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Distribution of chemical compounds in different soil layers of rhododendron forest

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ABSTRACT

The distribution of chemicals in forest soil layers is poorly understood. Hence, using GCMS we identified the occurrence of main chemical compounds and their relative composition in 3-different soil layers (litter, humus and subsoil layers) of rhododendron forest. We found that the total contents of main chemical compounds in rhododendron forest followed the order litter > humus > subsoil layers. In all these 3-layers, the primary chemical was palmitic acid. The higher fatty acids and alcohols significantly differed among the layers. The mannitol and pentadecanoic acid showed the largest differences and their contents were highest in humus layer. This study showed that the litter layer has more allelochemicals than humus and subsoil layers. These results can be very important for ecosystem management and protection of rhododendron forest.

Key words: Allelochemicals, allelopathy, chemical compounds, GCMS, palmitic acid, phenolics, rhododendron forest, soils, soil layers.

INTRODUCTION

Allelopathy affects the natural regeneration of forest communities (19,23,47). The allelopathic compounds in soil exerts important influence on the soil environment and plant growth (3). Because these compounds can strengthen the competitiveness of one specie over another, or that of one individual plant over others, thus leading to altered community and population structures (7,18,34). The allelopathy either promotes or inhibits the growth and development of neighbouring plants under a plant canopy by releasing chemicals into the environment (6,22,26,36,37).

Forest soil generally has thick litter and humus layer, so it is necessary to study the distribution of allelochemicals in these layers to understand the status and functioning of forest soils. However, further in-depth empirical research on both the humus and mineral layer is still needed (8,33), especially in less studied forests. Forest soils have litter and humus layers and the allelochemicals distribution in soil-related layers is very important for functioning of forest soils. A thick litter layer inhibits the seedling emergence and establishment and adversely affects the natural regeneration of forest (1,21,26,28,33,41,44). However, the allelochemical research on litter layer is still needed (14,40), especially in natural forests.

Baili Rhododendron Natural Reserve, northwest of Guizhou Province (China) is the largest natural rhododendron forest in the same latitude and low-middle altitude areas. At present, rhododendron forest, a subtropical evergreen broad-leaved forest, failed to regenerate naturally thus finding out the distribution of allelopathic substances in different

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soil layers can further clarify the causes of degradation and thus help to protect this specific forest. However, compared with other forest types, relatively little is known about the chemical composition of the litter, humus subsoil layers in the rhododendron forest. More study is needed to better understand the identity of ecologically important chemical compounds. This study aimed to investigate the distribution of chemical compounds in 3- ground layers (litter layer, humus layer and subsoil layer) of wild rhododendron forest at Baili Rhododendron Natural Reserve, northwest of Guizhou Province, China. Therefore, this study can provide deep understanding of forest allelopathy through the soil chemicals and the information to protect the secondary subtropical forests and the ecosystem management.

MATERIALS AND METHODS

I. Study site and soil sampling

Baili Rhododendron National Forest Reserve (105°52'-106°03' E, 27°10'-27°20' N) is located in northwest Guizhou Province, China. Elevation : 1060-2200 m. Annual Mean temp : 11.8°C and annual rainfall : 1000-1100 mm. The forest communities are dominant rhododendron species (e.g., *Rhododendron delavayi* Franch., *Rhododendron agastum* Balf. f. et W. W. Smith, and *Rhododendron irroratum* Franch.); other plants include tree seedlings, shrubs and herbs. The soils are mainly haplic acrisol with clay top.

In wild rhododendron forest (dominated by one species of rhododendron, e.g., *Rhododendron delavayi*, *Rhododendron agastum*, and *Rhododendron irroratum*) (Figure 1 A, B, C), samples of 3-layers (i). Litter (12 cm thick), (ii). Humus (0-10 cm in depth) and (iii). Subsoil (S, 10-20 cm in depth)] were collected under the 50-years old tree canopy in December 2016 from the nine plots (each 10 m × 10 m) (Figure 1D). Each rhododendron forest (e.g., *Rhododendron delavayi* Franch., *Rhododendron agastum* Balf. f. et W.W. Smith and *Rhododendron irroratum* Franch.) fixed 3- plots, from each plot 3- samples (litter, humus and subsoil) were taken. The subsoil samples were collected from a pit [30 cm × 30 cm × 20 cm (length × width × depth)] dug along the four side directions and mixed to form a composite sample. These soil samples were air-dried, then ground into powder with pulverizer and stored at 4 °C for further analysis of chemicals.

II. Preparation of samples

The pulverized samples (200 mg) were each placed in 10-ml centrifuge tube, to which 80 μL of adipic acid (235.2 $\mu g g^{-1}$) and 5 ml methyl alcohol-water-chloroform solution (v:v:v = 5:3:2) were added. The mixed solution was centrifuged for 10 min at 6000 RPM to allow sampling of 0.5-ml supernatant liquid in a 5-ml centrifuge tube after 30 min of ultrasound-assisted extraction. The supernatant liquid was dried in nitrogen for the derivation treatment and the dried samples were added the 200 μL methoxyamine-hydrochloride solution at 20 to 50 $\mu g\ min^{-1}$, and then reacted in water bath at 37 °C for 90 min after being swirled for 1 min. 100 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the mixed solution, swirled again for 1 min, then reacted in the water bath at 37 °C for 60 min. After 1 h at room temperature, the solution was filtered through 0.45-μm filter membrane and assayed using GC-MS.

III. Instruments, reagents and chemical Analysis

The instruments used was GC-MS (7890A-5975C, Agilent, USA) equipped with a multifunctional automatic sampler with CTC. The main chemical reagents and standard

substances were : trichloromethane (chromatographic grade, TEDIA) and methyl alcohol (chromatographically pure, TEDIA), with adipic acid serving as the internal standard (purity ≥ 99.5 %, Sigma-Aldrich).

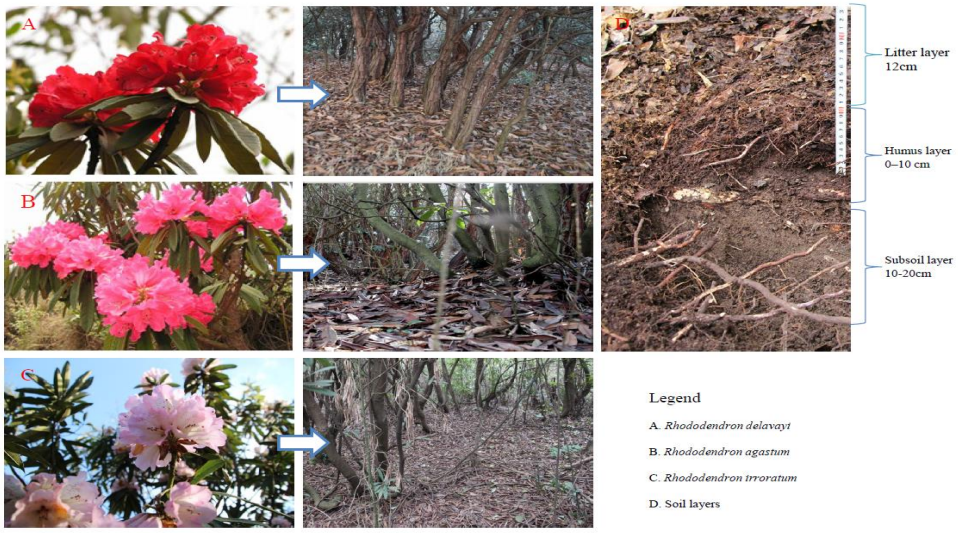

Figure 1. Rhododendron-dominated communities in Baili Rhododendron Natural Reserve

IV. Analysis of chemical compounds in different soil layers

The GC was conducted on HP-5 MS capillary gas chromatography columns (60 m × 250 μm × 0.25 μm), with its injection port temperature, sample size, split ratio, and column flow velocity set to 280 °C, 1 μL, 10:1, and 1.0 mL min^{-1}, respectively. Temperature programming was conducted as follows: the temperature was held at 60°C for 4 min, then increased to 280°C at 5°C min^{-1}, and held at that temperature for 5 min. The MS was done under the following conditions: temperature of the ion source, temperature of the quadrupole, ionization energy, and temperature of the transmission line were respectively 230°C, 150 °C, 70 EV, and 280°C; full-scanning mode with the mass range of 36 to 600 aum for the collection with a solvent delay of 15 min. The MS libraries used to identify the compounds were NIST08 and Wiley08.

By retrieving the peaks from the total ion chromatograms (TICs) in the MS computer data system and checking the standard mass spectra in Nist08 and Wiley08, the chemical components of each phase were determined (Figure 2). Then, the contents of each substance were measured for their respective amounts by using the internal standard method, in which adipic acid served as a reference without considering calibration factors; that is, $F = 1.00$ for all compounds. The formula used was :

$$m_i = F \times m_{IS} \times A_i/A_{IS},$$

Where, m_i : Amount of identified compound, m_{IS} : Amount of added internal standard, A_i and A_{IS} are the peak areas of the identified compound and internal standard, respectively.

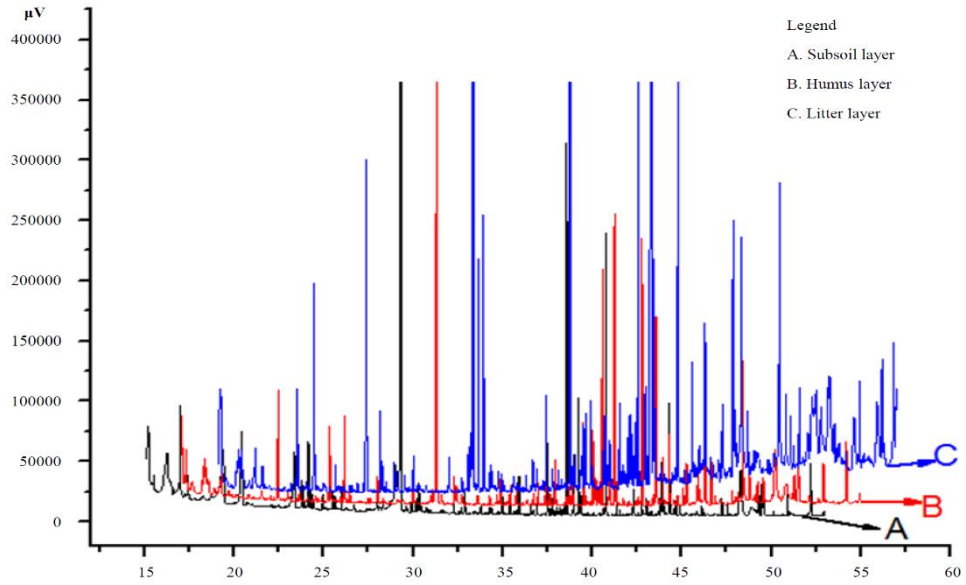

Figure 2. GC-MS chromatogram of chemicals in three different soil layers of rhododendron forest

Statistical analysis

For data analysis and figure drawing, we used software programs: SPSS v19.0 (IBM, USA), and Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). Partial least squares discrimination analysis (PLS-DA) was used to analyze the relationship between allelochemicals and soil samples to verify difference between the samples by SIMCA v13.0 (Umetrics, Umeå, Sweden). At the same time, the variable importance plot (VIP) is used to measure the influence intensity and explanatory power of each allelochemical on the classification and discrimination of the samples, to assist in the screening of the allelochemicals (chemicals with VIP >1.0 were selected as differentially allelochemicals) (46).

RESULTS AND DISCUSSION

Identification of chemicals in different layers

Total 27 primary chemicals were identified in litter, humus and subsoil layers of rhododendron forest. In the subsoil layer, the main chemicals were : palmitic acid, lactic acid, glycolic acid, stearic acid, and 3-picolinic acid. The main chemicals in the humus layer were : palmitic acid, glycolic acid, lactic acid, glycerol and inositol, while the litter layer had : palmitic acid, glycerol, inositol, stearic acid, and glycolic acid (Table 1). The total quantity of all chemicals in litter, subsoil, and humus layers was 709.88, 493.44, and 347.49 ng g^{-1}, respectively (Figure 3). These results showed that the main chemical components in the 3-layers of rhododendron forest differed considerably.

Table 1. Qualitative and quantitative analysis of primary chemicals in different layers

No.	Compound name	Retention time /min	Formula	Match	RSD / %	Relative concentration (ng g^{-1})		
						Litter layer	Humus layer	Subsoil layer
1	Lactic acid	17.05	$C_3H_6O_3$	96	11.2	9.74 ± 1.90	67.25 ± 14.48	61.03 ± 7.92
2	Glycolic acid	17.47	$C_2H_4O_3$	93	11.8	56.07 ± 4.15	71.48 ± 15.07	38.55 ± 3.69
3	Glycerol	23.36	$C_3H_8O_3$	92	12.0	132.13 ± 12.13	43.47 ± 3.85	21.65 ± 1.85
4	3-Picolinic acid	23.82	$C_6H_5NO_2$	98	11.4	14.65 ± 1.25	30.27±3.21	24.33 ± 3.82
5	Phenylacetic acid	23.97	$C_8H_8O_2$	84	5.9	3.39 ± 0.25	3.16 ± 0.55	1.57 ± 0.14
6	Succinic acid	24.33	$C_4H_6O_4$	94	13.5	3.90 ± 1.18	3.98 ± 0.30	2.17 ± 0.27
7	Glyceric acid	24.96	$C_3H_6O_4$	86	6.7	3.95 ± 0.77	4.49 ± 0.22	3.08 ± 0.44
8	Malic acid	29.05	$C_4H_6O_5$	96	11.8	4.36 ± 0.45	5.58 ± 0.24	3.66 ± 0.55
9	Proline	29.93	$C_5H_9NO_2$	97	12.3	12.20 ± 5.42	10.54 ± 3.78	18.12 ± 1.03
10	3-Hydroxybenzoic acid	30.84	$C_7H_6O_3$	92	14.2	0.81 ± 0.11	0.97 ± 0.06	1.10 ± 0.20
11	Threonic acid	30.95	$C_4H_8O_3$	89	11.4	0.79 ± 0.09	1.12±0.08	0.81 ± 0.15
12	4-Hydroxybenzoic acid	32.29	$C_7H_6O_3$	94	10.0	2.60 ± 0.18	2.65 ± 0.12	1.89 ± 0.23
13	4-Hydroxyphenylacetic acid	32.57	$C_8H_8O_3$	97	11.1	0.49 ± 0.06	0.60 ± 0.04	0.45 ± 0.08
14	Protocatechuic acid	36.59	$C_7H_6O_4$	90	12.3	23.65 ± 3.31	9.36 ± 0.47	6.17 ± 0.64
15	Myristic acid	36.92	$C_{14}H_{28}O_2$	98	10.1	14.64 ± 1.15	8.41 ± 0.41	3.77 ± 0.22
16	Pentadecanoic acid	38.18	$C_{15}H_{30}O_2$	84	9.0	4.57 ± 0.19	7.49 ± 0.85	1.89 ± 0.20
17	Palmitic acid	40.79	$C_{16}H_{32}O_2$	93	8.4	189.27 ± 10.14	92.08 ± 3.33	81.02 ± 5.36
18	Inositol	42.31	$C_6H_{12}O_6$	97	10.9	61.16 ± 7.17	34.41 ± 2.63	6.97 ± 1.10
19	Mannitol	43.2	$C_6H_{14}O_6$	90	9.1	3.96 ± 0.57	15.34 ± 2.16	1.96 ± 0.40
20	Linoleic acid	43.84	$C_{18}H_{32}O_2$	93	11.2	20.06 ± 1.98	2.48 ± 0.22	1.26 ± 0.11
21	Oleic acid	43.91	$C_{18}H_{34}O_2$	92	10.5	28.60 ± 1.85	9.91 ± 0.84	5.57 ± 0.77
22	*α*-Linolenic acid	44.02	$C_{18}H_{30}O_2$	95	12.8	3.77 ± 0.14	3.63 ± 0.45	1.60 ± 0.07
23	Stearic acid	44.32	$C_{18}H_{36}O_2$	93	10.2	57.66 ± 1.59	29.30 ± 1.15	31.18 ± 2.66
24	Eicosanol	46.14	$C_{20}H_{42}O$	89	13.5	2.71 ± 0.09	4.38 ± 0.49	4.12 ± 0.67
25	Eicosanoic acid	47.57	$C_{20}H_{40}O_2$	84	11.8	19.62 ± 0.72	5.71 ± 0.23	3.02 ± 0.60
26	Behenyl alcohol	49.31	$C_{22}H_{46}O$	95	10.6	12.56 ± 0.50	15.29 ± 1.14	14.22 ± 2.17
27	Docosanoic acid	50.92	$C_{22}H_{44}O_2$	97	9.8	22.59 ± 0.90	10.09 ± 0.49	6.34 ± 1.30
	Major Classes of Primary Chemicals							
(i)	Higher fatty acids					171.51	77.02	54.63
(ii)	Organic acids					96.84	187.33	135.2
(iii)	Phenolic acids					216.81	105.66	90.62
(iv)	Amino acids					12.20	10.54	18.12
(v)	Alcohols					212.52	112.88	48.92
	Total					709.88	493.44	347.49

Note: 1) Means with their standard errors (n = 9) in brackets; 2) Matching rate refers to that between ion source EI and the standard MS library NIST08. When the matching rate with NIST08 was < 50%, Wiley08 was instead applied; 3) RSD refers to the mean of each sample after triplicate testing; 4) Relative content represents that relative to adipic acid, the interior standard (assuming that the correction factor was 1).

Using GC-MS, 5-types of chemicals were identified in litter, humus and subsoil layers and some of them are known allelochemicals (35). Figure 4 shows, the contents of five types of chemicals: higher fatty acids, organic acids, phenolic acids, amino acids, and alcohols in different layers. Among them, organic acids and phenolic acids were dominant in the subsoil and humus layers, while, phenolic acids and alcohols were dominant in litter layer. The chemical components in litter layer significantly differed from those in humus and subsoil layers, except the amino acids. Higher fatty acids and alcohols differed significantly in 3-layers. The relative content of phenolic acids and alcohols in litter layer were about 2 folds than in subsoil or humus layer (Figure 4).

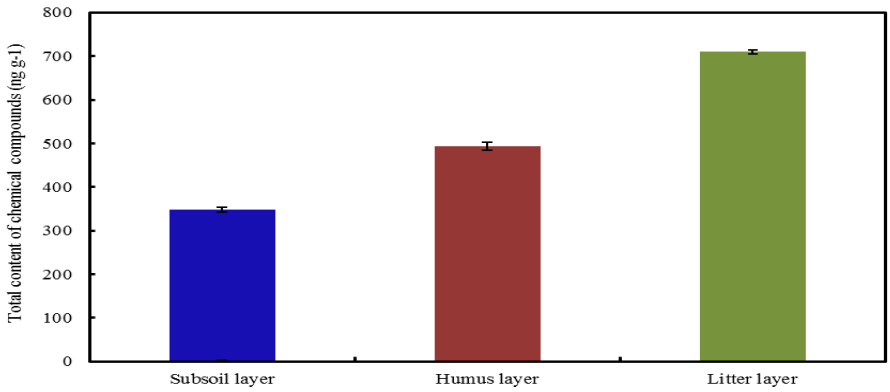

Figure 3. Total content of chemicals in different layers of rhododendron forest
Note: Error Bars are Standard Errors of the means, n = 9.

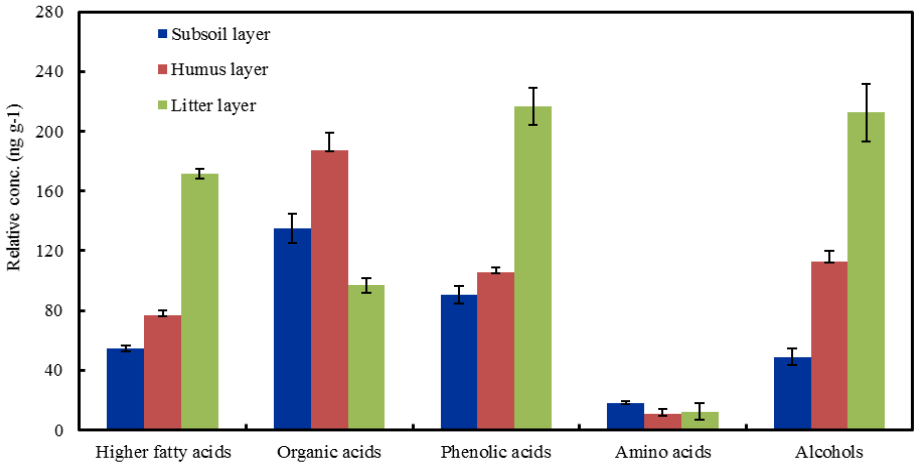

Figure 4. Relative concentrations of chemicals in different layers in of rhododendron forest
Note: Error Bars are Standard Errors of the means, *n* = 9.

Distribution of chemical compounds in soil layers

PLS-DA scatter diagram for the chemical contents, showed significant differences between the subsoil layer and other two layers, but, there was no significant difference between litter and humus layers (Figure 5A). This indicated certain non-random differences among the chemicals' distributions in the soil of the rhododendron forest. Generally, the R^2 intercept and Q^2 intercept limits for a valid model should be less than 0.4 and -0.05, respectively (11). Our intercepts for R^2 and Q^2 were 0.070 and -0.288, confirming the validity and reliability of the model (Figure 5B).

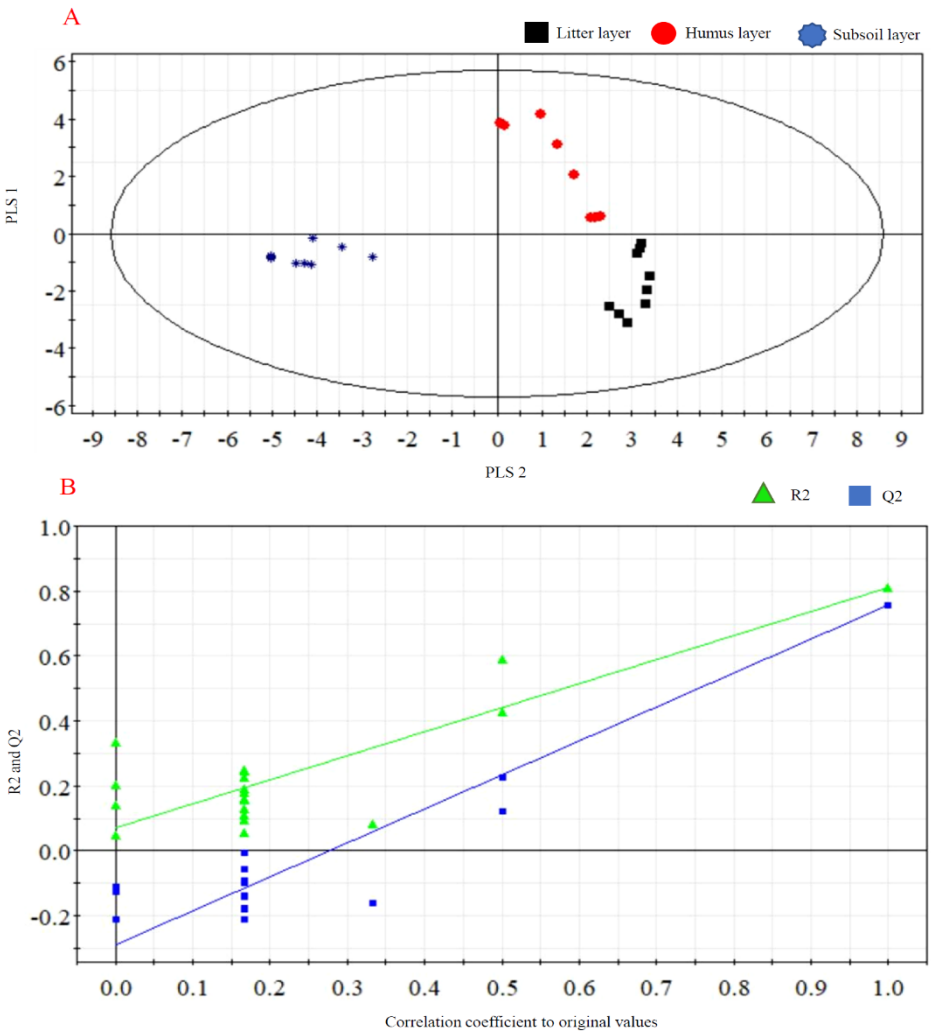

Figure 5. PLS-DA based on the chemical compounds in three soil layers of rhododendron forest. PLS-DA scores plots (A) and the permutation tests (B)

Variable importance for the projection (VIP) can quantify the contribution of each variable to the sample classification and VIP values > 1.0 presumably contributed to a clear differentiation of the samples (30). Since the VIP values of 12 substances > 1.0, this showed significant differences in rhododendron forest (Figure 6). Mannitol and pentadecanoic acid showed the largest difference (VIP-values >1.5) and their contents were ranked as humus > litter > subsoil layers.

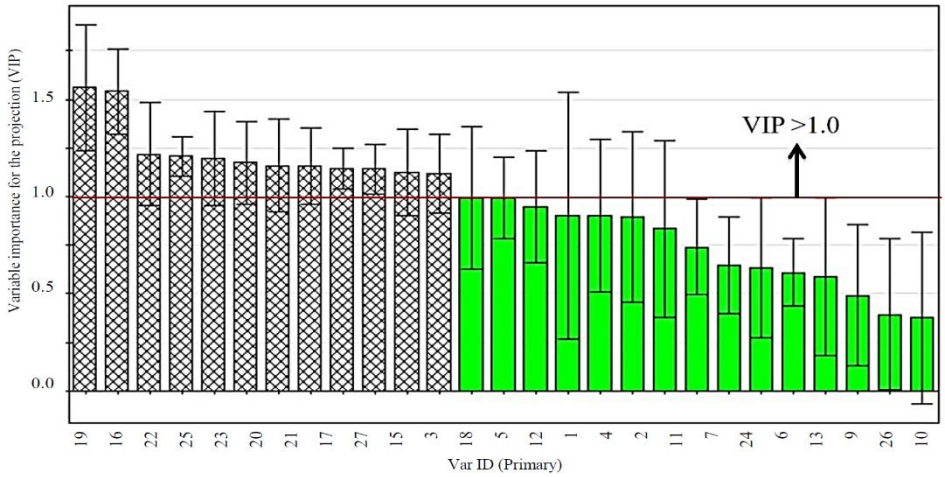

Figure 6. VIP of chemicals in three layers of rhododendron forest. The numbers of Var ID correspond to the numbers in Table 1.

Earlier studies found that the chemicals and soil microorganisms in the wild rhododendron forest community inhibits the seed germination (9,31,43). Moreover, some chemicals in the litter leachates inhibits the seed germination and seedlings growth (31) and also exerts allelopathic effects on soil microorganisms in wild rhododendron forest (9,43). Since the allelochemicals produced by dominant plant species continuously accumulates in the subsoil, thereby they influence the surrounding soil properties and growth of understory vegetation (21,26,38). By analyzing the contents of chemical compounds in different layers of rhododendron forest in China, we identified 5- types of chemicals in all litter, humus and subsoil layers. The total concentration of chemical compounds followed the order : litter > humus > subsoil. The litter layer chemical compounds significantly differed from humus or subsoil layers (except amino acids). This study also determined the differences in the chemical compounds among soil layers. Moreover, the VIP values of 12 chemicals were > 1.0, which significantly distinguished the chemicals in 3-soil layers. Litter and humus layers are important for forest regeneration (13,39). Allelochemicals in the litter, humus and subsoil are important in plant-soil interactions and some of these compounds could influence the humus and soil properties (8,24). However, various layers have different effects. For instance, seed germination and seedling growth can be inhibited by compounds in litter and humus layers, but the subsoil affects the root growth of seedlings (29,42). In this study, chemicals in litter layer seem to be more important in forest soil ecosystems.

Chemical compounds in plant-soil system and their effects on forest regeneration

In this study, we identified 27 compounds in rhododendron forest in China, with phenolic acids and alcohols as the main allelochemicals in litter layer and mannitol, pentadecanoic acid (VIP > 1.5) and phenolic acid as the major constituents of soil humus (Figure 4). Alcohols, fatty acids, organic and phenolic acids are very important in soil processes, because they affect the forest regeneration (15,16). The main allelochemicals in

litter layer were phenolic acids and alcohols. Mannitol and pentadecanoic acid showed the largest differences among other chemicals (VIP > 1.5). Pentadecanoic and palmitic acids are important compounds in plant roots, litter and microbial residues in soils (17,48). They are substrates for microbial metabolism serving as intermediates for biogeochemical reactions in soils (25,32). The plants and their ectomycorrhizas can affect the allelochemicals in forest soil (oxalic, citric, malonic, succinic, acetic, formic and lactic organic acids) (16) i.e. they, can increase the concentrations of some simple organic acids (glycolic acid). Generally, phenolic acids and alcohols allelopathic effects inhibits the seed germination and seedling growth of plants (2,4,5,6,20,27,35,45).

The allelochemicals may inhibit the natural regeneration of rhododendron species and finally reduced the understory biodiversity. The litter layer plays vital role in forest ecosystems and it contributes the largest nutrients and carbon input of forest soils (12,49). However, litter layer is also the major source of plant-derived allelochemicals (alcohols and phenolic acids) in subsoil, which may inhibit the forest regeneration. Water-soluble chemicals in the litter layer are released through the rainwater as leachates and enter in the subsoil, where they can be decomposed by microorganisms (10,49) showing the toxic effect on growth of understory plants species.

CONCLUSIONS

Chemical compounds found in litter, humus and subsoil layers negatively affected the natural regeneration of rhododendron forest. Palmitic acid was the primary chemical in all three soil layers, whereas mannitol and pentadecanoic acids were also important chemicals in these soil layers. All allelochemicals were mainly present in litter layer, where they may inhibit the forest regeneration. However further experiments on the action of the particular chemical compounds on the plant species, needs to be evaluated. These findings may have implications for forest management by removing the source of chemicals from litter layer promoting the natural regeneration of rhododendron forest.

ACKNOWLEDGEMENTS

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