the production of NH₄⁺ and NO₃⁻, which did not compensate for the complexation by tannins (Fierer et al., 2001; Castells et al., 2003). In our experiment, this was perceived as a negative net N mineralization or an apparent NH₄⁺ and NO₃⁻ immobilization. This hypothesis is further supported by NMP results (at 30°C) which were significantly lower in soils amended with tannins, most probably due to complexation of organic compounds (Fierer et al., 2001).

The high concentration of protocatechuic acid and C mineralization during the first step of freezing treatment suggests that the added tannins were metabolized despite very low temperatures. C mineralization was strongly affected by the temperature shift and no long-term effect of tannin addition was detected, possibly due to the shortage of easily decomposable tannin (Kraus et al., 2004). The absence of relationship between N immobilization and C mineralization between days 15 and 45 suggests a decoupling between both processes, as reported by Mutabaruka and colleagues (2007). This is probably because N immobilization is driven by both abiotic and biotic factors, whereas C mineralization depends on biotic controls.

**Impact on microbial diversity**

There have been several studies of microbial diversity fingerprints for bacterial communities in mesocosm experiments (Hewson et al., 2003; Hendrickx et al., 2005; Lejon et al., 2007), but none, in alpine soils, were carried out on the three main groups of microorganisms as we did here. We determined three distinct patterns, one for each microbial community. Previous studies showed that the bacterial SSCP patterns are specific for a given bacterial community (Godon et al., 1997; Mohr and Tebbe, 2006; Zinger et al., 2007a). Here, the bacteria profiles showed a high baseline suggesting a large number of rare phylotypes (Loisel et al., 2006), preventing the detection of minor changes. We found effects supported by bootstrapping for most treatments. However, because of the high baseline masking the community shifts (Fig. 3), the branch length remained very short between treatments (Fig. 2). Therefore, minor relevant changes in bacterial diversity cannot be detected and a more detailed study is needed to assess the tannin impact on bacterial communities.

For the crenarcheotes, freezing and tannin amendment resulted in a reduction in the number of peaks. Possibly, the convergence of both factors led to the disappearance of some crenarcheote phylotypes. However, the effects on nutrient cycling were likely to be negligible, as no decrease in population biomass or in C mineralization was detected.

Fungal communities, whose biomasses were found to increase during winter (Schadt et al., 2003), showed strong responses to all treatments. Tannin amendment associated with low temperature maintained a relatively high diversity whereas freezing temperatures alone led to a decrease in fungal richness. This result suggests that
some wintertime fungal strains may be able to benefit from the addition of tannins, as it has been already shown for bacterial communities (Chowdhury et al., 2004) or fungal populations (Mutarbukra et al., 2007). Previous studies on alpine meadows also suggested that a strong supply of allelochemical-rich litter in the fall may select wintertime populations able to grow on phenolic compounds (Lipson et al., 2002; Schmidt and Lipson, 2004). Unexpectedly, the phylotypes reacted differently in response to the addition of these compounds, depending on the thermic regime. This interaction may be related to the presence of psychrophilic fungi which were excluded at relatively high temperature. Another explanation is that the available labile C, which decreased at lower temperatures, created a selective pressure in favour of fungal strains which metabolize more resistant C substrates (Bradley et al., 2000).

**Ecological implications**

CO₂ efflux measurements showed that there were significant levels of microbial activities even well below 0°C (Brooks et al., 1998). In snow-covered ecosystems, litter decomposition occurs principally during the winter (Hobbie and Chapin, 1996) and recent studies indicate that winter microbial communities degrade phenolic compounds (vanillic and salicylic acids) better than summer microbial communities (Schmidt et al., 2000; Lipson et al., 2002). However, because of inconsistent snow cover during winter, dry meadows frequently experience very low temperatures (< −5°C) reducing soil microbial activity and litter decomposition rates (F. Baptist, unpubl. results, O'Lear and Seastedt, 1994). Furthermore, high concentrations of tannins in the fresh litter of *D. octopetala*, which is a dominant species in this ecosystem, potentially contribute to a decrease in N mineralization by complexing soil organic compounds (Northup et al., 1995; Hattenschwiler and Vitousek, 2000). Severe soil edapho-climatic conditions probably act by inhibiting microbial activity. However, we detected no toxic effects of compounds extracted from *D. octopetala* on microbial activity which indicates that plants producing phenolic compounds may select microbial populations able to use these compounds, or at least able to withstand them (Schmidt et al., 2000). Changes in phylogenetic composition coupled with higher C mineralization and protocatechuic acid contents showed that freezing temperatures selected psychrophilic fungi. These may be able to degrade *D. octopetala*’s tannins, and their activities are potentially initiated by a decrease in temperature. However, this particular effect of temperature remains unclear and could also be related to a decrease in labile C availability.

This study illustrates how soil and climatic conditions interact with soil microorganisms to enhance the metabolism of the tannins released by the plants which dominate alpine ecosystems. The degradation of recalcitrant compounds, during winter, produces a less recalcitrant litter which becomes available by the time plant growth starts. This limits nutrient immobilization thanks to a reduced litter C/N ratio (Schmidt and Lipson, 2004). Consequently, the microbial catabolism of these compounds during winter is of functional importance. A variation in snowfall might affect microbial functional diversity with cascading biogeochemical consequences on ecosystem processes and carbon sequestration. Nevertheless, further investigations remain necessary to identify the exact role of microorganisms in tannin catabolism and their vulnerability to climate change.

**Experimental procedures**

**Field site**

The study site was located in the Grand Galibier massif (French south-western Alps, 45°0.05’N, 06°0.38’E) on an east facing slope at 2520 m. The growing season lasts around 168 ± 6 days and the mean soil temperature is 7.7 ± 1.5°C in summer and −2.2 ± 1.7°C in winter. The mean soil temperature reaches very low values (< −5°C) during relatively long periods because of inconsistent snow cover. Dry meadow soils are classified as typical alpine rankers. The bedrock is calcareous shales. The dominant plant community in the field site consist mainly of *K. myosuroides* (Cyperaceae) and *D. octopetala* (Rosaceae). Fifteen soil cores were sampled in October 2005 using sterilized (ethyl alcohol 90°) PVC pipes (h = 10 cm, d = 10 cm) and tools, to avoid contamination. In the laboratory, the plants were separated from the soil cores which were covered with perforated plastic bags and stored at 0°C until the beginning of the experiment.

**Experimental design**

On day 0, three soil cores were destructively harvested and used as controls (Table 3), six soil cores were amended with 19 ml of a tannin solution (with a mean of 749 mg of C/core or 32.4 mg C g⁻¹ soil C, tannin treatment, T) and six cores with sterilized water (water treatment, W) to reach similar gravimetric soil moisture contents (34.2 ± 1.7% and 34.1 ± 1.6% for cores amended with tannins and water respectively). Three W cores and three T cores were incubated at −6°C for 2 weeks (freeze treatment, F) and then at 0°C for four more weeks. The six remaining cores were kept at 0°C (stable temperature treatment, S) during the whole period. To limit temperature gradients inside the incubators, the soil cores were regularly rotated. At the end of the first period (day 15), half of each soil core (3 S/T, 3 S/W, 3 F/T, 3 F/W) was harvested for a first analysis (longitudinal section). To limit disturbance, the harvested soil was replaced by a sterile and closed bag full of sand. The remaining soil cores were placed back in the incubator for four more weeks at 0°C and then were harvested for final analysis.
Table 3. A. Soil characteristics of the cores. B. Initial parameters estimated on the three control soil cores.

(A) Soil characteristics

| Soil water content (%) | 32.9 (3.7) |
| Bulk soil density on < 2 mm fraction (g cm⁻³) | 0.24 (0.04) |
| Organic matter (%) | 16.9 (4.3) |
| pH (H2O) | 5.1 (0.1) |
| pH (KCl) | 4.1 (0.1) |

Grain size analysis
- Clay (< 2 μm) | 9.7 (0.5)
- Silt (2–50 μm) | 41.4 (1.0)
- Sand (50–2000 μm) | 48.6 (1.2)

(B) Initial parameters (T₀)

- Microbial biomass (mg C g⁻¹ C) | 170.6 (37.3)
- Bacterial count (10⁶ cells g⁻¹ C) | 1.45 (0.21)
- Fungal biomass (μg ergosterol g⁻¹ C) | 130.2 (14.0)
- Tannin (mg g⁻¹ C) | 0.30 (0.10)
- NO₃ (μg N g⁻¹ soil) | 0.10 (0.02)
- NH₄ (μg N g⁻¹ soil) | 3.25 (0.10)
- Nₑₙ (μg N g⁻¹ soil) | 914.2 (65.7)

Potential mineralization
- NH₄ production (μg N g⁻¹ soil day⁻¹) | 12.1 (2.9)
- Nₑₙ production (μg N g⁻¹ soil day⁻¹) | 145.9 (40.9)

n = 3 ± standard error of the mean.

At each sampling time (day 0, day 15 and day 45), the soils were sieved (2 mm) and further analysed to determine the tannins and phenolic acid contents, microbial and fungal biomasses, bacterial counts, microbial diversity and nitrogen mineralization rates. The CO₂ efflux was measured between each harvest.

Soil water content, pH_HCl, pH_KCl, bulk soil density and texture were determined following standard methods (Robertson et al., 1999). The SOM content was determined by loss-on-ignition and the C mass was calculated by dividing SOM fraction by 1.72. In order to determine bulk soil density, the stones mass was determined and converted to stone volume using mean stone density of 2650 kg m⁻³ (Hillel, 1971).

Tannin extraction and phenolic analysis

Dryas octopetala leaves were collected at the end of July, and air-dried. Tannins were extracted from about 300 g of ground leaves, using liquid sequential extractions and a final purification on Sephadex LH-20 (Preston, 1999). The elemental composition of the dried final fraction was obtained by CHN analysis (C %: 62.4; N %: 0). The addition of tannins was performed with a solution of 15.85 g of purified tannins dissolved in 250 ml of distilled water. Proanthocyanins (here after referred to tannins) were quantified in the soil extracts by spectrophotometry, after hydrolysis with butanol/HCl using the proanthocyanidin assay (Preston, 1999). The calibration curves were prepared with a previously purified proanthocyanin fraction from D. octopetala. Phenolic acids were obtained (5 g FW) by a double ethanolic extraction (ethanol 70%) under reflux. Aliquots (20 μl) of the ethanolic solution filtered at 0.5 μm, were used for high-performance liquid chromatography (HPLC) analysis on a RP C18 μBondapak column (4.6 mm × 250 mm) monitored by a Waters 600 Controller with a UV detection at 260 nm (Waters 996 PDA). Phenolic acids were separated using a linear gradient from 0 to 20% of solvent B (acetonitrile) in solvent A (acetic acid 0.5% in distilled water) in 45 min, at 1.5 ml min⁻¹. Standards of common phenolic acids (including protocatechuic) were obtained from Sigma-Aldrich (L’Isle d’Abeau, France).

Nitrogen mineralization

Nitrogen was extracted from fresh soil samples with 2 M KCl. The soil extracts were analysed for ammonia (NH₄⁺) and nitrate/nitrite (NO₃⁻/NO₂⁻) contents using an FS-IV autoanalyser (OI-Analysis, College Station, TX). The TDN content in the soil extracts was measured after oxidation with K₂S₂O₈ at 120°C. The DON contents in the soil extracts (μg N g⁻¹ dw) were calculated as: [DON = TDN – (N-NH₄⁺) + (N-NO₃⁻/NO₂⁻)]. The net nitrogen mineralization (MINnet) was calculated between day 15 and day 45 was calculated as: MINnet = [(N-NH₄⁺) + (N-NO₃⁻/NO₂⁻)]day15 – [(N-NH₄⁺) + (N-NO₃⁻/NO₂⁻)]day45 / dw / 30. MINnet was not calculated between day 0 and day 15, because the day 15 did not originate from the same soil cores as those for day 0. The NMP was determined from subsamples, using anaerobic incubations (Waring and Bremner, 1964). This protocol allows comparisons of relative organic matter degradability in different soils. Under optimized conditions (dark, 7 day, 30°C, anaerobic) organic N in fresh soils was mineralized and accumulated as NH₄⁺. The difference between the NH₄⁺ in the fresh soil (t₀) and after the anaerobic incubation (tₜ) gave the N mineralization potential: NMP (mg N-NH₄⁺ g⁻¹ dw day⁻¹) = [(N-NH₄⁺)t₀ – (N-NH₄⁺)tₜ] / dw / 7.

Soil CO₂ efflux

Throughout the experiment, CO₂ efflux measurements were conducted just after tannin amendment (day 0), one before temperature shift (day 15) and three times between days 15 and 45 on all soil cores. The cores were enclosed in a hermetic Plexiglas™ chamber equilibrated to 400 p.p.m. prior to measurements. The chamber was connected to a LiCor 6200 gas exchange systems (LiCor, Lincoln, NE, USA). Data recording lasted 3–5 min, depending on the signal fluctuations, and the soil temperature was monitored.

Microbial community analyses

Microbial biomass and ergosterol determination. Microbial carbon biomass was determined by the fumigation-extraction method (Jocteur Monrozier et al., 1993; Martins et al., 1997) adapted from Amato and Ladd (1988). Duplicated soil samples (10 g) were fumigated for 10 days with chloroform. Total organic nitrogen was extracted with 20 ml of 2 M KCl from both the non-fumigated and fumigated soil samples (T₀, Tₜ₀), microbial nitrogen biomass being determined from the difference between the two treatments. After reaction with ninhydrin, the absorbance (570 nm) of all samples was deter-
mined by spectrophotometry using leucin as standard. The microbial carbon biomass calculated using a conversion factor of 21 (Amato and Ladd, 1988; Martins et al., 1997). The soil ergosterol content was evaluated as an indirect estimate of the soil fungal biomass (Nylund and Wallander, 1992; Gors et al., 2007). Ergosterol was extracted from 5 g of soil (FW) with 30 ml of 99.6% ethanol by shaking for 30 min at 250 r. p.m. The soil solution was filtered and immediately submitted to HPLC under isocratic flow of 1.5 ml min⁻¹ of MeOH, on a Lichrosorb RP18 column (250 × 4.6 mm, 5 μm). Calibration curves at 282 nm were recorded with standard ergosterol solution from Sigma-Aldrich (L’Isle d’Abeau, France).

Bacterial counts. The soil bacterial counting was conducted using the method described by Martins and colleagues (1997). Briefly, 10 g of soil (duplicated) was blended in 50 ml of sterile NaCl 0.9%. After flocculation of the soil particles, an aliquot of the soil suspension (1 ml) was collected and used to enumerate the bacteria after successive dilutions. One millilitre of the diluted suspension was filtered on 0.2 μm polycarbonate membrane filters (Millipore). Bacteria were then stained using a sterile solution (filtered at 0.2 μm) of 4′,6-diamidino-2-phenylindole (DAPI) and enumerated by direct counting with a motorized epifluorescent microscope (Axioskop, Zeiss) under UV excitation (Hg lamp) with a filter set for DAPI (365 nm) at 1000-fold magnification.

SSCP analysis of microbial diversity. DNA extraction and PCR: the protocols for fungal and prokaryotic signatures have already been described in Zinger and colleagues (2007a,b). Briefly, the soil DNA was amplified using microbial community-specific primers and submitted to capillary electrophoresis-SSCP (CE-SSCP). The 16S rRNA gene was used as the prokaryotic specific marker. The bacterial primers were W49 and W104-FAM (Zumstein et al., 1990). The soil DNA extraction was performed using the Aperio extraction protocol (Ozyme, St Quentin en Yvelines, France) using 250 mg (fresh weight) of soil per core sample, according to manufacturer’s instructions. The DNA extracts from the same-condition cores were then pooled to limit the effects of soil spatial heterogeneity. The PCR reactions (25 μl) were set up as follows: 2.5 mM of MgCl₂, 1× of AmpliTaq Gold™ buffer, 20 g l⁻¹ of bovine serum albumin, 0.1 mM of each dNTP, 0.26 μM of each primer, 2 U of DNA polymerase (Applied Biosystems, Courtaboeuf, France) and 1 μl of DNA (1–10 ng DNA). The PCR reaction was performed as follows: an initial step at 95°C (10 min), followed by 30 cycles at 95°C (30 s), 56°C (15 s) and 72°C (15 s), and final step at 72°C (7 min). The PCR products were visualized on a 1.5% agarose gel. Then, amplicons of each microbial community were then pooled for each sample to perform multiplex CE-SSCP. Capillary electrophoresis-SSCP: 1 μl of the PCR product was mixed with 10 μl formamide Hi-Di (Applied Biosystems, Courtaboeuf, France), 0.2 μl standard internal DNA molecular weight marker Genescan-400 HD ROX (Applied Biosystems, Courtaboeuf, France), and 0.5 μl NaOH (0.3 M). The sample mixtures were denatured at 95°C for 5 min and immediately cooled on ice before loading on the instrument. The non-denaturing polymer consisted of 5% CAP polymer, 10% glycerol and 3100 buffer. Capillary electrophoresis-SSCP was performed on an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems, Courtaboeuf, France) using a 36-cm-long capillary. The injection time and voltage were set to 22 s and 6 kV. Electrophoresis was performed for 35 min. The CE-SSCP profiles were normalized in order to control for differences in the total fluorescence intensity between profiles.

Statistical analysis. We tested for differences between the temperature and tannin amendment treatments using Mann-Whitney rank sum test (P < 0.05) (Statistica 5.0, Statsoft, (1995) Statistica 5.0 Software. Statsoft, Tulsa, USA). Paired differences between days 15 and 45 sampling were tested using the Wilcoxon signed rank test (P < 0.05). The normalized profiles of SSCP were analysed by Neighbour-Joining analysis based on a matrix of Edwards distances (Edwards, 1971). The robustness of the resulting tree was assessed using 1000 bootstraps. The data analysis was performed using the Ape package of the R software (RDevelopment-CoreTeam, 2006).

Acknowledgements

We gratefully acknowledge Olivier Alibec, Mathieu Buffet, Alice Durand, Niu Kechang, Claire Molitor and Tarafa Mustafa for their help in the field and in the laboratory. Logistical supports were provided by the ‘Laboratoire d’Ecologie Alpine’ (UMR 5553 CNRS/IJUF, University Joseph Fourier) and the ‘Station Alpine Joseph Fourier’, the alpine field station of the University Joseph Fourier. This study was supported by CNRS and MicroAlpes project funding (ANR). We thank Sophie Rickebusch, Stéphane Reynaud and Michel Tissut for their helpful comments on an earlier version of this manuscript. We also thank the technical platform MOME of the Enviroional Pole, Rhône-Alpes Region.

References


Chowdhury, S.P., Khanna, S., Verma, S.C., and Tripathi, A.K. (1971). The robustness of the resulting tree was assessed using 1000 bootstraps. The data analysis was performed using the Ape package of the R software (RDevelopment-CoreTeam, 2006).

Acknowledgements

We gratefully acknowledge Olivier Alibec, Mathieu Buffet, Alice Durand, Niu Kechang, Claire Molitor and Tarafa Mustafa for their help in the field and in the laboratory. Logistical supports were provided by the ‘Laboratoire d’Ecologie Alpine’ (UMR 5553 CNRS/IJUF, University Joseph Fourier) and the ‘Station Alpine Joseph Fourier’, the alpine field station of the University Joseph Fourier. This study was supported by CNRS and MicroAlpes project funding (ANR). We thank Sophie Rickebusch, Stéphane Reynaud and Michel Tissut for their helpful comments on an earlier version of this manuscript. We also thank the technical platform MOME of the Envirional Pole, Rhône-Alpes Region.

References


Chowdhury, S.P., Khanna, S., Verma, S.C., and Tripathi, A.K.


