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Forestry Research Methods

P. PAVLOVIĆ*, L. DJURDJEVIĆ, S. JARIĆ, O. KOSTIĆ, D. PAVLOVIĆ,
M. PAVLOVIĆ and M. MITROVIĆ

Department of Ecology, Institute for Biological Research "Siniša Stanković",
University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
E. Mail: ppavle62@yahoo.com

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ABSTRACT

Field studies to test the effects of natural levels of allelopathic compounds on coexisting native species are rare because allelopathic effects are difficult to quantify and evaluate under the field conditions. Field studies are conducted to demonstrate the effects of allelochemicals on ecosystem-level (through effects on litter decomposition, activity of decomposers, soil C and N cycling, soil humus, soil nutrients availability, soil water relation and soil respiration, plant mycorrhizal fungi and plant pathogens) and also to test allelopathic potential of allelochemical compounds from fresh leaves, leaf litter and soil of donor species on individuals of the same species (autotoxicity) and on coexisting species (growth, photosynthesis, respiration, chlorophyll and water relations of target plants) in forest community, through simultaneous measurements of allelochemical content in fresh leaves, leaf litter and in soil in experimental and control plots. This paper describes some field research methods and techniques to screen and monitor the effects of allelochemicals in forest ecosystems.

Keywords: Allelopathic effects, forestry, plants, research methods, soil.

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*Correspondence author

1. INTRODUCTION

Although the study of allelopathy in agricultural and horticultural systems has long history, allelopathy research in forested ecosystems is recent (75). In such ecosystems, the soil plays an important role as the matrix through which potential allelochemicals pass, therefore, the influence of allelochemicals on different components of the soil ecosystem and their role in shaping community structure has been studied by numerous authors (80, 101, 35, 33, 34, 88, 17, 18, 60, 115, 114, 28, 29, 30, 31, 76, 120). Reviews by Blum *et al.* (14), Dalton (26), Huang *et al.* (54), Inderjit (55), Novak *et al.* (87), and Schmidt and Ley (109) discussed the influence of abiotic and biotic soil factors on phytotoxic levels of allelochemicals.

The demonstration of allelopathic interactions has three components: (a) an ecological component [demonstration in nature], (b) a chemical component [isolation, identification and characterization of allelochemicals involved] and (c) physiological component [identification of interference mechanism at the biochemical, physiological, cellular, and molecular level] (64). The ideal approach to study the allelopathic effects under field conditions is to do bioassay *in situ*. In such *in situ* bioassay, limitations imposed by the complex environment on the interpretation of bioassay must be considered (61). Likewise, bioassays *in situ* are necessary in allelopathic studies to link the physiological monitoring of target species and biochemical monitoring of growth medium (61). Slow progress in this area is due to difficulty of designing field experiments to prove that a chemical produced by one plant directly affects a neighboring plant. In addition, *in situ* studies to determine the presence and effect of allelochemicals in forest soil are very problematic, owing to their low concentrations, little persistence and possibility of chemical alterations by soil microorganisms (40,63,91,123). At the same time, it is logical to conduct experiments to investigate the role of other mechanisms that may be implicated, such as a resource competition. It may also be possible to conclude from separate or joint studies that a given growth response is explained by both resource competition and allelopathy, although simulation of nature is complex and impossible, there is a need to design experiments that provide the best evidence for allelopathic interactions (64).

Field studies that test the effects of natural levels of allelopathic compounds on coexisting native species are exceptionally rare because allelopathic effects are difficult to quantify and evaluate under the field conditions. Field studies are conducted to demonstrate allelochemicals effects on ecosystem-level (through effects on litter decomposition, activity of decomposers, soil C and N cycling, soil humus, soil nutrients availability, soil water relation and soil respiration, plant mycorrhizal fungi and plant pathogens) and alsto to test allelopathic potential of allelochemical compounds from fresh leaves, leaf litter and soil of donor species on individuals of the same species (autotoxicity) and on coexisting species (growth, photosynthesis, respiration, chlorophyll and water relations of target plants) in forest community, through simultaneous measurements of allelochemical content in fresh leaves, leaf litter and in soil in experimental and control plots.

2. EXPERIMENTAL METHODS (PLANTS)

In forested ecosystems, allelopathic effects can be induced by canopy trees on other individuals of same species by autotoxicity and also on other tree species causing forest decline. Trees and understory plants can also affect each other allelopathically causing alterations in forest structure and composition. The most adverse allelopathic effects have been reported in relation to the influence of the understory on tree regeneration both in natural and managed ecosystems (119, 84, 74).

Multiple physiological effects, cell division, cell differentiation, ion and water uptake, photosynthesis, respiration, enzyme function, and consequently plant growth and production (3, 5, 59, 51), have been observed with many phenolics allelochemicals.

Experiment 2.1. Plant growth

Experiments are done to assess the effects of different plant parts and soil leachates on plant growth. Soil samples can be collected from the rhizosphere of the allelopathic plant and then seeds of target species can be sown in that soil. Compare the germination rate and seedlings growth of target plants with control. Soils samples from adjacent fields or from sites in the same fields without the allelopathic plant should be used as control.

To estimate the potential toxicity of donor species in forest, several experiments should be done. In all the experiments, seeds of target species should be used to assess the inhibitory effects of treatments on germination and elongation of radical and stem. Experiments include pot trays with the forest floor in the experimental plots. At each site, trays should be placed in a small gap/clearing (as control) and under a closed canopy as treatment.

Materials and equipments required

Plant material (fresh leaves, leaf litter), seeds of target plant, soil, Petri dishes, filter paper, distilled water

Procedure

Soil sampling

(i). Collect adequate amount of rhizosphere soil from top 15 cm, as per Jacob *et al.* (66). Remove visible plant remains from the soil samples.

(ii). Take a definite quantity of soil and shake with distilled water for 2 h. Filter the sample through Whatman No. 1 paper or centrifuge at 3,000 rpm for 30 min and use filtrate/supernatant immediately for bioassay or collect the leachates and store at 4^{o}C before assaying. Prepare controls without soil.

Soil leachates

To test possible inhibitory effects of leachates from the organic layer of soil from the experimental plots under donor species on germination and growth of target species, following procedure can be used:

Procedure

(i). Take Petri dishes with 10 cm dia., place two filter papers and moist with 5 ml soil leachate.
(ii). Prepare controls in same manner without leachate but with 5 ml distilled water.
(iii). Sow 100 seeds of target species per Petri dish, seal them with Parafilm and leave at room temperature for 4 days.
(iv). Prepare controls in same manner without leachate but with 5 ml distilled water.
(v). Replicate each treatment thrice.
(vi). After 4 days, calculate the germination rate and measure the shoot and root length.

Calculations

Simultaneous calculation of several germination indices has been proposed to provide a better interpretation of allelochemical activity on seed germination (Jacob *et al.* 66):

Total germination (G_T)

$$G_T = \frac{NT \times 100}{N}$$

Where, NT: Number of germinated seeds for each treatment at the end of assay and N: Total number of seeds

Leaf leachates

(i). Collect fresh leaves from donor plants trees, wash them with tap water and then with distilled water to remove dust and soil particles.
(ii). Prepare the leachates in distilled water.
(iii). Filter the leachate through Whatman no.1 filter paper. The filtrate should be immediately assayed.
(iv). Prepare controls in same manner without leachate but with 5 ml distilled water.
(v). Sow 100 seeds of target species per Petri dish, seal them with Parafilm and leave at room temperature for 4 days.
(v). Replicate each treatment thrice.
(vi). After 4 days, calculate the germination rate and measure the shoot and root length.

Pot culture

(i). Sow the test plant seeds in experimental pots containing the soil mixture. Pots should be sufficiently large to prevent pot bound condition of roots.
(ii). Place the pots in greenhouse or glasshouse.
(iii). Apply measured and equal quantity of leachates to the pots, to maintain sufficient and uniform moisture and prevent over desiccation of soils in the pots.

(iv). Pots containing the same soil mixture but irrigated only with tap water are considered as control. Correct the osmolality of the distilled water used in control, if needed, as suggested by Jacob (66).
(v). Conduct the measurements of germination (%), root and shoot length.

Observations

(i). Seed germination rate (%)
(ii). Root length of seedlings (cm)
(iii). Stem length of seedlings (cm)
(iv). Concentration of allelochemicals in soil and plant material
(v). Chemical composition of allelochemicals in soil and plant material

Statistical analysis

The effects of allelochemicals can be analyzed by one-way or two-way ANOVA depending on complexity of the experimental design. Previously to analysis of variance it is necessary to verify normality and homogeneity of variance. When data are homogenous we will proceed to one- or two-way ANOVA and LSD or Tukey HSD test to determine main differences in effects between groups. When homogeneity of variance cannot be achieved, the nonparametric Kruskal-Wallis in the event of a treatment effect should be used.

Precautions

(i). If major morphological changes are not apparent within the experimental period after the plants have been exposed to allelochemicals, the effects may be overlooked. In some cases, natural variation in seed germination may in some cases pose some challenges to the design of experiments due to a low and inconsistent germination of target species. Sometimes seed germination may be delayed, cotyledon and root size diminished or radicle or seedling development abnormal e.g. in form of twisted growth.
(ii). The effect on population size may be apparent only after a relatively long period of time when some of the seedlings in a population are inhibited.

Exeriment 2.2. Autotoxicity

Autotoxicity occur when a plant species releases chemical substances that inhibit or delay germination and growth of the same plant species (94,2). This mechanism reduces the competition between the individuals of same species thus inhibition of growth of other plants increases the availability of nutrients. Earlier research suggested that allelochemicals, mostly phenolics are involved in the phytotoxicity with ferulic, vanillic, cinnamic and syringic acids identified as phytotoxins (105, 106). For example, Alías *et al.* (2) found autotoxic effects of a species widely represented in the communities of the Iberian Peninsula, *Cistus ladanifer* L. by leachates from leaves that inhibited its own seed germination and cotyledon emergence. Because of its importance in forestry culture, autotoxicity suppose to be studied in forest trees by using leaves, litter, roots and soil leachates containing allelochemicals which can affect germination as well as seedlings growth, photosynthesis and photosynthetic pigments. Experiments are conducted to investigate inhibitory or stimulatory effects of allelochemicals on seed germination.

Materials and equipments required

Seeds, fresh leaves, soil, Whatman filter paper, Petri dishes, refrigerator, culture chamber

Reagents

Distilled water, de-ionized water

Procedure

(i). **Soil sampling:** Prepare soil samples, collected from randomly selected points, up to 10 cm depth (where most of the seeds can be found). Label the samples collected beneath the plant and those collected from areas without test plants. In the laboratory, the soil samples has to be air-dried, sieved through 2 mm pore-size mesh, and keep in dark at room temperature until use.

(ii). **Leaf leachates:** Prepare aqueous solutions from the leaves, maintain 100 g of fresh leaves with constant shaking in 1 L of distilled water for 24 h. Than prepare two further concentrations, corresponding to 1/2 and 1/4 that of the original solution. They were filtered and then keep it in refrigerator at 4 ^{0}C during use.

(iii). **Germination trial 'a'***:* Sowing on paper- Put the seeds (4 replicates of 50 seeds for each trial) on Whatman no. 118 filter paper, in Petri dishes. Within 2-day interval water the seeds with 5 ml of either the different concentrations of the aqueous solution of leaf leachate (1; 1/2; 1/4), or with de-ionized water for the controls.

(iv). **Germination trial 'b':** Sowing on soil- Conduct trials both on soils collected beneath the plant and those collected from areas without test plants. Sow fifty seeds were on 25 g of soil in Petri dishes. Every 2 days, water with 5 ml of either the three concentrations of the aqueous solution of leaf leachate (1; 1/2; 1/4, 4 replicates for each trial), or deionised water for the controls.

(v). Both trials maintain for 10 days in a culture chamber under a photoperiod of 12 h light and 12 h dark at 20 ^{0}C or any other optimal temperature range for germination of the test plant species.

Observations

Count the number of germinated seeds and the number of emerged cotyledons every day.

Statistical analysis

The non-parametric Mann–Whitney U test is suitable to establish significant differences between treatments and the controls. The Kruskal–Wallis test can be used to test for significant differences among treatments. Differences are considered significant for $P<0.05$.

Precautions

(i). In case of the low percentage of seed germination under natural conditions use any valid technique to break dormancy and promote germination.
(ii). In case that germination occurs in autumn or winter than test autotoxicity with the samples collected during two seasons, in the middle of each of these two seasons.

Experiment 2.3. PSII photosynthetic efficiency

The efficiency of photosynthesis of the whole plant is crucial to agriculture and forestry ecosystems when analyze their productivity. Photosynthetic efficiency in leaves is frequently altered in the presence of allelochemicals thus the field bioassay aim to estimate the inhibitory effects of allelochemicals from leaf litter and soil by measuring the response of target plant. One of the best-characterized phytotoxic mechanisms induced by allelochemicals is the inhibition of photosynthesis through interactions with components of PSII (36, 34, 49, 103). Photoinhibition of photosynthesis typically reduces the quantum yield of PSII photochemistry and Chl fluorescence. Allelopathic effects observed and measured in the field have to be tested in the laboratory with allelochemicals isolated from litter or soil. The technique of chlorophyll fluorescence measurement *in situ* and *in vivo* is widely used in the field at a particular site in forest ecosystem where negative influence is suspected, thus provide monitoring of a large number of plants or repeating of experimental settings in the field.

Materials and equipments required

Intact plant leaves of a target plant, Portable Plant Stress Meter or any other type of Chlorophyll Fluorometer

Procedure

(i). Select highly accessible, representative experimental plots with same age class, similar soils, parent material and climate.
(ii). Select experimental plots with target species
(iii). Select control plots with the same characteristics with target species and without a donor species.
(iv). Collect leaf litter and soil samples in adequate intervals (e.g. for species with short life cycles in 10 days intervals; for seedlings in 30 days).
(v). Conduct the measurements on fully developed leaves of target species.
(vi). Replicate adequately and record observations.
(vii). Conduct measurements (prior to measurement, close gently the black shutter by pushing it in direction from cuvette joint (arrow direction). Snap-on the cuvette on a leaf and let it be there for 20-30 min, to dark adapt measuring spot to maximize the oxidation of the primary quinone electron acceptor pool of PSII and to enable the full relaxation of any rapidly recovering fluorescence quenching (A). After approximately 20-30 min, place fiber optic cable in the cuvette (B). Keep fiber optical cable placed 2-3 mm from the black shutter in cuvette (C). Open the black shutter and push the fiber optical cable gently against the leaf. Adjust the chlorophyll excitation time to 2 s or more, if necessary, and actinic light with a photon flux

density to 200 or 400 μmol m^{-2} s^{-1}. Measure by pushing the Run-key on the PSM and chlorophyll fluorescence parameters (Fo, non-variable fluorescence; Fm, maximum fluorescence; Fv=Fm-Fo, variable fluorescence; $t_{1/2}$, half the time required to reach maximum fluorescence from Fo to Fm; and photosynthetic efficiency Fv/Fm) will be displayed. Store the measured results by Save-key).

Figure 1. Measuring of PSII photosynthetic efficiency in filed conditions

Observations

(i). Allelochemicals content in leaf litter under donor species in experimental plots.
(ii). Allelochemicals content in the soil under donor species in experimental plots.
(iii). Parameters of photosynthetic efficiency (Fv/Fm, Fo, Fm, Fv, $t_{1/2}$) of target species in experimental plots.
(iv). Allelochemicals content in the soil from control plots
(v). Parameters of photosynthetic efficiency (Fv/Fm, Fo, Fm, Fv, $t_{1/2}$) of target species in control plots.
(vi). Microclimate parameters (radiation, temperature, relative humidity) both in experimental and control plots.
(vii). Intensity and duration of rainfall.
(viii). Read, write or store into computer following chlorophyll fluorescence parameters: Fo, non-variable fluorescence; Fm, maximum fluorescence; Fv=Fm-Fo, variable fluorescence; $t_{1/2}$, half the time required to reach maximum fluorescence from Fo to Fm; and photosynthetic efficiency Fv/Fm.

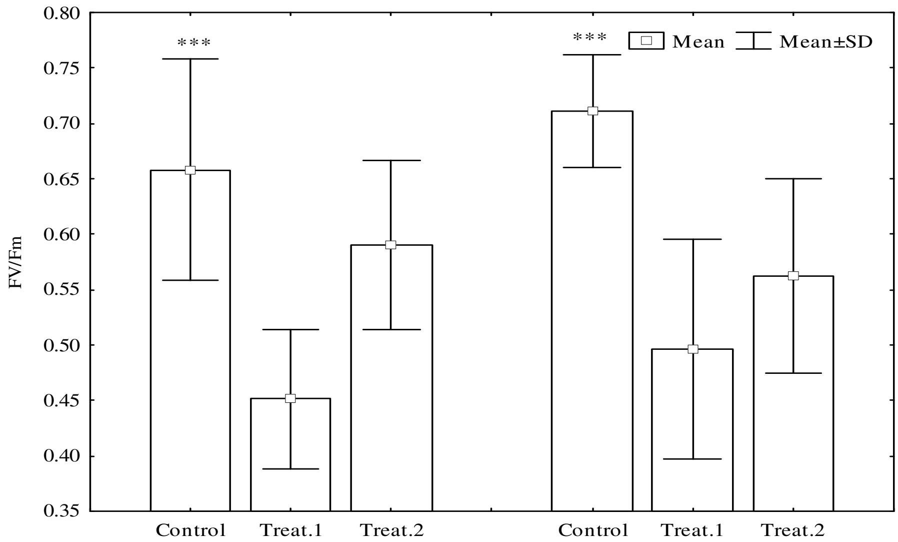

Figure 2. Inhibitory effects of allelochemicals on photosynthetic efficiency

Calculations

For general comparison it can be useful to use the general optimum range of photosynthetic efficiency parameters (Fv/Fm, 0.750–0.850) of plants, empirically obtained by Björkman and Demmig (10). By that range different types of vascular plants from different taxonomy groups, living forms, and site types were covered. A decrease in this ratio indicates that photoinhibition occurs in plants used for the study.

Statistical analysis

The effects of released allelochemicals on photosynthetic efficiency and related parameters can be analyzed by one-way or two-way ANOVA depending on complexity of the experimental design. Previously to analysis of variance it is necessary to verify normality and homogeneity of variance by the Kolmogorov-Smirnoff test and the Levene test respectively. When data are homogenous we will proceed to one- or two-way ANOVA and LSD or Tukey test to determine main differences in effects between groups. When homogeneity of variance cannot be achieve, the nonparametric Kruskal-Wallis test followed by *post hoc* Man-Whitney test should be used in the event of a treatment effect.

Precautions

(i). When taking measurements always use fully expanded leaves
(ii). Create experimental setting in accordance to the natural conditions of the studied forest because it depends on how many tree species dominate in the forest canopy

Experiment 2.4. Net Photosynthesis and Respiration

Photosynthesis is the driving force of plant productivity and the ability to maintain the rate of photosynthetic carbon dioxide and nitrate assimilation under environmental stresses is fundamental to the maintenance of plant growth and production. Plant growth and productivity are usually correlated to both total leaf area and photosynthetic rate per unit of leaf, and the factors that depress photosynthesis will reduce the plant growth. Respiration is essential for growth and maintenance of all plant tissues, and plays an important role in the carbon balance of individual cells, whole plants and ecosystems. Through the processes of respiration, solar energy conserved during photosynthesis and stored as chemical energy in organic molecules is released in a regulated manner for the production of ATP, the universal currency of biological energy transformations, and reducing power (e.g. NADH and NADPH) (48).

Specific allelochemicals have shown interferences with stomatal function, carbon fixation and distribution, and respiration (33, 123). Despite of the fact that carbon photosynthesis is defined as the carbon dioxide assimilation and is expressed as it is always a calculated parameter. The driving force is the amount of photosynthetically active radiation (PAR) per surface and per unit time. So, photosynthesis measurement is determined by measurements of concentrations, gas flow and other parameters like temperature or water vapour pressure, which are usually measured simultaneously (118). The field experiment aims to determine the inhibitory effects of allelochemicals from the leaf litter and soil under the donor plants on net CO_2 assimilation (P_n) and respiration of target plants.

Materials and equipments required

Fully expanded plant leaves, a portable LI-6200 CO_2 analyzer, LI-6400 or LI-6400XT portable photosynthesis system with integrated gas exchange/fluorescence or other type of gas analyzers.

Procedure

(i). Select experimental plots with target species.
(ii). Select control plots with the same characteristics with target species and without a donor species.
(iii). Collect leaf litter and soil samples of donor species for analyses in adequate intervals (e.g. for efemeroid species in 10 days intervals; for seedlings in 30 days).
(iv). Conduct the measurements with photosynthesis system, under field conditions on fully developed leaves of target species by placing the leaf in chamber and conduct the measurement. For each leaf sample data on CO_2 assimilation together with photosynthetic photon flux density (PPFD), air flow, leaf and air temperature and relative humidity conditions has to be measured within the chamber using the sensors housed in the leaf chamber. During the measurement air flow through desiccant is controlled in order to keep constant concentration of CO_2, temperature and relative humidity within the chamber (for LI-6400XT the CO_2 Injector system provides a constant CO_2 input from 50 to 2000 μmol mol^{-1}).
(v). Conduct measurements of dark respiration using the same analyzer, in the closed leaf chamber covered with aluminium foil by using the same analyzer, in the closed

leaf chamber covered with aluminium foil. Measurements of photosynthetic and respiratory rates in leaves are usually expressed as rates of CO_2 exchange per unit time per unit leaf area (μmol CO_2 m^{-2} s^{-1}).

(vi). Replicate adequately and record observations.

(vii). Express the gas exchange values of each leaf on a leaf area basis. Leaf area can be determined by using the variety of methods for measuring (by measuring manually, by scanning leaves and use programs for area estimation or with equipment, e.g. LI-3000A Portable Leaf Area Meter).

Observations

(i). Assimilation of CO_2 (μmol m^{-2} s^{-1}) of target species in experimental plots
(ii). Assimilation of CO_2 (μmol m^{-2} s^{-1}) of target species in control plots
(iii). Respiration rate (μmol m^{-2} s^{-1}) of target species in experimental plots
(iv). Respiration rate (μmol m^{-2} s^{-1}) of target species in control plots
(v). Leaf area (cm^2)
(vi). Photosynthetic photon flux density (PPFD), air flow, leaf and air temperature and relative humidity conditions both in experimental and control plots
(vii). Allelochemicals content in leaf litter/soil under donor species in experimental plots
(viii). Allelochemicals content in the soil from control plots
(ix). Microclimate parameters (radiation, temperature, relative humidity) both in experimental and control plots

Statistical analysis

The effects of released phenolics on net photosynthesis, respiration and related parameters can be analyzed by one-way or two-way ANOVA depending on complexity of the experimental design. Previously to analysis of variance it is necessary to verify normality and homogeneity of variance by the Kolmogorov-Smirnoff test and the Levene test respectively. When data are homogenous it can be proceed to one- or two-way ANOVA and LSD or Tukey test to determine main differences in effects between groups. When homogeneity of variance cannot be achieve, the nonparametric Kruskal-Wallis test followed by post hoc Man-Whitney test can be used. To clarify which part of the variation in the net photosynthesis rate is due to the distinct treatments, it is suggested to conduct an ANCOVA analysis for the variables: photosynthetic rate and treatments, introducing as covariables the parameters: irradiance, air and leaf temperature, carbon dioxide, relative humidity and plant phenology.

Precautions

(i). Always conduct measurements on fully expanded leaves
(ii). Plant phenology data is important because leaf maturation and aging may decrease the photosynthesis
(iii). Consider that there are anatomical and physiological differences between sun and shade leaves in plants
(iv). Calibrate the instrument at the start of each sampling date

(v). Choose the leaf quantity and desiccant flow rate to keeps the humidity constant

Experiment 2.5. Root respiration

Root respiration often comprises from one third to more than one half of total soil CO_2 efflux in forest ecosystems (19,78). However, the ecophysiological characteristics of fine roots of mature forest plants are poorly understood because of difficulties of measurement. Root respiration rates can be measured in the field at ambient soil temperatures and its measurement dates should include seasonal dynamics in forest. Fahey and Yavitt (38) proposed a root in-growth approach to measure respiration of woody plant roots *in situ*. The field experiment is conducted to test effects of allelochemicals on root respiration of target plants.

Materials and equipments required

Target plant roots, Li-Cor 6200 infrared gas analyzer, Li-Cor 6000-09 soil respiration chamber (Li-Cor Biosciences, Lincoln, NE) or any other type of analyzer, balance

Procedure

(i). Install randomly root in-growth chambers at each experimental and control plots.
(ii). Conduct measurements of CO_2 emissions from chambers using a gas analyzer. equipped with soil respiration chamber by connecting to the LI-6200 portable leaf photosynthesis system. Air from the chamber circulates to the gas analyzer in the LI-6200. The CO_2, concentration in the chamber is monitored for 45 s and the rate of increase is computed every 15 s. Soil respiration rate is determined by average of the three trials.

Observations

(i). Allelochemicals content in leaf litter under donor species in experimental plots
(ii). Allelochemicals content in the soil under donor species in control plots
(iii). Root respiration rate (nmol gDM^{-1} s^{-1}) of target species in experimental plots
(iv). Root respiration rate (nmol gDM^{-1} s^{-1}) of target species in control plots
(v). Root dry mass (g m^{-2})
(vi). Soil atmosphere CO_2 concentrations
(vii). Soil temperature
(viii). Microclimate parameters (radiation, temperature, relative humidity) both in experimental and control plots

Statistical analysis

The effects of phenolics on root respiration can be analyzed using analysis of variance (ANOVA) with means separated by Duncan's multiple range test together with using the t-test to compare experimental groups.

Precautions

(i). The target plant roots must not suffer damage during the respiration measurement
(ii). Conduct all measurements after at least 2 days after the rainfall
(iii). Keep the soil chamber shaded to avoid overheating
(iv). If measurements are made on a bare soil surface with no canopy, variations in the measured fluxes can occur due to dynamic pressure fluctuations at the pressure vent outlet caused by wind effects.

Experiment 2.6. Chlorophyll

The physiological status of forest canopy foliage is influenced by a range of factors that affect leaf pigment content and function (24). Together with measurements of plant gas exchange and water content, analysis of leaf photosynthetic pigment content is frequently undertaken in plant physiological studies (92). Changes in leaf chlorophyll content provide an indicator of maximum photosynthetic capacity, leaf developmental stage, productivity and stress (71). In stressed vegetation, leaf chlorophyll content decreases, thereby changing the proportion of light-absorbing pigments and leading to less overall absorption (129). Allelochemicals may reduce chlorophyll accumulation in three ways: the inhibition of Chl synthesis, the stimulation of Chl degradation, and both (32, 52, 101, 126). Experiments are conducted to investigate inhibitory or stimulatory effects of allelochemicals on photosynthetic pigments.

Materials and equipments required

Intact leaf, Chlorophyll meter (Minolta SPAD 502- Minolta) or any other chlorophyll fluorescence fluorometer

Procedure

(i). Calibrate the instrument by conducting the measurement without leaves.
(ii). Conduct measurement of chlorophyll content (μmol chlorophyll per m^2 of leaf).
(iii). Provide a valid number of replicates.

Observations

(i). Chlorophyll content (μmol chlorophyll per m^2 of leaf)
(ii). Allelochemicals content in leaf litter/soil under donor species in experimental plots
(iii). Allelochemicals content in the soil from control plots
(iv). Data on microclimate conditions both in experimental and control plots

Statistical analysis

Use t-test if there are only two independent variables (control and treatment) and (one- or two- way) ANOVA if there are more than two that depends on the complexity of the experimental design.

Precautions

(i). For filed measurements provide water-resistant instrument so it can be used in the field studies even in the rain
(ii). Provide instrument which measure chlorophyll concentrations without a distraction of plant tissues thus allows field measurement of a large number of samples
(iii). Provide control set of data
(iv). It is important to remember that results of *in situ* analysis will not be as accurate as results from the certified leachateive analysis procedure
(v). The limitations of the *in situ* method should be carefully considered before making chlorophyll determinations

Experiment 2.7. Water relations

Water availability in higher plants is the most important limiting factor of photosynthesis and in consequence of the plant productivity. Disruption of plant water relations induced by allelochemicals is the primary mode of action that reduces the overall plant growth (67, 3, 6). These effects include decreases in absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turger pressure, and osmotic potential (5). Numerous studies showed that alellochemicals caused a lowering of relative water contents in leaves of all target species with 10% decrease in relative water content correlated with a decline in leaf water potential (34, 5, 107). Sometimes decreases in tissue water content may be more important than decreases in water potential or pressure potential in terms of influencing growth. Then, it is not enough to study water relations in plants to know only water content but is often important to measure both, tissue water content and water potential for plants growing under field conditions (47). Phenolic acids such as *p*-coumaric, caffeic, ferulic, and salicylic acids often cause water stress in plants by causing stomata closure and other constraints on plant water relationships (4, 34, 3, 6, 5). On the other side, water stress (caused by reduced rainfall or salinity) can increase allelochemical production (98).

Relative water content

The relative water content (RWC) is a useful indicator of the state of water balance of a plant and also it can be used to test effects of allelochemicals on water relation of target species. For general comparison, following relations can be used: a RWC among 100-90 % is related to closing of the stomata in the leaf and a consequently lead to the reduction in the cellular expansion and growth; contents of 90-80 % are correlated with alterations in the relative rates of photosynthesis and respiration; and levels of RWC below 80 % imply usually water potential of the order of -1.5 MPa or less, and this would produce changes in the metabolism with decrease of photosynthesis and increase of the respiration (47). Measurements are conducted to investigate inhibitory effects of allelochemicals on relative water content of target plants.

Materials and equipments required

Fully-expanded leaves or shoots of target plant, Eppendorff tubes, balance, stove, distilled water

Procedure

González and González-Vilar (47) suggested following procedure:

(i). Use small discs of leaf tissue pieces for RWC measurement.
(ii). Weigh and label with a number 0.2 ml Eppendorff tubes and label with the same numbers 1.5 ml Eppendorff tubes.
(iii) Fill the 1.5 ml tubes with cold de-ionised water.
(iv) Put four sections of a same leaf in a tube.
(v) Seal the tubes and place in ice to prevent growth and evaporation.
(vi) Weigh the tubes with tissue sections inside to give a value for tissue fresh weight (FW).
(vii) After weighing, transfer samples to a 1.5 ml Eppendorff tubes containing de-ionised water with the same number of the small tube.
(viii) Take away the tissue sections from the de-ionised water. Remove carefully the excess of water on the leaf surface with tissue paper.
(ix) Transfer every sample to their original tubes (0.2 ml) and reweigh. This gives a measure of fully turgid fresh weight.
(x) Open the tubes and place them in a water bath (70°C, for 48 hr).
(xi) After drying reweigh the tissue to obtain a value for dry weight (DW).

Calculations

Calculate relative water content (RWC) by using the following equation:

$$RWC = 100x\ (FW-DW)/(TFW-DW)$$

SW- turgid mass after rehydrating the leaves, FW-fresh weight, DW-dry weight of leaves

Statistical analysis

The effect of allelochemicals on RWC can be analyzed by using analysis of variance (ANOVA) with means separated by Duncan's multiple range test. Use t-test to compare experimental groups.

Precautions

(i). The relationship between RWC and water potential varies significantly with nature and age of plants. Therefore, for comparison it is essential to notice above parameters
(ii). The plant must not suffer damage during the RWC measurement
(iii). Small discs or tissue pieces are useful for determination of RWC to avoid a great heterogeneity of the leaf and to get a good correlation between RWC and some physiological processes.
(iv). The procedure to get full saturation needs to reach constant weight in the tissue. Young leaves, which are still suffering expansion, absorb water for a substantially longer period (usually several days). Young tissue is respiring up and consumes part of its dry weight. This can result in a significant error.
(v). To avoid this problem it is necessary to reduce the time for saturation of the tissue by reducing the sample size as much as you can manage it and keep away the sample

from physiological activity by physical inhibition of growth and respiration during determination. Saturation of the tissue portions at 4°C inhibits satisfactorily the growth.

Transpiration

Inhibitory allelopathic effects of different allelochemicals on transpiration and stomatal conductance have been observed and described (67, 6, 100). Interference with plant-water balance appears to be one mechanism of action of allelochemical phenolics causing a lower stomatal conductance and lower water potential plants (51). Porometers are used to measure the transpiration, diffusive resistance and stomatal conductance in the field conditions. Field bioassays aim to estimate inhibitory effects of donor species allelochemicals by measuring transpiration rate of target species leaves.

Materials and equipments required

Target plant leaves, Li-COR 1600 Steady-State Porometer with a variety of easily interchangeable apertures and chambers (Broadleaf Aperture, Narrow Aperture for grasses and small leaves, or Square Chamber for small leaves, leaflets, or conifer needles)

Procedure

(i). Select highly accessible, representative experimental plots with same age class, similar soils, parent material and climate.
(ii). Select experimental plots with target species.
(iii). Select control plots with the same characteristics with target species and without a donor species.
(iv). Collect leaf litter and soil samples in adequate intervals.
(v). Install the instrument in the definitive measuring place.
(vi). Keep the instrument to ambient conditions, at least for 30 min to adjust the cuvette in environmental temperature and humidity.
(vii). Conduct the measurements on fully developed leaves of target species.
(viii). Store the data in data memory or transfer data to a PC with a data transfer program for later analysis.
(ix). Replicate adequately and record observations.

Observations

(i). Allelochemicals content in leaf litter under donor species in experimental plots.
(ii). Allelochemicals content in the soil under donor species in control plots.
(iii). Leaf conductance (mmol m^{-2} s^{-1}) of target species in experimental plots.
(iv). Leaf conductance (mmol m^{-2} s^{-1}) of target species in control plots.
(v). Transpiration rate (mmol m^{-2} s^{-1}) of target species in experimental plots.
(vi). Transpiration rate (mmol m^{-2} s^{-1}) of target species in control plots.
(vii). Leaf temperature (^{o}C).
(viii). Photosynthetic Active Radiation (PAR, µmol m^{-2} s^{-1}).
(ix). Relative humidity RH (%).

Statistical analysis

The effect of allelochemicals on transpiration can be analyzed by using one-way analysis of variance (ANOVA) with means separated by Duncan's multiple range test. Use t-test to compare experimental groups.

Precautions

(i). The plant must not suffer damage during the transpiration measurement
(ii). When conduct measurements always use fully expanded leaves
(iii). Plant phenology data is important because leaf maturation and aging may influence the transpiration
(iv). Prior to measurement a previous habituation (for 30 min) to the environmental parameters is necessary
(v). The chamber must be installed prior to measurements. The selection of the leaf chamber and the aperture (size and form) will depend on the plant species that will be measured (e.g. grass leaves or conifer needle)
(vi). The sensor head has to be cleaned, without presence of pollen or dust because it can increase the water vapor absorption with unusually increased values
(vii). Prior to measurements try to work facing the sun to prevent shading the leaf. However, direct sun must be avoided during the measurement in order to protect the chamber against abrupt temperature changes
(viii). Conduct measurements when the instrument is at or near the null point relative humidity and the diffuse resistance value is stable. A steady state condition or null balance is achieved when the humidity in the cuvette is constant. In this condition, the dry air from the instrument balances the water vapor contributed by leaf. The null adjust meter indicates if more or less air is required to reach a steady state condition.
(ix). Maintain the natural orientation of the leaf during the measurement
(x). Be aware that stomata might close when placed into a ventilated cuvette (air saturation deficit, plant water relations, light intensity, etc. may affect closure of stomata)
(xi). Data on leaf transpiration is completed with data about edaphic water availability, atmospheric humidity, temperature, radiation and wind that are usually measured with the common techniques to measure transpiration because environmental factors can influence not only the physical process of evaporation and diffusion but also the stomatal aperture and closure on the leaf surface.

Total water potential and osmotic potential

Water potential in leaves is frequently altered in the presence of allelochemicals (104). In field conditions, measurement of water potential using pressure chamber apparatus is the most common method to determine the water status of higher plants. Reduction in growth is often accompanied by decreases in leaf water potential (Ψ). González (46) described a detailed procedure for determination of water potential and stated that components of water potential depend on the plant species and vary with growth environment (turgor potential Ψp, osmotic potential $\Psi\pi$, matric potential Ψm). Leaf turgor potential (Ψp) is calculated as the difference between Ψ and $\Psi\pi$. Thus, environmental factors must be considered during experiment and also during the interpretation of obtained results. Osmotic potential measurement is not possible to conduct in field conditions because it has to be done with samples frozen in liquid nitrogen. Experiments are conducted to investigate changes in water and osmotic potential caused by allelochemicals.

Materials and equipments required

Fully-expanded leaves or shoots of target plant, Scholander Pressure Chamber, Vapor-pressure osmometer

Figure 3. Sholander pressure chamber

Procedure

(i). Prior to measure, fill the chamber with compressed gas until the distribution of water between the living cells and the xylem conduits is returned to its initial, pre-excision, state. This can be detected visually by observing when the water returns to the open ends of the xylem conduits that can be seen in the cut surface. The pressure needed to bring the water back to its initial distribution is called the balance pressure and is readily detected by the change in the appearance of the cut surface, which becomes wet and shiny when this pressure is attained.
(ii). Conduct measurement of water potential (Ψ) with a Scholander chamber in the following manner: excise the leaf or shoot to be measured from the plant and partly sealed it in a pressure chamber.
(iii). Collect leaf samples for osmotic potential measurements and put them in a freezer or in a liquid nitrogen and trabnsfer them to a laboratory for analysis. Osmotic potential measurements have to be conducted in laboratory with samples frozen in liquid nitrogen, with an aid of vapor-pressure osmometer.

Observations

(i). Allelochemicals content in leaf litter under donor species in experimental plots.
(ii). Allelochemicals content in the soil under donor species in control plots.
(iii). Water Potential Ψ (MPa) of target species in experimental plots.

(iv). Water Potential Ψ (MPa) of target species in control plots.
(v). Osmotic Potential Ψ_s (mmol kg^{-1}) of target species in experimental plots.
(vi). Osmotic Potential Ψ_s (mmol kg^{-1}) of target species in control plots.
(vii). Microclimate parameters (radiation, temperature, relative humidity) both in experimental and control plots.

Statistical analysis

The effect of phenolics on water and osmotic potential can be analyzed by using analysis of variance (ANOVA) with means separated by Duncan's multiple range test. Use t-test to compare experimental groups.

Precautions

(i). When conduct measurements always use fully expanded leaves
(ii). Before excision, the water column in the xylem is under tension. When the water column is broken by excision of the organ (i.e., its tension is relieved allowing its Ψ_p to rise to zero), water is pulled rapidly from the xylem into the surrounding living cells by osmosis. The cut surface consequently appears dull and dry.
(iii). Osmotic potential measurements have to be conducted in laboratory with samples frozen in liquid nitrogen.
(iv). The sample should be freshly cut (additional cutting is not allowed)

3. EXPERIMENTAL METHODS (SOIL)

At the ecosystem level, allelochemicals are able to inhibit or decrease not only growth performance of tree species but also influence function of other organisms. When allelochemicals enter the soil organic matter pool, they affect several aspects of ecosystem functioning due to effects on rate of litter decomposition and soil C and N recycling (102, 73, 23, 53, 81, 69, 111, 119, 125, 75) and on composition and activity of the decomposer community (by inhibiting microbial activity and N release from litter and humus). Allelochemicals can also influence function of free or symbiotic bacteria in forest ecosystems and, consequently processes such as identification, ammonification or bacterial pathology (23, 110). Allelochemical polyphenols may influence nutrient turnover by direct toxic effects on soil organisms involved in the decomposition and N transformations and also by inhibiting the crucial enzymes for the microbial activity in N turnover (120). Alternatively, polyphenols could control the pool and the form of nutrients available for plants and/or microbes (86, 112).

Experiment 3.1. Litter decomposition

Litter decomposition of plant species has been studied for decades. Factors that regulate decomposition rate values are climatic factors (temperature, precipitation and evapotranspiration; litter quality (N content, C:N ratio, lignin content, lignin:N ratio; vegetation and litter types; and geographical variables (latitude and altitude) (1, 8, 44, 93, 121, 113). Release of allelochemicals during litter decomposition can potentially affect the structure of plant communities (101) therefore Bonanomi *et al*. (16) studied the dynamical patterns of litter phytotoxicity under controlled and standardized conditions. Above authors proposed experimental conditions of this decomposition method that can be considered comparable to those found in the field, because the soil microbial community always operates either in thin water films surrounding solid particles or inside the soil aggregates (79). The experiment is conducted to to investigate if this allelochemical affects the viability

Material required

Leachate of dry plant leaves and roots, leachate of soil collected under plants, target plant, seeds, plastic litterbags, growing chamber, greenhouse, Petri dishes

Procedure

Sample collection

(i). Select species to study and collect samples (for each species, 20 leaves and thin roots of diameter < 2 mm).
(ii). Collect soil samples under plants of each examined species.
(iii). Chop with scissors (size < 1 cm) plant samples dried at +40 ^{0}C for 5 d, and then store at room temperature.

Litterbag decomposition

(i). To assess phytotoxicity dynamics, use the litterbag method to simulate field decomposition conditions.
(ii). Collect samples of the coexisting species from forest community.
(iii). Fill plastic litterbags (mesh size 1 mm) with 6 g of dry plant material.
(iv). Place litterbags in large trays (100 x 100 cm and 30 cm deep) fill with soil from the same stand
(v). Keep in a greenhouse in controlled temperature conditions and wet daily (for soil to reach the field capacity) to speed up the decomposition process.
(vi). Collect litterbags after period of 10, 30 and 90 days of decomposition.
(vii). Dry the bags in the laboratory (+40^{o}C for 5 d) and mix the material with distilled water in a beaker at 5% of dry weight and shake for 5 h.
(viii). Treat the aqueous suspensions according to same protocol of laboratory litter decomposition, i.e. centrifuge (4300 rpm for 10 min), sterilize (microfiltration with 0.22-µm pore filter), dilute by distilled water to three concentrations (from 5 to 50 g L^{-1}) and store at −20^{o}C until bioassay

When conduct filed experiment, the standard litterbag technique can be used to assess the decomposition characteristics of tree leaf litter in field conditions by using following procedure described by Jacob *et al.* (2011).

Procedure

(i). Collect freshly fallen leaf litter of the tree during normal leaf fall period by placing suitable litter traps erected on supports beneath the canopy of the trees.
(ii). Air-dry the litter to constant moisture. Determine dry weight equivalents by oven drying the air-dried litter samples at 70^0C for 48 h.
(iii). Set apart triplicate samples of the oven-dried litter for the initial biochemical analysis.
(iv). Set apart triplicate samples of the oven-dried litter for the initial biochemical analysis.
(v). Make litter bags of size 25 cm x 20 cm from nylon nets (mesh size 2 mm) and fill 20 g of air dried leaf litter in each bag. The 2 mm mesh size prevents losses of litter due to breakage, but permits access of decomposers. Seal the bags by stitching the open ends.
(vi). Prepare 200 such bags and place beneath the tree on the soil surface or incorporate in soil according to the objective of the study (surface/incorporated). Place the litter-bags to mimic the position of natural litter when freshly fallen.
(vii). Fasten the bags to the ground with 10-15 cm long stakes beneath the canopies of the trees.
(viii). In an adjacent open area, demarcate control plot (10 m x 5 m) and place 200 litter bags filled with 100 g of the litter either on the surface or incorporated.
(ix). Draw samples at fortnightly intervals from beneath the tree canopy and control plot. Retrieve five bags during every sampling for analysis. Continue sampling until 95 % decomposition has occurred.
(x). Sieve the retrieved samples to remove soil and other extraneous material, dry at 70^0C for 48 h and weigh to assess the dry weight loss during decomposition.
(xi). Analyse the samples of green leaves of each species, freshly fallen leaf litter and the residual litter mass collected during each sampling for nutrients and allelochemical content.

Observations

I. Leaf litter

(i). Initial nutrients and allelochemicals content in the leaf litter.
(ii). Dry weight of the leaf litter sampled at fortnightly intervals.
(iii). Nutrient and allelochemicals content in the leaf litter sampled at fortnightly intervals.

II. Soil

(i). Initial nutrients and allelochemicals content in the soil.
(ii). Initial soil physical (bulk density, porosity, hydraulic conductivity, clay content) and chemical properties (pH, organic matter, cation exchange capacity, available nutrients content).
(iii). Soil moisture content at fortnightly intervals.
(iv). Soil microbial population at fortnightly intervals.

III. Bioassay

Objective: To tests the root elongation with target plant, which you recognized as sensitive to allelochemicals in studied forest community.

(i). Conduct the experiments in a growing chamber at constant temperature and in dark conditions.
(ii). Place seeds on Petri dish over sterile filter paper with 4 ml of test solution.
(iii). Perform the experiment using three or more different concentrations of leachates (for example, from 5 to 50 g L^{-1}).
(iv). Provide 10 replicates for every solution and the control on distilled water.
(v). In order to test effects of pH and CEC conduct a second experiment according to the same design by using pH (4.4, 5.5, 6.3, 7 and 8.8) and EC (2, 4.6, 7.6, 9 and 13.1 mS cm^{-1}) levels. Obtain pH levels by using a 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer adjusted with 1M NaOH. For the EC values use 20, 50, 80, 100 and 150 mmol L^{-1} of NaCl solution.
(vi). Arrange Petri dishes in accordance to a totally randomized design.
(vii). Measure the length of seedlings root after 36 h from germination.

Figure 4. Litter decomposition experiment in forest ecosystems (Litter bag method)

Observations

(i). Root elongation of the test plant under leachates at different concentrations and control.
(ii). Seedlings root length under different pH levels.
(iii). Seedlings root length under different EC levels.
(iv). Percentage of inhibition of root length (measure with a ruler, and express as percentage length of seedlings calculated by reference to the length of control plants).

Statistical analysis

Perform three-way ANOVA two times to test the main effects and interactions of conditions of decomposition (aerobic *vs* anaerobic), plant material (leaf *vs* root or species functional groups) and decomposition time on the inhibition of root growth of test plants. Apply two-way ANOVA to test the effects of single species examined and decomposition time on the inhibition of root growth for both laboratory and litterbag leachates. In addition, it is possible to apply two-way ANOVA to test the effects of conditions of decomposition (aerobic *vs* anaerobic) and decomposition time on leachates pH and EC. Apply one-way ANOVA to test the effects of pH or EC on test plant root length.

Precautions

(i). For target plant use species co-occurring in natural conditions instead of test plant
(ii). Prior to measurements correct the redox potentials according to pH levels.

Experiment 3.2. Decomposers

Decomposition of organic matter in forest ecosystems is performed mainly by fungi and has been in the focus of research for several decades (117, 7, 9). Soil microorganisms are important components of forest ecosystems because they play a central role in organic matter decomposition and nutrient cycling. They affect soil nutrient content and related primary production, which is particularly relevant in forest ecosystems (11). Poor natural regeneration of forests occur due to inhibition of tree germination and seedling growth by allelochemical compounds mostly phenolics in the humus layer as well as mediation of soil microorganisms that could metabolize the inactive phenolics of understory plants into biologically active compounds (and *vice versa*) (89, 90). Thus, Souto *et al*. (115) studied the effects of phenolics from soil humus on the microbial populations in the soil and proposed following procedure for testing of effects of allelochemical phenolics. The objective is to identify and quantify the allelochemical phenolic compounds in humus and to test these on the microbial populations. Experiment should be conducted in two seasons (winter and summer).

Materials and equipments required

Humus, agar, sterile flasks, High-Performance Liquid Chromatograph (HPLC), sterile flasks

Reagents

Phenolics mixture (Sigma Chemical Co.), de-ionised water, diethyl ether, ethanol, HCl, acetic acid

To identify and quantify the allelochemical phenolic compounds in humus and to test their effects on the microbial populations the following procedure can be used:

Procedure

Experimental design

(i). Collect humus samples and incubate after the addition of a sterile phenolic mixture or sterile demineralized water (as control).

(ii). Add phenolic mixture or water in control, for second time, after three days of incubation and continue the incubation for three more days. At the end of this period, inoculate the samples in media for identification and quantification of microorganisms. Than, at intervals of 0, 3, and 6 days, analyze content of phenolics in humus samples in order to describe the fate of the chemicals according to the microbial activity.

I. Humus sampling

(i). Collect the samples from the forest with domination of allelopathic species for which you consider as inhibitor in canopy gaps.
(ii). Collect randomly the humus samples from humic layer which contain most of the microorganisms.
(iii). Prepare a collective sample in the field by mixing and passing through a 5 mm sieve.
(iv). Store the samples at +4^{o}C during transportation to the laboratory.

II. Phenolics analysis

(i). Immediately after arrival at the laboratory, shake gently three subsamples of 20 g of humus (fresh weight) in 80 ml of de-ionised water for 18 hr (dark, 15^{o}C).
(ii). After the filtration, leachate the phenolics from the solution acidify by 2 N HCl (to pH 2.0) with diethyl ether and redissolve in ethanol before analysis by HPLC.
(iii). Place a short column of Novapak 18 C (50 mm x 3.9 mm ID) immediately before a 300-mm x 3.9-mm-ID column filled with mBondapak 18 C. Carry out a linear gradient elution at a flow rate of 1.5 ml/min. Use 0.5% acetic acid in distilled water as solvent A, and acetonitrile with 0.5% acetic acid as solvent B. Use a gradient from 0% to 20% B over 45 min, followed by 15 min reequilibration with A.
(iv). Identify and quantify the phenolic compounds by comparing retention times, wavelength detection, and peak areas to those of standard compounds (as suggested by Gallet, 41).

III. Degradation of phenolic compounds by humus microorganisms

(i). Add 2 ml of phenolic solution to six different flasks containing 20 g fresh wt of intact humus and to three flasks containing autoclave-sterilized humus (20 g fresh wt). Add de-ionised water to another six flasks containing unsterilized humus (control).
(ii). Incubate at 28^{o}C in darkness for three days.
(iii). After the incubation, leachate the phenolics for analysis, in three of the flasks with unsterilized humus given the phenolic solution, and in three of the control flasks. Supply the remaining three flasks of the two treatments again with phenolic solution or de-ionised water, and leave it for another three days to incubate before analyzing the phenolic content.
(iv). Analyze the samples in flasks with the sterile humus for phenolic content only after six days of incubation.

IV. Bioassays

(i). Incubate subsamples of humus (20 g) in sterile flasks in dark at 28°C. Just before the start of the incubation, in three replicates add 2 ml of sterile de-ionised water solution (by filtration through a 0.22 mm membrane) containing a naturally occurring phenolics mixture (Sigma Chemical Co.). The solutions should vary with the season as follows: (1) for winter experiment use 7.7 x 10^{-5} M *p*-hydroxybenzoic acid, 7.8 x 10^{-5} M *p*-hydroxybenzaldehyde, 1.8 x 10^{-3} M vanillic acid, and, 1.5 x 10^{-3} M vanillin; (2) for summer experiment use 1.5 x 10^{-5} M protocatechuic acid, 7.8 x 10^{-6} M *p*-hydroxybenzoic acid, 1.4 x 10^{-5} M vanillic acid, and 1.58 x 10^{-4} M ferulic acid. In the remaining three subsamples, add 2 ml of sterile de-ionised water. After three days of incubation, add another 2 ml of the respective solution.
(ii). For determination of effects of phenolic compounds on the density of humus microbes, add 10 times more concentrated phenolics than the natural concentration previously determined in the humus. After a total of six days of incubation, quantify microbial populations by the most probable number method (proposed by Alexander and Clark, 1965) and by counting colonies growing on agar media. Shake each replicate of humus in 180 ml de-ionised water for 25 min (10^{-1} dilution), to obtain 10-fold serial dilutions (until 10^{-7} dilution).
(iii). Conduct the determination of following microbial groups: nitrogen fixers; proteolytic microorganisms, ammonifiers, and starch hydrolyzers; denitrifying bacteria; *Nitrosomonas* and *Nitrobacter*, bacteria and fungi; and cellulose hydrolyzers.

Observations

(i). Phenolics content in humus.
(ii). Number of dinitrogen fixers colonies.
(iii). Number of proteolytic microorganisms colonies.
(iv). Number of ammonifiers and starch hydrolyzers colonies.
(v). Number of denitrifying bacteria colonies.
(vi). Number of *Nitrosomonas* and *Nitrobacter* colonies.
(vii). Number of bacteria and fungi colonies.
(viii). Number of cellulose hydrolyzers colonies.

Statistical analysis

Use one-way analysis of variance (ANOVA) with $P < 0.05$. When data are not normally distributed, use the nonparametric Mann-Whitney U test ($P < 0.05$).

Precautions

(i). Perform the experiments two times, once during winter and again in the summer because dynamics of soil microbial populations can vary depending on weather conditions
(ii). Conduct the experiments with humus samples collected in winter and summer because of seasonal fluctuations in soil microbial population

(iii). Conduct the experiments with humus samples collected in winter and summer because of seasonal fluctuations in content of allelochemical phenols
(iv). Instead of methods for quantification of microbial populations proposed here you may use other methods

Experiment 3.2.1.1: Effects of leaf litter on fungal biomass and sporulation in field experiments.

These were tested by Bruder *et al.* (20) by using following procedures.

Materials and equipments required

Litter, litter bags, nets for litter collection, Petri dishes, GF⁄ F filters, shaker, plastic tubes, membrane filter (5 lm pore size), microscope, scanner, leachateion cartridges, High-pressure Liquid Chromatograph (HPLC)

Reagents

Formaldehyde, methanol ⁄KOH, distilled water, Triton X-100 (0.01 % solution), 0.1 % Trypan blue in 60% lactic acid

Procedure

(i). Organize a field study set up as randomized complete block design.
(ii). Collect tree litter upon abscission in the autumn by using nets installed under trees in a forest.
(iii). Construct litter packs of approximately 6 g, from litter dried at 40 ^{0}C, previously weighed, than wetted and placed in tetrahedral mesh bags and place in the examined forest. The tetrahedral shape and mesh size (4 mm) of the bags ensured access of a natural decomposer community to the litter and minimized artefacts resulting from unnatural moisture retention in the bags during dry periods. Leaching losses of litter upon wetting are determined by soaking dried and weighed litter batches in running tap water for 24 h, retrieving them and drying them to constant weight at 65 ^{0}C (conversion factors to account for differences in residual moisture between litter dried at 40 and 65 ^{0}C are determined from the same litter batches).

I. Litter mass remaining

(i). Collect litter samples from the field, separate by leaf species and gently clean with tap water.
(ii). Cut leaf discs (14 mm diameter) immediately after cleaning and preserve for analyses of fungal sporulation activity and biomass (avoid the central vein of tree leaves as much as possible).
(iii). Dry the remaining litter material to constant mass (65 ^{0}C) and weight to the nearest 0.01 g.

II. Fungal sporulation activity

Objetive: To assess the fungal sporulation activity by identifying and counting aquatic hyphomycete spores released from Leaf samples (10 leaf discs per sample) and litter in short-term laboratory incubations.

(i). Place leaf discs in a Petri dish containing 20 mL of filtered (GF⁄ F filters) streamwater.
(ii). Induce sporulation by gentle shaking on an orbital shaker at 10 ^{0}C.
(iii). After 24 h, remove leaf discs frozen at -20 ^{0}C.
(iv). Transfer the spore suspension to a plastic tube and preserved with 3 mL of 37% formaldehyde, than rinse Petri dish with 2 mL of distilled water to collect spores adherent to the Petri dish. Add the rinsing water to the tubes, and adjust the volume with distilled water to a total of 50 mL.
(v). Add 1 mL of Triton X-100 (0.01 % solution) before stirring the suspension for at least 20 min and filter 5 or 10 mL through a membrane filter (5 lm pore size), staing the spores with 0.1 % Trypan blue in 60% lactic acid and identifying and counting 200 of them at 200x in randomly chosen microscopic fields.
(vi). Scan the entire filter if there are < 200 spores.
(vii). Calculate daily spore biomass.

III. Fungal biomass

(i). Weight freeze-dried leaf discs weighed (±0.05 mg) and preserve in methanol ⁄KOH at 20 ^{0}C.
(ii). Leachate the lipids at 80 ^{0}C for 30 min under constant stirring. Transfer the leachates to solid-phase leachateion cartridges, acidified (pH 2–3) and pass it through the cartridges by applying a gentle vacuum. Before eluting ergosterol with isopropanol, rinse the cartridges and dry under a stream of air.
(iii). Monitor efficiency of leachateion of ergosterol by running standards in parallel. Purife ergosterol in the eluate and quantify by high-pressure liquid chromatography (HPLC; detection wavelength: 282 nm, flow rate: 1.5 mL s $^{-1}$, column temperature: 33 ^{0}C, injection volume of: 20 µlL).

Observations

(i). Daily spore biomass.
(ii). Mean dry-mass density.
(iii). Spore production (expressed on a mycelial biomass basis, in ng spore biomass per mg mycelial biomass per day).

Calculations

Daily spore biomass production calculation is based on species-specific spore biovolumes of the fungal species dominating the community in terms of spore numbers (accounting for >99% of all spores) and an average dry-mass density of 500 fg µm^{-3} (according to Findlay and Arsuffi, 39)

Calculate biomass of the mycelial from ergosterol content by applying a mean conversion factor of 5.5 µg ergosterol per mg of fungal dry mass (as described by Gessner and Chauvet, 43).

Statistical analysis

Differences between treatments can be tested with Tukey's HSD test following the ANOVA.

Experiment 3.3. Mycorrhizal fungi

Lack of adequate natural regeneration in forest gaps dominated by understory species resulting from singletree harvesting of dominant tree species is a serious concern in boreal forests. For example, natural regeneration of spruce in the *Vaccinium myrtillus*-dominated patches is almost nonexistent (88). Allelopathic phenolics, particularly catechol, *p*-hydroxyacetophenone, *p*-hydroxybenzoic, and protocatechuic acids, present in the humus were inhibitory to seed germination and seedling growth of spruce (89). A number of authors have attributed decreases in mycorrhizal formation to allelopathy, with dramatic consequences on tree water and nutrient uptake and consequently tree growth rates (33). It was noticed that phenolics have inhibitory effects on growth (17) and respiration (18, 90) of some spruce mycorrhizal fungi. Thus, Souto *et al*. (114) tested allelopathic effects of phenolics on growth and respiration of two mycorrhizal fungi.

Materials and equipments required

Humus, mycorrhizal fungi, nutrient medium, agar, dark incubator, Hansatech instrument with a Clarck-type oxygen electrode, Petri dishes, Erlenmeyer flasks

Procedure

I. Humus sampling

(i). Collect the samples from the forest with dominance of allelopathic species, which you consider inhibitor in canopy.
(ii). Collect randomly the humus samples from humic layer which contain most of the microorganisms, prepare a collective sample, 20 g of humus, in the field by mixing and passing through a 5 mm sieve.
(iii). Store the samples at +4^{o}C during transportation to the laboratory.

II. Phenolics analysis in humus

(i). In laboratory, shake three replicates of 20 g of humus (fresh weight) in 80 ml of acidified de-ionised water (adjusted to pH 3.8 by 2 N HCl) for 18 hr (dark, 15^{o}C).
(ii). After filtration, leachate phenolics from the acidified solution with ethyl ether and redissolve it in ethanol before HPLC analyzes. Place a short column of Novapak 18 C (50 mm x 3.9 mm ID) immediately before a 300- mm x 3.9-mm-ID column, filled with mBondapak 18 C. Carry out a linear gradient elution at a flow rate of 1.5 ml/min. Use 0.5% acetic acid in distilled water as solvent A, and acetonitrile with

0.5% acetic acid as solvent B. Use a gradient from 0% to 20% B over 45 min, followed by 15 min reequilibration with A.

(iii). Perform an identification and quantification of phenolic compounds by comparing retention times, wavelength detection, and peak areas to those of standard compounds (Gallet, 41).

III. Bioassays with phenolics mixtures

(i). In accordance to the natural concentration of phenolics in the humus, prepare two chemical mixtures with protocatechuic, *p*-hydroxybenzoic, vanillic, and ferulic acids (Sigma Chemical Co.).

(ii). The first mixture is a source of phenolics in fungi growth medium at quantities identical to those supplied in the experiment with humic solutions. The second, more concentrated mixture is for addition phenolics in amounts similar to those detected in the soil.

IV. Fungal material

(i). Select two mycorrhizal species because they are usually associated with the main autotrophic plants involved in the allelopathic interaction.

(ii). Carry out experiments with symbiont of the main allelochemical-producing plant and the symbiont of the target plant. Grow the fungi in a solid or a liquid modified Melin-Norkrans (MMN) nutrient medium.

V. Bioassays

(i). Prepare the same growth media for both growth and respiration experiments.

(ii). Prepare a first set with humic solution obtained as described above (500 g of fresh weight humus in 1 L of de-ionised water).

(iii). Add 800 ml of sterile humic solution (obtained by means of 0.22-mm filtration) to 800 ml liquid or 800 ml solid MMN in order to prepare 10 Erlenmeyer flasks containing 150 ml of growing medium (five replicates per species) or 20 Petri dishes containing 20 ml of the above medium (10 replicates per species).

(iv). Prepare a second set as described above but replace the humic solution with a buffered (pH 3.8) phenolic mixture containing 3.7×10^{-5} of protocatechuic, 1.72×10^{-5} of *p*-hydroxybenzoic, 3.8×10^{-5} of vanillic, and 4.91×10^{-4} g of ferulic acids, i.e. the same quantity of phenolics as in the first set. Because the first set preparation (and consequently the second one) implied a dilution (1/4) of phenolics compared to quantities naturally occurring in humus, prepare a third set as above but with a fourfold more concentrated phenolic mixture, still buffered (pH 3.8).

(v). Prepare respective controls for each set of experiments, with buffered (pH 3.8) de-ionised water instead of allelopathic solutions.

(vi). Inoculate the Erlenmeyer flasks and Petri dishes with a 5-mm-diameter agar disk cut from a fungal colony. Place in a dark incubator at ±26^{o}C for five weeks.

(vii). Measure a mycelium diameter for each plate, in one week interval.

(viii). After five weeks incubation, measure an oxygen utilization of fungal mycelium in flasks of controls and set A, polarographically with a Hansatech instrument (King’s Lynn, Norfolk, England), with a Clarck-type oxygen electrode. Set the fungal sample (1± 0.2 g fresh wt) in the measuring cell containing 1 ml of de-ionised, oxygen-saturated water (250 nmol/ml at 25^{o}C). After several minutes, oxygen consumption became stable, at a level corresponding to respiration than dry the sample at 105^{o}C for 48 hr to obtain its dry weight and to express respiration values as nanomolar O_2 per gram dry weight per minute. Measure the respiration of two samples per flask, with 10 replicates for each test (one fungus against one allelopathic solution).

Observations

(i). Phenolics content in humic solutions.
(ii). Mycelium diameter.
(iii). Oxygen utilization of fungal mycelium.

Statistical analysis

Compare the result on growth experiment and for respiration by using the Mann-Whitney U test for $P<0.005$.

Table 1. Statistical significance according Mann-Whitney U test for P <0.05 (bottom) of *Hebeloma crustuliniforme* when the growing media is supplied with humic solution (A), phenolic mixture as in A (B), or phenolic mixture as in the humus (C). *Significantly different for P < 0.05 (Souto et al. 2000)

Week	A	B	C	Control
A		*	*	
B				*
C				*

Experiment 3.2.1.2. To investigate the possible inhibitory allelopathic effects of alellopathic species on on ectomycorrhizal growth

These were investigated with following procedure (82):

Materials and equipments required

Leaf litter of allelopathic species from its site, leaf litter of tree species from forest sites without allelopathic species, Hagem's media, Petri plates, autoclav

Procedure

I. Sample preparation

(i). Conduct the decoction experiments by using solid Hagem's media modified by Cripps and Miller (25) with and without the addition of chopped leaf materials collected during summer and stored frozen until used.

(ii). Cut the leaves into fine pieces and incorporate into the media, then put it in autoclav for 18 min at 250°C.
(iii). Pour warm media on Petri plates, with constant agitation to evenly distribute the leaf materials among the plates.
(iv). After cooling, inoculate the decoction media plates and controls with the mycelium of ectomycorrhizal fungi initially grown on solid Hagem's media for 1 mo at 20°C. Cut the uniform squares of mycelium and agar from the plates, near the actively growing margin of the fungal mycelium, and use to center inoculate the bioassay plates. Immediately after inoculation, two perpendicular lines should be drawn on the lid of the plate.

II. Ectomycorrhizal bioassay experiment 'a'

(i). In this experiment include litter from allelopathic species and ectomycorrhizae species.
(ii). Conduct measurements over a period from 28 to 37 d at approximatelly one week intervals.
(iii). Conduct measurements 6 weeks after inoculation.

III. Ectomycorrhizal bioassay experiment 'b'

(i). In this experiment include different types of litter (alleopathic species, and tree species from forest without the allelopathic species).
(ii). Use mycorrhizae species.
(iii). Conduct measurements of radial growth of fungi along the pre-determined lines from the edge of inoculum block.

Observations

(i). Radial growth of fungi (mm).
(ii). Concentration of allelochemical compounds in litter from allelopathic plant.
(iii). Chemical composition of allelochemical compounds in litter from allelopathic plant.

Statistical analysis

Differences between mean radial growth on control plates vs. decoction media plates can be analyzed by using Students *t* tests for each fungal strain on each measurement date for the first experiment. Use analysis of variance for the second experiment to examine the main effects of inoculum species and the leaf material added.

Precautions

Provide an adequate number of replicates for statistical evaluation of results obtained in the study

Experiment 3.2.1.3. Comparison of responses of ectomycorrhizal fungi to allelopathic effects of species dominant in the area and from area without the allelopathic species.

It can be studies by following procedure proposed by Zeng and Mallik (130):

Materials and equipments required

Humus, fresh leaves, leaf litter, Melin–Norkrans' nutrient solution, Whatman No. 42 filter paper, Millipore filters, malt leachate, granulated agar, distilled water, Petri dishes, autoclave, Liquid Chromatograph (HPLC)

Reagents

Ferulic acid, *o*-coumaric acid, and *o*-hydroxyphenylacetic (o-HPA) acid, methanol, formic acid, acetone

Procedure

I. ECM Fungal Cultures

(i). Use modified Melin–Norkrans' nutrient solution (MMN) for growing the ectomycorrhizal fungi.
(ii). Inoculate Petri dishes containing the MMN, allelopathic plant leachates, and phenolic acids with 4 mm diam agar plugs cut from the edge of 2 weeks old ectomycorrhizal fungal colonies.
(iii). Inoculate the plates at room temperature (20–25 ^{0}C).
(iv). Measure diameters of the fungal colonies in five days intervals.
(v). Provide at least three replicates.

II. Donor species leaf leachates

(i). Collect fresh leaves (mature, 1year old), litter (dry leaves on the ground), and humus (0–10 cm) from an area with dominance of alellopathic species, from 10 random locations.
(ii). Prepare composite samples of fresh leaves, litter, and humus were made by mixing the 10 samples. Remove coarse roots and branches from the humus samples prior to mixing.
(iii). Soak twenty g of fresh leaves and litter were separately in 100 ml distilled water for 24 h at room temperature (20–25-C). Obtain approximatelly 0.2 g FW equivalent of leaves or litter per ml of leachate.

III. Donor species humus leachate

(i). Drain 200 ml distilled water at a slow rate for 24 hr through 100 g fresh humus in a glass chromatography column (30 mm) at room temperature.
(ii). Adjust the filtrate of humus leachate to 100 ml, to obtain concentration of 1 g FW equivalent of humus per ml of leachate.
(iii). Pass the water leachates of leaves, litter, and humus through Whatman No. 42 filter paper and then sterilize by passing through two layers of 0.45-mm Millipore filters.

IV. Effects of Donor species water leachates on ECM Fungi

(i). Prepare leachates as following: 0.1 g FW/ml leachate by adding 50 ml original leachate (0.2 g FW/ml) into 50 ml autoclaved MMN medium containing 1.5 g agar at 50–60 ^{0}C; 0.05 g FW/ml leachate by adding 25 ml original leachate and 25 ml sterilized water into 50 ml autoclaved MMN agar medium, and 0.01 g FW/ml leachate by adding 5 ml original leachate and 45 ml sterilized water into 50 ml autoclaved MMN medium.
(ii). Use humus leachate in concentrations of 0.25 and 0.05 g FW/ml prepared as following: mix the original leachates of humus (25 ml, 1 g FW/ml) and 25 ml sterilized water with 50 ml autoclaved MMN medium containing 1.5 g agar at 50–60 ^{0}C to obtain 0.25 FW/ml of humus leachate; 0.05 g FW/ml humus leachate was prepared by adding 5 ml original leachate and 45 ml sterilized water into 50 ml autoclaved MMN agar medium; prapre the control from half-strength MMN medium.
(iii). Pour the medium into 9 cm diam Petri dishes (20 ml medium in each dish). Cut a disk of inoculum (diam 4 mm) from each of the five ECM fungi from the edge of a 2- to 3-wk-old colony and inoculated in a Petri dish.
(iv). Keep the culture for 28 d at 20–25 ^{0}C.
(v). Conduct measurements on diameters of the fungal colonies.

V. Effect of pure phenolics on ECM Fungi

(i). For measurements of ECM fungal isolates responses to the allelopathic effects of the dominant species use phenolic acids from allelopathic species.
(ii). Obtain 2-mM solution of phenol acids by dissolving them in sterile hot water (80 ^{0}C).
(iii). Sterilize phenolic solutions by passing through two layers of 0.45-mm Millipore filters.
(iv). Mix equivalent volumes of a 2-mM phenolic solution and a double concentration MMN agar to obtain a medium with 1 mM phenolic acid and full-strength MMN. Obtain the equimolar mixture of the phenolic acids by mixing equivalent volumes of their respective 1 mM media for a final concentration of 0.33 mM for each acid.
(v). Obtain the control of only MMN medium.
(vi). Inoculate the Petri dishes containing the above-mentioned media with the ectomycorrhizal fungi with four replicates for each treatment.
(v). Inoculate the plates at 20–25 ^{0}C.
(vi). Conduct measurements of the diameters of the fungal colonies after 20 days.

VI. Phenolic reduction by ECM Fungi in pure culture

(i). Prepare a fungal culture on MMN agar medium in Petri dishes, at the center with one piece of 4 mm mycelial plug. (ii). Maintain the cultures at 20–23 ^{0}C, for 20 days.
(iii). Place two U-shaped glass rods (diam 2 mm on the surface of the medium. Than, spott acetone solutions of the phenolic compounds (10 mM, 200 ml) on a piece of

Whatman No. 3 filter paper (diam 12 mm). When the acetone completely evaporate, add 200 ml sterile water to the filter paper. Place filter papers over each rod.

(iv). Provide at least three replicates per treatment.

(v). Ten days later, take filter papers soak in 5 ml methanol for 24 hr. Concentrate the methanol leachates to 2.5 ml at 40 ^{0}C under reduced pressure and passed through Whatman No. 1 filter paper.

(vi). Analyze phenolic compounds in the filtrates by using a Varian Prostar HPLC equipped with a Chrompak column (250- 4.6 mm) and PDA detector (Model 330) monitoring the absorbance of the elution at 280 nm. Authors proposed following setting of the solvent system: 0–9 min, 10% methanol and 90% water containing 2.5% formic acid; 9–20 min, 40% methanol and 60% water containing 2.5% formic acid; 20–25 min, 60% methanol and 40% water containing 2.5% formic acid; 25 min, 100% methanol. Flow rate is to be 1.5 ml/min, and temperature of 32-C. For standards use pure compounds and identify the phenolic acids by comparison of retention times and UV spectrum.

VII. Detoxification bioassay

Objective: It is done to test whether inoculation of tree seedlings with ECM fungus reduce the phytotoxicity of phenolic acids from allelopathicspecies.

Procedure

(i). Since full-strength MMN medium itself inhibits seedling growth of tree associated species, all media should be a half strength MMN.

(ii). Prepare a fungal culture in MMN liquid medium (half strength) containing 0.2 and 0.5 mM examined phenolic acid. Each Petri dish (10 cm) should contained 15 ml liquid medium. Culture and inoculation should be conducted as described above, with noninoculated culture as the control.

(iii). Inoculate the plates at room temperature (20–23 ^{0}C).

(iv). Add 5 ml sterile water, after 15 days.

(v). Decante the culture solution from the dishes after 30 days, and pass through Whatman No. 42 filter paper.

(vi). Adjust the filtrates with sterile water to initial volume and used for the phytotoxicity test with tree seedlings. Each dish contain 20 surface-sterilized tree seeds placed on a piece of Whatman 3 filter paper and add 5 ml culture solution. Use the half-strength MMNmedium was used as negative control, and half-strength MMN medium with 0.2 and 0.5 mM phenolic acid, as positive controls.

(vii). Conduct the bioassay at 20–25 ^{0}C, with a 16 h photoperiod. Add 2 ml sterile water to each Petri dish during incubation.

(viii). Measure the lengths of tree primary root and shoot, after 15 days.

Observations

(i). Diameters of the fungal colonies (mm).

(ii). Length of tree primary root (mm).

(iii). Length of tree primary shoot (mm).
(iv). Concentrations of phenolic compounds.

Statistical Analysis

In case of normal distribution of data use one-way analysis of variance (ANOVA), and treat differences among means were tested at P = 0.01 with Duncan's multiple range test.

Experiment 3.4. Nutrient dynamics

Once in the soil, allelochemicals can affect soil nutrient dynamics by forming complexes with proteins and delaying organic matter decomposition and mineralization (21,53, 69, 81) and by increasing the soil microbial activity and N immobilization (13, 85, 116). These processes result in a decrease of the inorganic N available for plant uptake. Condensed tannins seem more involved in slowing degradation processes when they form stable complexes with proteins and inorganic N (85), whereas lower molecular weight phenolic acids are easily degraded by microbes (12,13,116). The ecological relevance of phenolic compounds can be of especial interest in N-limited ecosystems with slow decomposition, such as boreal ecosystems, where slow growing species with high concentrations of carbon-based secondary compounds predominate (97). The size of carbon (C) stocks in the soil is largely controlled through the antagonistic processes of input and decomposition of soil organic matter (SOM). Carbon input originates mainly from leaf litter, root litter, and root exudates. Approximately 85%–90% of organic-material decomposition in soils are mediated microbially, and about 10%–15% of the energy of organic C (OC) are utilized by soil animals (37,124). It is well accepted that phenolic compounds are an important source of C for heterotrophic organisms in forest ecosystems (108,114) and therefore soil phenols may limit N availability simply as a result of rapid immobilization of NH_4^+ and NO_3^-. De Luka *et al.* (27) conducted studies in order to determine whether net N mineralization and nitrification change are related to concentrations of free polyphenols in the forest floor.

Litter decomposition provides nutrients that sustain ecosystem productivity, but litter may also inhibit root proliferation. In order to assess roles of phytotoxicity and N immobilization in litter inhibitory effects, Bonanomi *et al.* (15) conducted a litter-bag decomposition experiment in which activated carbon (AC) was used to separate nutrient immobilization from phytotoxic effects (AC has been widely applied in allelopathic studies to adsorb and neutralize phytotoxic organic molecules while having a limited impact on mineral nutrients (83). Litter inhibitory effects were assessed by two bioassays: seed germination and root proliferation bioassays. Litter decomposition in open fields mainly depends on organic matter quality, water availability and temperature (45). Therefore, to analyze only the effect of litter quality, Bonanomi *et al.* (15) worked under controlled conditions, thus avoiding variations caused by changes in water availability and temperature. Experiments were carried out using litter-bags (9).

I. Litter decomposition experiment

(i). Decomposition experiments should carried out in microcosms placed in a growth chamber to simulate field decomposition conditions.

(ii). Fill large (20x20 cm) terylene litterbags (mesh size 2 mm) with 6 g of dry leaf litter and place inside square trays (30 cm deep; 100 cm wide).
(iii). Add a microbial inoculum, obtained by mixing 10 g of soil taken from the fields from which litter was collected (top 10 cm) and 90 g of water, in order to improve the start up of the decomposition process. Spray the microbial inoculum over the litter bag.
(iv). Keep microcosms in a growth chamber under controlled temperature (18 ± 2 ^{0}C : 24 ± 2 ^{0}C, night : day) and irrigate (watered every 7 d to holding capacity with distilled water) conditions.
(v). Detremine the litter water-holding capacity by soaking 5 g of litter in distilled water for 24 h, shaking off excess water, weighing the saturated material, drying in a oven to a constant weight and reweighing.
(vi). Harvest litter bags after 30, 90 and 180 d of decomposition and dry the bags in the laboratory (40 ^{0}C until a constant weight) and weight the remaining material.

II. Leachates

(i). Mix the dried litter material with distilled water in a beaker at 5% of dry weight (50 g l^{-1}) and shake for 5 h.
(ii). Centrifugate the aqueous suspensions (2395 g for 10 min), sterilize (microfiltration with a 0.22-lm pore filter), dilute with distilled water to three concentrations (50, 17 and 5 g g l^{-1} and store at -20 ^{0}C until used in the bioassay.

III. Seed germination bioassay

Objective is to assess how decomposition influences the inhibitory effects of different plant litter types.

(i). Place twenty seeds in 9 cm Petri dishes over sterile filter papers with 4 ml of test solution. Every solution plus the control with distilled suppose to be replicated for five times for each plant litter species.
(ii). Arrange the Petri dishes in a growing room in a completely randomized design
(iii). Measure the seedling root length 36 h after germination.
(iv). Express the data as inhibition of root growth (i.e. per cent difference in root length) in comparison to the control.

IV. Root proliferation bioassay

Objective is to determinate the capability of target species seedling roots to colonize the different litter types independently from the germination process.

(i). Apply a mineral nutrient solution and ⁄ or activated carsbon (AC) for assessment of the roles of both nutrient immobilization and phytotoxic effects by placing a 2-cm-wide sterile filter paper strip immediately above a wider (5-cm) strip in square Petri dishes (size 12x12x1.5 cm).
(ii). Separate the two strips by 5 mm of free space.

(iii). Place the target species seeds (five in each dish) over the 2-cm strip, and apply the different nutrient solutions, AC and litter to the lower 5-cm-wide strip in order to to prevent these materials from coming into contact with the seeds during germination.
(iv). Arrange the Petri dishes at a 45^0 slope on a horizontal surface so that the positive geotropism of roots would allow growth down along the plate.
(v). Cover the plates with opaque sheets when roots are under observation.
(vi). Prepare six relications for each treatment
(vii). Arrange the Petri dishes in a growing room according to a totally randomized design
(viii). Measure the seedling shoot and root lengths 10 d after germination.
(ix). Express the data as root and shoot growth (i.e. per cent difference in root length) compared with the control.

Bioassays should include a) the control, with sterile distilled water; b) the addition of a complete nutrient solution (half strength (2.15 g l^{-1} Murashige and Skoog Basal Salt Mixture; Sigma-Aldrich Co.), to assess whether the inhibitory effect is attributable to nutrient immobilization; c) the addition of AC (Sigma-Aldrich Co.) at 0.2 g per dish, to assess whether the inhibitive effect was attributable to phytotoxic compounds; and d) the combined application of the nutrient solution and AC (Authors applied all above treatments without (controls) and with litter on the lower strip; the upper strip with the seeds is only with distilled water).

Litter chemical analyses

Determine total C and N contents in litter by flash combustion of microsamples (5 mg litter) in Elemental Analyser.

Observations

(i). Shoot and root lengths (mm).
(ii). Total C and N in litter.
(iii). Concentration of allelochemicals in litter.
(iv). Decomposition rate k.

Calculations

Calcualte the seedling shoot : root ratio on the basis on shoot and root length measurements.

The litter negative exponential decay constant (k) should be calculated according to Berg and McClaugherty (2008) as follows:

$$M_t = M_0 e^{-kt}$$

Where, M_0: Initial litter mass; M_t: Mass remaining after a certain time t; k: Decay rate constant

For each plant spp, litter decay rate (k) should be calculated for 3-decomposition periods (0-30, 30-90 and 90-180 d).

Statistical analysis

Litter mass loss and changes in N content can be statistically evaluated by one-way ANOVA with levels of significance at $P < 0.05$ and $P<0.01$.

Experiment 3.5. Soil respiration

The influences of allelopathy in natural and managed ecosystems on plant productivity and crop production has been studied (101, 104). The decline in the growth of repeated plantation woodlands may have been caused by some factors which are directly involved in, such as soil fungi, allelopathic chemical effects, and also by indirect effects, such as interactions between allelopathic chemicals and soil microorganisms, as well as through soil nutrient dynamics. For example, Zhang (131) found that soil leachates from replanted woodlands significantly inhibited soil non-pathogenic fungi growth, reduced soil respiration activities, and net soil nitrogen mineralization rates. Thus, author concluded that the allelochemicals from soil leachates, rather than pathogenic fungi, are the key factor regulating the productivity and nitrogen cycling in repeated plantation woodlands. Therefore, Yuste *et al*. (127) suggested the experiment procedure to test effects of allelochemicals on soil respiration based on field respiration measurements:

Materials and equipments required

LI6400-09 soil chamber connected to an LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA), soil thermistor, frequency domain reflectometry sensors (Theta Probe model ML2-X; Delta-T Devices, Cambridge, UK), reflectometer (Moisture Point, model 917; E.S.I. Environmental Sensors Inc., Victoria, Canada)

Procedure

Field measurements

(i). Define both experimental and control measuring plots (20 x 20 m^2).
(ii). Define the understorey soil respiration area, in the the vicinity of the trees, 3 m away from the trunk.
(iii). Define the control area (open soil respiration far from the tree influence, at least 20m away from trees.
(iv). Use collars with a height of 4.4 cm and a diameter of 11 cm inserted into the soil for measuring soil respiration. Yuste *et al*. (127) suggested 30 collars to cover the spatial variability of soil respiration.
(v). Conduct measurement of soil respiration (three to four rounds in a day).
(vi). Measure soil temperature at 5cm in soil profile with a soil thermistor next to each collar
(vii). Measure volumetric soil moisture content, at several depths (5, 10, 20 and 50 cm) in the soil with frequency domain reflectometry sensors.
(viii). Prepare profiles of soil moisture (0-15, 15-30, 30-45 and 45-60 cm) by using using an enhanced time domain reflectometer. It is possible to instal moisture sensors at 10 cm, both in the control plot and experimental plot for continuously measuring soil moisture at 5 min intervals with the aid of variable dataloggers.
(ix). Collect samples of soil, litter for analysis of allelochemicals.

Observations

(i). Soil respiration.

(ii). Soil temperature.

(iii). Volumetric soil moisture content.

(iv). Content of allelochemicals in soil.

Calculations

Soil respiration was calculated from the initial slope in CO_2 concentration increase as a function of time (normally 40 s time interval) within the closed loop (72):

$$Fc = (dCO_2/dt - a) \times 1/t/Vs$$

Where Fc: Total soil CO_2 evolved from the soil sample during the sampling interval (mmol), dCO_2/dt : Change in CO_2 concentration (ppm) within the system during the sampling interval, t: Sample interval (s), a: Intercept of linear function and Vs:Volume of system (L). Volume of system was calculated by injecting in the system a known quantity of CO_2 and applying the following dilution function

$$Vs = R \times T/P \times \Delta CO_2/ppm_f$$

Where, Vs: Unknown volume of system (L), R:Universal Gas Constant (8.31×10^{-3} L kPa $mol^{-1}K^{-1}$), T: Observed temperature (K) and P: Air pressure (kPa) at measurement time, DCO_2 : Known injected quantity of CO_2 (60mL of air with 600 ppm concentration of CO_2 50.94 mmolCO_2) and ppmf the final CO_2 concentration within the closed system.

Before CO_2 addition, the system was flushed with N to achieve zero CO_2 concentration. A second syringe was installed as buffering volume avoiding overpressure in the system when injecting the 60mL air. Volume of system was also corrected by pore space of soil sample in the closed system based on the bulk density of sample and the density of mineral particles (2.65 g cm^{-3}), assuming free pore space for dry samples and saturated pore space for wet samples.

Statistical analysis

In case of normal distribution of data use one-way analysis of variance (ANOVA), and treat differences among means were tested at P = 0.01 with Duncan's multiple range test.

Precautions

(i). Soil respiration suppose to be measured twice a month at both control and experimental plots

(ii). Prior to measure the volumetric soil moisture content, calibrate the reflectometry sensors by using the gravimetric method.

Experiment 3.4.1. Effects of plant litter quality on soil microbial attributes and plant biomass decomposition.

It may be tested by Jacinthe *et al.* (65) with following procedure:

Materials and equipments required
Leaf litter, soil, glass jars, inductively coupled plasma atomic emission spectroscop ICP, CHNS analyzer, distilled water, plastic bags

Reagents

Ammonium acetate

Procedure

I. Sampling

(i). Collect soil and plant biomass samples.
(ii). Select experimental plots in forest stands (separated by 50-500 m from each other).
(iii). In each plot (plant community), establish a triplicate study plots (10 m × 10 m).
(iv). To carry out the microbial assay, collect composite soil samples (0-10 cm; both underneath and between plants) from each study plot (6 sampling points per plot), transport them to the laboratory in plastic bags and store at 4°C.
(v). Use dry soil subsamples for determination of soil chemical properties: soil pH (1:2 soil to water ratio); exchangeable bases (extraction with 1 M ammonium acetate pH 7 and quantification by using inductively coupled plasma atomic emission spectroscopy; soil organic C and N (determined by dry combustion at 850°C, by using CHNS analyzer).

II. Field experiment (plant biomass decomposition)

(vi). Collect subsamples of plant biomass for analyzes of lignin, cellulose, phenolics, and total C and N (CHNS analyzer or by any other analytical prodecure).
(vii). Incubation experiment involve soil types from selected communities and types of plant matter.
(viii). For each soil type, a control (with no plant biomass added) has to be included. Each treatment supposed to run in triplicate.
(ix). A content of approximately 25 g of fresh soil supposed to be mixed with 0.5 g of leaves (shred to 2 to 3.44 mm size) and incubate (900 mL glass jars, 19°C) for 21 days.
(x). Jar headspace supposed to be sampled periodically, to determine CO_2 concentration.

Observations

(i). Rate of CO_2 production in experimental plots.
(ii). Rate of CO_2 production in control plots. The rate of CO_2 production in the controls is used as a measure of basal soil respiration (BSR).
(iii). Concentrations of lignin, cellulose, phenolics, and total C and N in litter.
(v). Concentrations of allelochemicals in litter.
(vi). Concentrations of allelochemicals in soil.
(vi). pH in soil.

Calculations

The amount of CO_2 attributable to the decomposition of added plant biomass is computed as CO_2 produced in the treatment minus that produced in corresponding controls. Cumulative CO_2 (Ct) produced is fitted to the non-linear model Ct=Co $(1^{-\exp{-kt}})$ where k is the rate constant (d^{-1}) and Co is pool of readily decomposable C in the plant biomass (mg C g^{-1} plant biomass).

Statistical analyses

In case of normal distribution of data use one-way analysis of variance (ANOVA), and treat differences among means were tested at P = 0.01 with Duncan's multiple range test.

Precautions

Well absorbance readings should be normalized by dividing the absorbance of each well by the average color of all wells in a plate, as suggested by Garland (42) and Grayston *et al*. (50).

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