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Allelopathic potential of *Rhododendron formosanum* Hemsl in Taiwan

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ABSTRACT

Bidens pilosa was used as test species for greenhouse and laboratory experiments. The leaves powder of *R. formosanum* mixed in soil at 1% concentration, moderately suppressed the growth of *B. pilosa* seedlings. Aqueous leachates of *R. formosanum* flowers, leaves, litter and organic matter inhibited the radicle growth of *Ageratum houstonianum*, *Amaranthus inamoenus*, *Brassica chinensis*, *Bidens pilosa*, *Lactuca sativa* and *Ocimum basilicum*. The leaves aqueous extract contained phytotoxins [*p*-hydroxybenzoic acid, trans *p*-coumaric acid, syringic acid, vanillic acid, *cis* ferulic acid, methyl ferulate, coumarin, protocatechuic acid and (-)-catechin]. These findings and identification of phytotoxins suggests that lack of understory species beneath *R. formosanum* canopy was due to allelopathic effects.

Key Words: Allelochemicals, allelopathy, *Castanopsis cuspidata* var. *carlesii*, extract, leachate, phenolics, phytotoxin, *Rhododendron formosanum*.

INTRODUCTION

A variety of secondary plant metabolites released into environment from one plant may suppress or stimulate the growth of another plant or its own sharing the same habitat (39). The phenomenon called *Allelopathy* was coined from two Greek words by Molisch in 1937 (29). The scientific research on allelopathy has been increasingly recognized as playing an important role in plant interference (30) and has been elucidated as the mechanism in the processes of vegetation formation, such as dominance, succession, climax, and productivity (8,31,38,39). The metabolites are released into environment through volatilization, leaching, decomposition of plant residues in soil and root exudation (6,9,14,30). In different vegetation, the mode of release of allelopathic substances into the environment may be depended on the plants and environmental conditions. In the subtropical and tropical regions of Taiwan, the processes of leaching and decomposition of plant residues in soil are particularly pronounced (9,10,12,13,42).

Rhododendron species (Ericaceae family) are ubiquitously distributed in Taiwan. Some species are used for ornamental or horticultural purposes, while other species (*R. formosanum* and *R. rubropilosum* var. *taiwanalpinum*) are growing naturally in mountains (1000 m to 3000 m above sea level). It exhibits a pure stand and lacks understory species. Two study sites were selected, Yuan Yang Lake (2003 and 2007) and

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Sun Link Sea (2007 and 2008). A control plot was selected in the adjacent area of *Rhododendron* Sun Link Sea site, where field measurements on diversity and coverage of understory species were recorded. On the floor of *Rhododendron*, exceedingly low plant diversity and coverage of understory species was found than its adjacent vegetation, *Castanopsis cuspidata* var. *carlesii*, reflecting that the allelopathic interaction could be involved. It grows with a dense thicket and often forms a unique dominant stand without other competitive species. In 1976, Chou and Chen evaluated the phytotoxic potential of 25 woody species, including *Rhododendron* species. Of which, 15 species exhibited allelopathic potential. Chou has observed a unique pattern of lacking understory species on the *R. formosanum* floor for more than 2 decades during a long-term ecological research at Yuan Yang Lake. This phenomenon is quite similar to that reported by Nilsen *et al.* (35), who indicated that *R. maximum* exhibited the unique pattern of sparse diversity of understory plants in the northern Appalachian mountain of the USA. They conducted detailed study on *R. maximum* and concluded that the allelopathic effect is not manifest in field and is not likely to be an important cause for the inhibition of *R. maximum*. Additionally, *Rhododendron* species grown in northern America, Europe and Mainland China exhibited the similar pattern (Chou's personal observation). Yet, little attention has been paid to the allelopathic interaction of this unique pattern of *Rhododendron*, although various phenolics from *R. dabanshanense* in China were reported (44). The study aimed to evaluate the allelopathic potential of *R. formosanum* using bioassay methods, Pot culture and field studies. Additionally, natural products present in plant material were identified.

MATERIALS AND METHODS

Although *Rhododendron formosanum* is distributed widely from 1000 m to 3000 m in Taiwan but two following sites exhibiting relatively pure stand of plants were selected:

Site I. Yuan Yang Lake: It was described by Chou *et al.* (11).

Site II. Sun Link Sea: It is located in forest area (N 23^{o} 38' 19" and E 120^{o} 47' 40", Elevation: 1700 m, annual rainfall: 2000-2300 mm). At this site, the *R. formosanum* occupies a relatively pure stand of about 2 ha. with mean height of 15 m and stem diameter (10 to 15 cm) at the breadth height. Underneath the *R. formosanum*, it exhibits relatively low diversity of understory species and often lacks plants (Fig.1 A and B).

Field measurement of botanical composition

In the Sun Link Sea site, 3 quadrats, 10×10 m^2 each, were selected for botanical inventory, including the measurement of coverage of each understory species and counting the number of understory species per quadrat in the *R. formosanum* stand. In the adjacent area for comparison, same sizes of quadrats were also selected in *C. cuspidata* var. *carlesii* as control. Plant species were identified by T. W. Hsu and voucher species was deposited in Taiwan Endemic Species Research Institute.

Figure 1. An almost lacking understory plant on the floor of *R. formosanum* (A) as compared to many understory plants on the floor of adjacent woody plant, *C. cuspidata* var. *carlesii* (B), at the Sun Link Sea study site in April, 2008.

Sampling

The flowers, leaves, litter and organic matter of *R. formosanum* were collected in October 2003, 2007 at the Yuan Yang Lake site and in April 2007, 2008 at the Sun Link Sea site, respectively. Additionally, the upper 20 cm soil layer of *R. formosanum* floor was collected only from the Sun Link Sea site. All samples were allowed to air-dry at room temperature at the China Medical University laboratory.

Preparation of aqueous leachates and extracts

A leaching apparatus designed by Chou (7,26) was used to collect leachate (artificial rain drip) of *Rhododendron* plant parts as shown in figure 2. The apparatus consists of three layers: the upper layer container with needle holes filled with 4 liter distilled water, which passed through the holes to reach the middle container filled with 400 g plant parts, such as leaves, litter or organic matter. The rain drip washes through the plant material and plant leachate was collected in the bottom container without holes. The leachate was recirculated several times for about 6 hr, and then was filtered with Whatman 3 MM paper to clean up dust. The final leachate was stored in a freezer before bioassay or chemical analysis.

A series concentration [1 , 2 , 3 and 4 %] (4 g sample plus 96 ml double distilled water) of aqueous extract of *R. formosanum* flowers, leaves, litter, organic matter and their rhizosphere soil was obtained as per Chou (9) and Chou and Young (17). The extract of plant parts and soil were used in bioassay to determine the allelopathic activity

Figure 2. The leaching apparatus system: Upper part is a plastic tray, 55×40×15 cm with numerous needle-sized holes (2 cm between holes) was filled with tap water, which dripped through the holes to make an artificial raindrop, middle part is the same as upper tray, was filled with chopped plant material/organic matter to receive the artificial raindrop from upper tray, lower part is a plastic tray, like that of upper and middle tray, without holes to receive plant leachate from middle tray. The pump re-circulated the water from lower tray to upper tray

and leaves extract was also used for chemical identification of phytotoxins using various techniques (9).

The pH and osmotic concentration of plant leachate and extract from *R. formosanum* was measured with pH meter (Denver Instrument, UB-10, USA) and a cryoscopic osmometer (Osmomat 030, Gonotec), respectively.

Bioassays

Three bioassay techniques used were (i). Standard sponge bioassay (SSB) and (ii). Root initiation bioassay (RIB) as described by Chou (9). For the SSB, a piece of 5×5 cm cellulose sponge, cleaned and dried before use, was soaked with test solutions of either plant extract or with distilled water as a control. This was placed in a plastic Petri dish (9 cm dia). A Whatman filter paper, soaked with the test solution, was placed on an identical sponge. Thirty test seeds presoaked for 2 h with either extract or distilled water as control, were placed on the filter paper. The Petri dish was then covered and sealed with Parafilm and placed in a 25±2 ^{o}C incubator in the dark, depending upon the test species. Seeds of lettuce (*Lactuca sativa*), Chinese cabbage (*Brassica chinensis*), *Bidens pilosa*, *Ageratum houstonianum*, *Amaranthus inamoenus* and *Ocimum basilicum* were used. The radicle

length of test plants was measured at 72 h after incubation. For RIB, five fresh cuttings of stolons were put in 80 ml leachate or extract, or distilled water (control) in 100 ml beakers, with 4 replications. The beakers were kept at 25 ^{o}C for 6 days in dark and the number of roots initiated and their dry weight of *Brachiaria mutica* adventitious root were measured. Inhibition (%) of each leachate and extract against distilled water control was calculated as under:

$$\% \text{ Inhibition} = \frac{\text{Length or number of (control} - \text{test)}}{\text{Length or number of control}} \times 100$$

Greenhouse pot experiments

Each pot (18 cm dia) was filled up with 1 kg sandy loam soil and then was separately mixed with or without leaves powder of *Rhododendron* at 10, 20, 30, 40 and 50 g (to make 1, 2, 3, 4 and 5 % mixture, respectively). Same soil without the leaves powder was used as control. Sixty seeds of *B. pilosa* were uniformly sown into each pot including control and irrigated daily. The experiment was conducted in greenhouse of China Medical University. Thirteen days after sowing, the seedlings of *B. pilosa* were harvested to measure the plant height and dry weight.

Isolation and identification of water-soluble phytotoxins

By using the same techniques (14), 20 g of air-dried leaves of *R. formosanum* were extracted with 200 ml dd water thrice. The aqueous extract was evaporated *in vacuo* to a syrup-like concentrated aliquot and then was partitioned with ethyl ether. The ethyl ether soluble fraction was evaporated to dryness in hood. The dry residue was re-dissolved in methanol to a final volume of 5 ml and an aliquot of 50 µl was spotted onto a strip of chromatography paper. An aqueous 2 % acetic acid solution was development solvent for paper chromatography (PC) to separate and identify phenolics by comparison with R*f* values of respective authentic standard. For analysis in HPLC, 20 g of ground leaves of *R. formosanum* was soaked in 500 ml dd water and was shaken for 2 h and half of the extract was concentrated to about 100 ml and partitioned with the same volume of ethyl ether thrice to give 83.5 mg residue. Also, 400 g of organic matter was leached for 8 h with 4 l dd water. The leachate was collected and concentrated to 100 ml and partitioned with ethyl ether thrice to obtain 12.1 mg residue. Twenty µl, 50 µl of the residue for leaves and organic matter ethyl ether residue were injected separately into HPLC at a concentration of 1 mg/ml, respectively. The analytical HPLC was run on a 4.6 × 250 mm Mightysil RP-18 GP column (Kanto Chemical Co. Inc.) eluted with a gradient consisting of water containing 0.1 % formic acid (A) and methanol (B) at a flow rate of 1.0 ml/min and monitored at 254 nm. The initial solvent composition of 5 % B in A was raised to 95 % B from 0 to 40 min. For preparative purpose, 200 g ground powder of dried leaves was extracted in 4.8 l distilled water for 2 h. Then the aqueous extract was concentrated to 150 ml and partitioned with ethyl ether with the same volume thrice to give 1.16 g residue which was subjected to pass through Sephadex LH-20 column (2.5 cm × 92 cm, MeOH) to give 284 mg pure compound, which was structurally elucidated by NMR spectroscopy. PC of the ethyl ether layer was employed by techniques described by Wang *et al.* (45), thin

layer and column chromatography were used as described by Chang *et al.* (5) and Mabry *et al.* (24).

Purified compound except phenolics was subjected to spectroscopic identification using ^{1}H-NMR and ^{13}C-NMR (Bruker Avance400) and MS (Bruker Daltonics Esquire HCT). Specific optical rotation was measured by polarimeter (Jasco P2000).

ICP-MS analysis

Air-dried soil sample (4 g) from underneath the floor (20-40 cm) of *R. formosanum* and *C. cuspidata* var. *carlesii* collected from Sun Link Sea site was mixed with 40 ml dd water and was shaken subsequently for 2 h. An aliquot of the aqueous extract (15 ml) was added to digestion vessel before addition of 3 ml 65 % nitric acid (Merck) and was digested for 6 h at 80 ^{o}C in Automated Synthesis System (Syrris, UK). In this study, a heating period of 6 h was employed to ensure the achievement of total decomposition. After the decomposition, the samples were diluted to 25 ml with purified water and preserved at 5 ^{o}C until analyzed. The concentration of macroelements (Na, K, Ca, Mg, Mn, Fe) were measured with an inductively coupled plasma-mass spectrometer (PerkinElmer ELAN DRC II ICP-MS) (23).

Statistical analysis of data

Bioassay data were statistically analyzed by Student's *t*-test, standard deviation, or the Duncan's multiple range test (20) and SAS for Windows V 6.1.2.

RESULTS AND DISCUSSION

Botanical composition of understory species

Although many *R. formosanum* vegetations exhibit unique pattern of dominance in the field, but the understory species varied with the habitats. In Yuan Yang Lake site, there was no understory specie due to dense stand of *Rhododendron* and thick organic matter (20 cm) A0 soil layer, while in Sun Link Sea site there were some understory species (Table 1). The two selected vegetations: *R. formosanum* and *C. cuspidata* var. *carlesii*, are quite close to each other. In *Rhododendron* stand, there was relatively pure stand and 12 understory species; while in *C. cuspidata* var. *carlesii*, there were 19 species. The coverage of understory species was higher in *C. cuspidata* var. *carlesii* (105.7 %) than in *R. formosanum* (13.4 %) (Table 1). The botanical composition of understory spp. between *Rhododendron* and *Castanopsis* was quite different (Table 1). In *Castanopsis* stand, several pteridophytes [*Acrophorus stipellatus* (13.1 % coverage), *Arachnides rhomboides* (18.1 %), *Diplazium kawakamii* (1.5 %), *Monachosorum henryi* (31.8 %), *Plagiogyria japonica* (4.7 %) and *Vandenboschia auriculata* (0.5 %)] were dominant. The remaining understory species were present in relatively low density, except in *Strobilanthes formosanus*. The total coverage of pteridophytes on *Castanopsis* floor was about 70 %, while that of remaining species was about 35 %, i.e. the pteridophytes shaded the tolerant species. Contrarily, the total percent coverage of understory species in *Rododendron* floor was 13.4 %, which was significantly lower than *C. cuspidata* var. *carlesii*. Assuming the coverage of *C. cuspidata* var. *carlesii* was 100 %, than

Table 1. Comparison of floristic composition and relative coverage of each understory species found on the floors of *C. cuspidata* var. *carlesii* and *R. formosanum* in the Sun Link Sea site

Species	Coverage (%) / Species	
	C. cuspidata var. *carlesii*	*R. formosanum*
Acrophorus stipellatus T. Moore	13.1	-
Arachniodes rhomboides (Wall. *ex* Mett.) Ching	18.1	-
Ardisia crenata Sims	1.1	-
Callicarpa formosana Folfe	6.8	-
Diplazium kawakamii Hayata	1.5	-
Elatostema lineolatum Wight var. *majus* Wedd.	1.2	-
Eurya crenatifolia (Yamam.) Kobuski	1.6	-
Hemiboea bicornuta (Hayata) Ohwi	2.0	-
Hydrangea angustipetala Hayata	0.5	-
Monachosorum henryi Chist	31.8	-
Nanocnide japonica Blume	0.2	-
Pileostegia viburnoides Hook. f. & Thomson	4.2	-
Plagiogyria japonica Nakai	4.7	-
Rubus formosensis Kuntze	0.3	-
Smilax sieboldii Miq.	0.5	-
Stauntonia obovatifoliola Hayata	0.1	-
Strobilanthes formosanus S. Moore	13.2	-
Symplocos caudata Wall. *ex* G. Don	4.5	-
Vandenboschia auriculata (Blume) Copel.	0.5	-
Barthea barthei (Hance) Krass	-	0.4
Cinnamomum subavenium Miq.	-	0.0
Cleyera japonica Thunb.	-	1.0
Damnacanthus angustifolius Hayata	-	3.5
Dendropanax dentiger (Harms *ex* Diels) Merr.	-	0.1
Machilus thunbergii Siebold & Zucc.	-	0.3
Myrsine stolonifera (Koidz.) Walker	-	0.6
Neolitsea acuminatissima (Hayata) Kaneh. & Sasaki	-	0.5
Plagiogyria dunnii Copel.	-	3.6
Rhododendron formosanum Hemsl.	-	1.6
Smilax lanceifolia Roxb.	-	1.7
Shortia rotundifolia (Maxim.) Makino	-	0.2
Average total coverage (%)	105.7	13.4
Relative coverage of understory (%)	100.0	12.7
Number of species	19	12

- : Absent

R. formosanum (12.7 %), reflecting that very less number of understory plants grew under the *Rhododendron* stand. This suggests that both physical competition and allelopathic interactions might be involved.

Regarding the physical competition, we determined the light intensity under both vegetations, the light intensity on sunny day ranged from 1000 lux to 800 lux, and on cloudy day from 600 lux to 300 lux. The light intensity, under the canopy of *C. cuspidata* var. *carlesii* was slightly higher than *R. formosanum*. Five shade tolerant epiphytic ferns [*Vittaria flexuosa*, *Lemmaphyllum diversum*, *Bulbophyllum* spp., *Mecodium polyanthos*

and *Lepisorus monilisorus*] were found in the *Rhododendron* stand at Sun Link Sea site. The variation in light intensity between the two vegetations was not significantly different, reflecting that the competition for light between the growth of understory species was not a limiting factor. Besides, the amounts of soil macroelements (Na, K, Ca, Mg, Mn and Fe), in *R. formosanum* and *C. cuspidata* var. *carlesii* were not significantly different (Table 2), suggesting that the physical competition for nutrients was not possible. This phenomenon was similar to Yuan Yang Lake site.

Table 2. Six macroelements of soil underneath the floor (20 to 40 cm depth layer) for *R. formosanum* and *C. cuspidata* var. *carlesii* determined by ICP-MS in ppm (mg/l)

Species	Na	K	Ca	Mg	Mn	Fe
R. formosanum	19.80	370.92	6.61	0.30	30.98	0.38
C. cuspidata var. *carlesii*	18.12	275.28	5.38	0.30	26.48	0.31

However, the understory species in *C. cuspidata* var. *carlesii* were different from *R. formosanum*, suggesting that biochemical interactions between the dominant plant and understory species might be variable. It is hypothesized that the allelopathic interactions between the two vegetations would be different, leading to variable suppression of understory species occurring on the floor. Thus, the allelopathic evaluation of *Rhododendron* plants was done in this study as under.

Greenhouse pot experiment

The seeds of *B. pilosa* were sown in pots containing different amount of leaves powder of *R. formosanum* mixed with soil to make 1, 2, 3, and 4 % mixture. Thirteen days after planting, the seedlings of *B. pilosa* were harvested to measure the plant height and dry weight. The inhibition of plant height and dry weight increased moderately with increase in mixed powder dose. The plant height inhibition was 23 % at 4 % concentration (Fig. 3A). The inhibition of dry weight of *B. pilosa* was 27 % at 1 % leaf-soil mixture and increased to 30 % at 4 % concentration (Fig. 3B). Thus *R. formosanum* leaves were phytotoxic to the growth of (*B. pilosa* most popular weed in Taiwan).

Osmotic concentration and pH of plant leachate and extract

To avoid the misleading inhibition caused by pH and osmotic concentration, all leachates and extracts were subjected to such measurements. The pH of leachate and extract ranged from 4.98 to 6.00, while the osmotic concentration of leachate was -2 to 14 millosmol (mosmol). The osmotic concentration of extract ranged was 9 to 30 mosmol for leaves, 9 to 39 mosmol for litter, -2 to 3 mosmol for organic matter and -1 to 1 mosmol for flowers, respectively (Table 3). The negative value for osmotic concentration was simply instrumental deviation. The pH and osmotic concentrations were in the threshold range and will not cause both pH and osmotic inhibition in bioassay (16).

Phytotoxicity of leachates

Aqueous leachate of *Rhododendron* leaves, litter and organic matter were bioassayed using 6 test plants (*B. chinensis*, *L. sativa*, *B. pilosa*, *O. basilicum*,

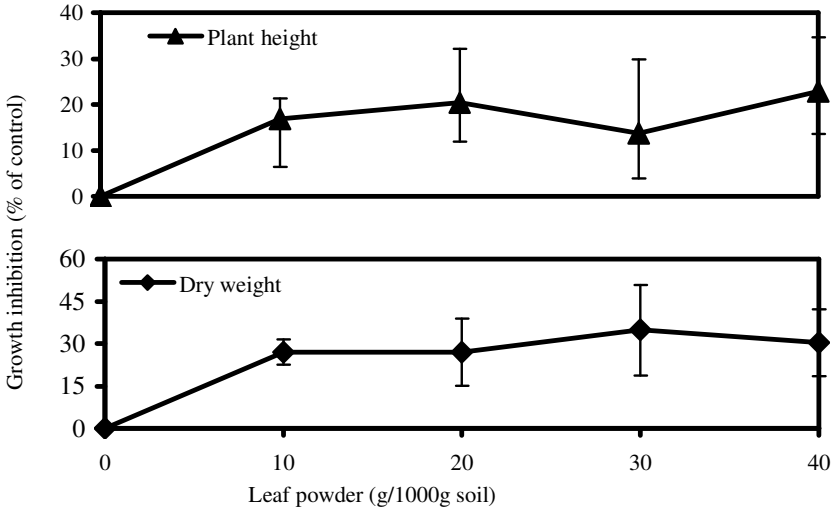

Figure 3. Inhibitory effects of *R. formosanum* leaves powder in gram mixed with 1000 g soil in pot set in a greenhouse on the growth of *B. pilosa*. The inhibition between treatments and control was significantly different at 5 % level using Student's *t*-test

Table 3. The osmotic concentration (mosmol) of leachate and aqueous extract from plant parts of *Rhododendron formosanum*

Plant parts	Leachate	Aqueous extract, %			
	10 %	1	2	3	4
Leaves	3	9	18	24	30
Litter	14	9	19	28	39
Organic matter	-2*	-2*	1	2	3
Flowers	N.D.**	-1*	0	1	1

*Negative value is due to instrumental deviation; **N.D. stands for not-determined

A. houstonianum and *A. inamoenus*). All leachate caused 5 to 40 % inhibition from leaves, 20 % to 100 % inhibition from litter and 50 % to 90 % inhibition from organic matter, showing remarkable inhibition in radicle growth regardless of tested species (Fig. 4). The inhibition was not due to the pH and osmotic concentration, because the pH range and osmolarity of organic matter leachate did not cause inhibition. The inhibition of *B. pilosa* was higher in litter and organic matter than in leaves. *A. houstonianum*, *A. inamoenus*, and *B. pilosa* species are often found in the open fields and are most notorious weeds in Taiwan, however, these species were absent in the understory species of *R. formosanum*.

Phytotoxicity of aqueous extracts

Aqueous extract concentrations (1, 2, 3 and 4 %) from *Rhododendron* flowers, leaves, litter, and organic matter were bioassayed against 6-test species. The

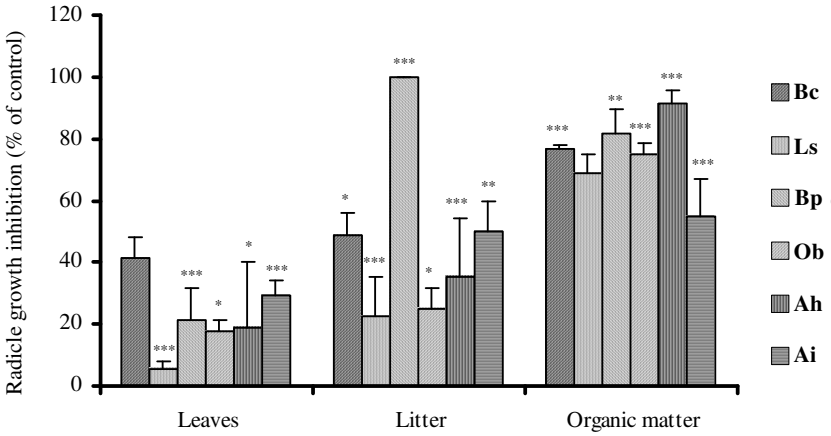

Figure 4. Effects of aqueous leachates of *Rhododendron* leaves, litter, and organic matter on the radicle growth of six bioassay species. Levels of statistical significance are expressed by asterisk: * <0.05, ** <0.01, *** <0.001 . The abbreviations of species names are: *Ageratum houstonianum* (Ah), *Amaranthus inamoenus* (Ai), *Brassica chinensis* (Bc), *Bidens pilosa* (Bp), *Lactuca sativa* (Ls) and *Ocimum basilicum* (Ob)

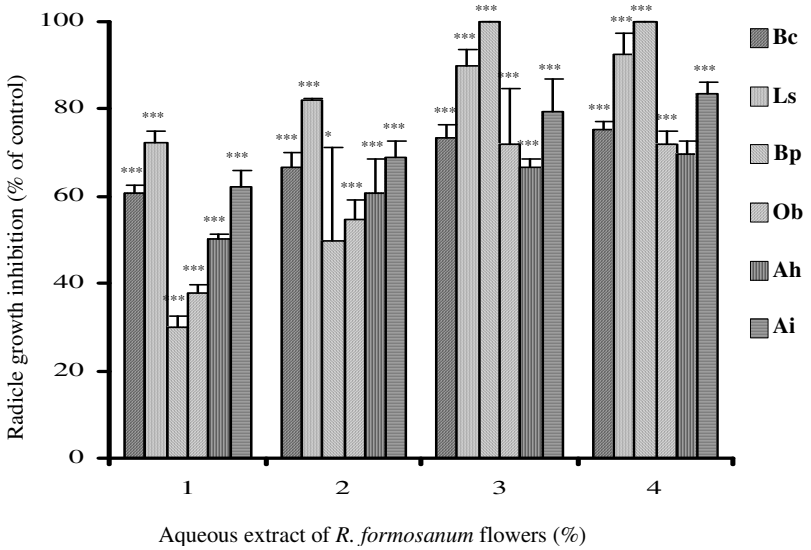

Figure 5. Effects of aqueous extracts concentration of *R. formosanum* flowers on radicle growth of six bioassay species. The abbreviations of legends see Figure 4.

flowers and leaves (1 % and above concentrations) significantly suppressed the radicle growth by 30-100 % (Fig. 5) and up to 100 % (Fig. 6) in *B. pilosa* and in 6-test spp. respectively over the control. Contrarily litter extract (up to 2 %) stimulated the radicle growth but was inhibitory above 2 % concentration (Fig. 7). Nevertheless, the inhibition of litter extract was irregular and not sure like flowers and leaves extracts. The organic matter extract slightly suppressed the radicle growth of *B. pilosa* at of 1 % concentration and was moderately suppressed at 2 %. This extract at 2 % and 3 % concentrations caused 22 and 50 % inhibition of *A. inamoenus* concentration, respectively. The other species were either suppressed or stimulated by the extract (Fig. 8).

Thus the radicle growth of most test species was suppressed at the lower concentrations as low as 1 % extract. Among the test species, *B. pilosa*, *A. houstonianum* and *A. inamoenus* common weeds in the field were sensitive to the aqueous extract of flowers, leaves, litter, and organic matter of *R. formosanum*.

Root initiation bioassay

In stolon cutting bioassay, the aqueous extracts of *Rhododendron* leaves suppressed the root length of *B. mutica*. After harvesting, the mean root length was 6.7 mm in control and was drastically reduced to 1.9 mm at 1 % extract and further to 0.2 mm at 8 % extract (Fig. 9). The root initiation was drastically suppressed to 75 % and 9.5 % at 1 and 8 % extracts, respectively.

Aqueous extract of *Rhododendron* soil

The aqueous soil extracts (1, 2, 3, 4 and 5 %) of *R. formosanum* were bioassayed against *L. sativa* and *B. chinensis* to find their allelopathic activity. All extracts stimulated the *B. chinensis* seedling growth (40-62 %), but were less stimulatory to *L. sativa*. The aqueous soil extracts stimulated the plant growth simply because the allelochemicals released into soil might be fixed by organo-mineral complex or humic acid. Thus, the allelochemical nature of compounds released through leaching or decomposing plant material might be lost (8,45).

Identification of hydrophilic phytotoxins in *R. formosanum*

Using PC and HPLC, phytotoxic phenolics were identified in the aqueous extract of *R. formosanum* leaves. These compounds were: *p*-hydroxybenzoic acid, *trans p*-coumaric acid, syringic acid, vanillic acid, *cis* ferulic acid, methyl ferulate, coumarin, protocatechuic acid and (-)-catechin (Table 4). From PC, a definite quantity of sample was applied on the chromatography paper and the relative quantity was evaluated by the colour response after viewing and/or spraying with diazotized *p*-nitroaniline followed by 10 % Na_2CO_3 (28). From HPLC chromatogram, the identity of each phenolic was done by comparing the retention time of authentic standard. Through mutual comparison between PC and HPLC profiles, due to various UV maximum of absorption and response factor for each phenolic, from which intensity was greater than 200 mv would be considered as existence, as shown in Figure 11. Through Sephadex LH-20 column chromatography, 284 mg of (-)-catechin out of 200 g *Rhododendron* leaves was isolated, $[\alpha]_D^{24}$ (*c* 1.0) -5.83, ESI-MS (negative mode) *m/z* 289, ^{1}H-NMR (400 MHz, MeOH-d_4) δ2.53 (dd, *J*=8, 16 Hz, 1H), 2.86 (dd, *J*=5.6, 16 Hz, 1H), 4.00 (m, 1H), 4.60

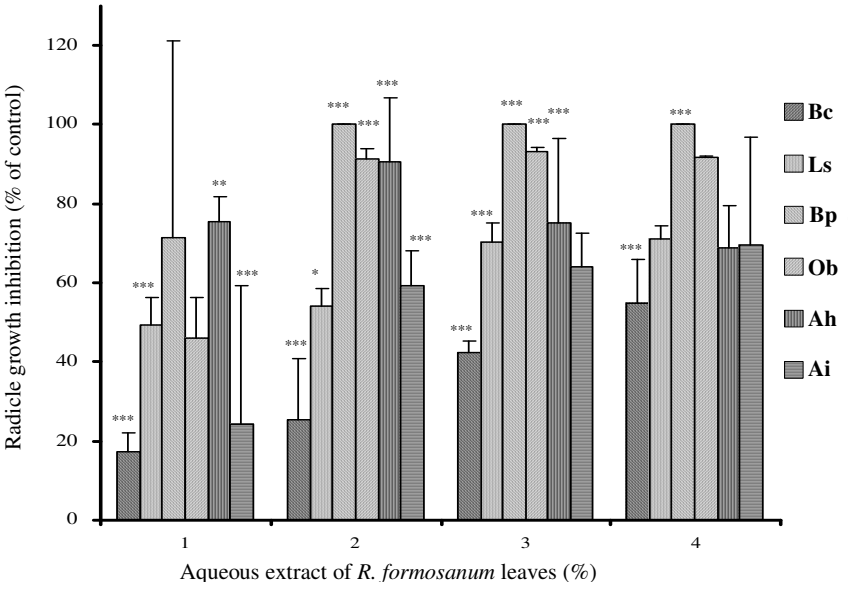

Figure 6. Effects of aqueous extract in a series of concentration of *R. formosanum* leaves on radicle growth of 6 bioassay species. The abbreviations of legends see Figure 4

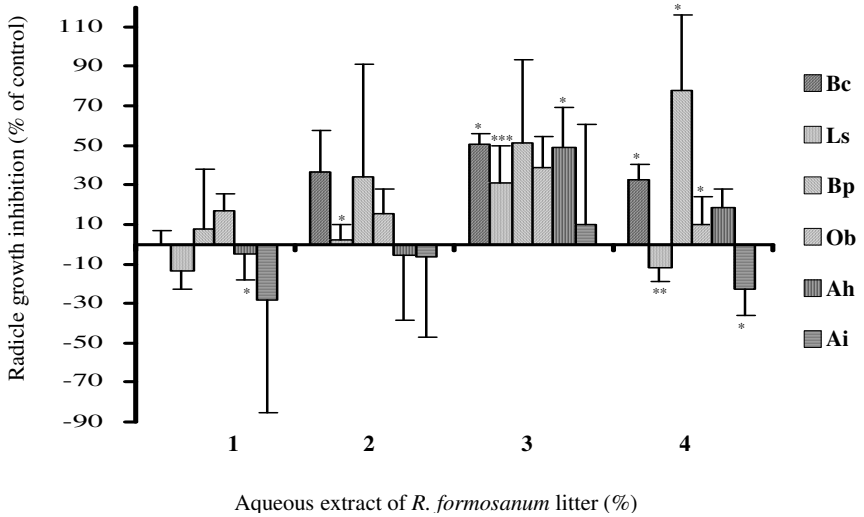

Figure 7. Effects of aqueous extract in a series of concentration of *R. formosanum* litter on radicle growth of 6 bioassay species. The abbreviations of legends see Figure 4. The positive value indicates inhibition and negative value indicates stimulation

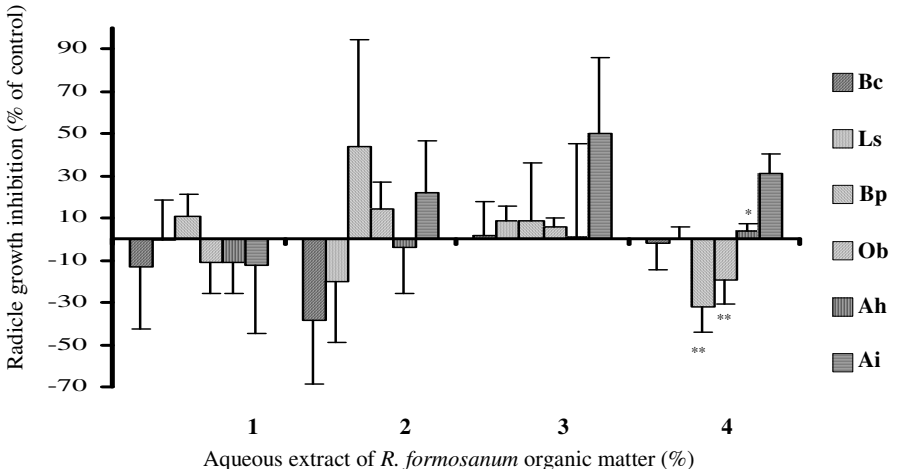

Figure 8. Effects of aqueous extract in a series of concentration of *R. formosanum* organic matter on radicle growth of 6 bioassay species. The abbreviations of legends see Figure 4. The positive value indicates inhibition and negative value indicates stimulation.

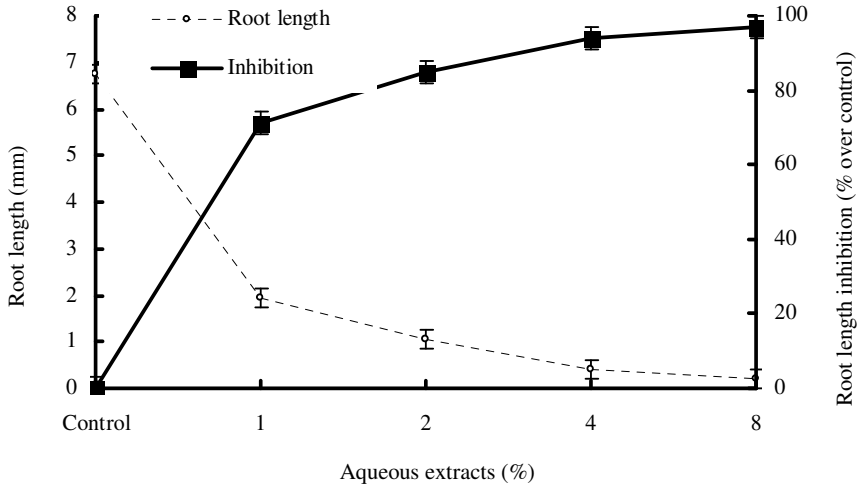

Figure 9. The inhibitory effect of aqueous extract, 0, 1, 2, 4 and 8 % of *R. formosanum* leaves on root length of root initiation of *B. mutica*. The inhibition was significantly different from distilled water control at 5 % level using Student's *t*-test.

(d, *J*=7.2 Hz, 1H), 5.90 (d, *J*=2.4 Hz, 1H), 5.97 (d, *J*=2.4 Hz, 1H), 6.72 (dd, *J*=2, 8 Hz, 1H), 6.78 (d, *J*=8 Hz, 1H), 6.86 (d, *J*=2 Hz, 1H). ^{13}C-NMR (100 MHz, MeOH-d_4) δ28.1(t), 68.6(d), 82.5(d), 95.5(d), 96.3(d), 100.8(s), 115.1(d), 116.1(d), 120.0(d), 132.0(s), 145.9(s), 145.9(s), 156.7(s), 157.3(s), 157.4(s). The identity of (-)-catechin was confirmed by comparison with the spectroscopic data and in agreement with specific optical rotation in literature (33). Some of the aforementioned compounds were also present in other species of *Rhododendron* (10). These phenolics have been confirmed as phytotoxins in many allelopathic potential plants (8,37,41).

Table 4. Phytotoxins isolated from the leaves of *Rhododendron formosanum* in Taiwan*

Compound	R*f* values in paper chromatography (2 % HOAc)			HPLC	
	Authentic standard	Isolated from *Rhododendron* leaves	Relatively quantitative comparison**	Compound present	Peak number
p-Hydroxybenzoic acid	0.63	0.66	+	+	(5)***
Chlorogenic acid	0.67			+	(4)
trans p-Coumaric acid	0.43			+	(10)
cis Ferulic acid	0.64	0.68	+++		
Methyl ferulate	0.61	0.61	+	+	(13)
Syringic acid		0.56	++		
Vanillic acid	0.56	0.60	++	+	(6)
Coumarin	0.73			+	(12)
Protocatechuic acid	0.63	0.56	+	+	(2)
(-)-Catechin	0.46	0.47	++	+	(3)

* The identification is primarily based on PC and HPLC.
** The amount of compound is based on the intensity of spot on the PC, showing +++ > ++ > +.
*** Data in parenthesis indicate the number of compound as compared to the authentic peak shown in Figure 11, the peak intensity that is larger than 200 mv in HPLC chromatogram is consider as +.

Much allelopathic researches have been done in different habitats of world (15,25,26,27,38,39,40), however, only few studies have been done on the allelopathic mechanism of *Rhododendron* plants (10,35).

A unique pattern of sparse understory species was found on the floor of *R. formosanum* in mountain areas at Yuan Yang Lake site in northern Taiwan and the Sun Link Sea site is in central Taiwan (Fig.1, Table.1). Both sites exhibit pure vegetation and a few species survived under the canopy of *R. formosanum*. We therefore, collected the leachates from the *R. formosanum* leaves, litter and organic matter for bioassay against 6-species (*L. sativa*, *B. chinensis*, *B. pilosa*, *A. inamoenus*, *O. basilicum* and *A. houstonianum*), the later 4 species were obtained from fields. All leachate from leaves, litter and organic matter significantly inhibited the radicle growth of 6-test species (Fig. 4). It was surprising that organic matter layer exhibited remarkable inhibition. Nilsen *et al.* (35) reported that the throughfall and leachate of the organic layer from *R. maximum* stand stimulated the germination of bioassay species over the distilled water control. The aqueous extracts of *R. formosanum* flowers (Fig. 5), leaves (Fig. 6), litter (Fig. 7) and organic matter (Fig. 8) above 2 % concentration inhibited the radicle growth of 6-bioassay

species. The inhibition was not due to pH and/or osmotic inhibition. Thus the phytotoxicity was primarily due to phytotoxic substances present in flowers, leaves, litter and organic matter.

In bioassay of root initiation, the aqueous extracts of *R. formosanum* leaves drastically inhibited (80 % at 1 % extract and 100 % at 4 % extract) the root initiation of *B. mutica* (Fig. 9). On the contrary, the aqueous extract of *R. formosanum* soil stimulated the growth of *B. chinensis* and *L. sativa* (Fig. 10). The stimulatory effects over distilled water control were not surprising to us, simply because the phytotoxic substances might already be decomposed or fixed into soil organic-mineral complex, resulting in losing their phytotoxicity (8,9).

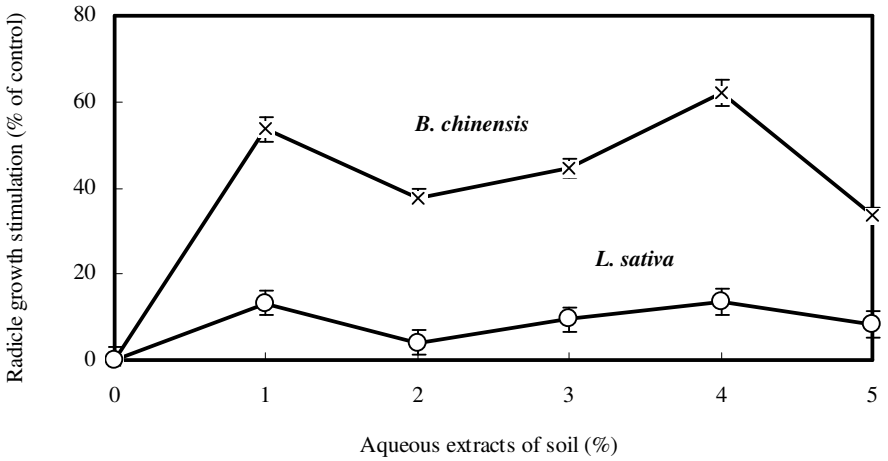

Figure 10. The stimulatory effect of *R. formosanum* aqueous soil extract, 1 % to 5 %, on the radicle growth of *L. sativa* and *B. chinensis*. The stimulation was significantly different from the distilled water control at 5 % level using Student's *t*-test

In decomposition studies in water, the phytotoxicity of decompositing leaves of *Rhododendron* drastically decreases 4-weeks after decomposition (Chou *et al.*, 2009 unpublished). It is known that the fresh leaves are the main source of allelopathic agent. Several Ericaceae species possess allelopathic potential (27), including *Rhododendron* species (34,35,36,43,44) and *Arctostaphylos* species (14).

As the allelopathic substances were mostly present in the *Rhododendron* leaves, a series of extractions were done by using both hydrophilic and hydrophobic solvents. In the aqueous extract of *Rhododendron* leaves, we found several phytotoxic phenolics: *p*-hydroxybenzoic acid, *trans p*-coumaric acid, syringic acid, vanillic acid, *cis* ferulic acid, methyl ferulate, coumarin, protocatechuic acid and (-)-catechin, whose identity was confirmed by comparing the retention time from PC, HPLC and/or from spectroscopic data of 1-D NMR. (-)-Catechin has recently been confirmed as an allelopathic compound

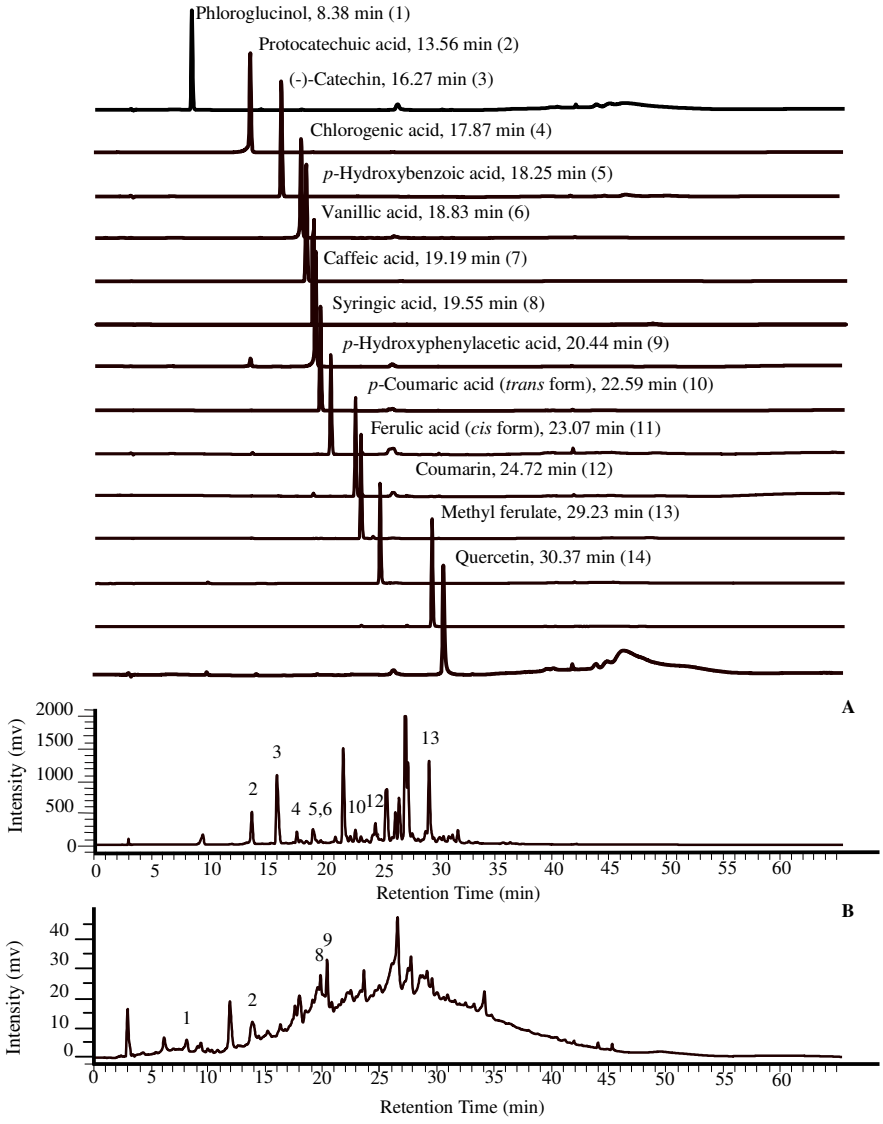

Figure 11. HPLC Chromatograms of ethyl ether layer of aqueous extract (A) and leachate (B) from leaves and organic matter of *R. formosanum* detected at 254 nm, respectively. The peak and its corresponding retention time for each of the standard is shown on the top for comparison and identification

in *Centaurea maculosa* soil (37) and in tropical weed *Sesbania virgata* for its enantiomer, (+)-catechin (41). However, the fate or role of (-)-catechin in soil with moisture is still unknown and controversial for the allelopathic effect (2,19). In our present study, the major constituent in aqueous extract of *R. formosanum* is (-)-catechin, *ca.* 0.14 % (w/w, dry weight) as per the HPLC profile (Figure 11 A). On the other hand, HPLC chromatogram of ethyl ether layer of organic matter (Figure 11 B), very trace or undetectable amount of (-)-catechin was found. Phloroglucinol, protocatechuic acid, syringic acid and *p*-hydroxyphenylacetic acid were tentatively identified according to the profile. It is purported that catechin is utilized as sole carbon source by *Rhizobium* spp., *Bradyrhizobium* spp., *Aspergillus* spp., *Streptomyces* spp., *Fusarium* spp. and *Pseudomonas* spp. (21,32). Catechin is degraded by microbes to protocatechuic acid or phloroglucinol via phloroglucinol carboxylic acid as an intermediate (32). Therefore, the underlying mechanism of its allelopathic activity is still elusive. Tentatively identified compound, such as chlorogenic acid, should be further confirmed. In addition, coumarin also found in the *Rhododendron* leaves is known allelopathic agent on seed germination and plant growth (1,3,4).

Regarding the hydrophobic substances present in the leaves of *R. formosanum*, we have isolated 18 compounds from the methanolic extract (22). Of 18 natural products, some exhibit inhibitory effect or stimulatory effect on test plants (18).

It is necessary to further investigate the biochemical processing of the hydrophobic and hydrophilic substances in soil to elucidate the real mechanism of allelopathic substances interacting with the growth of understory species. Extraction, isolation and structural determination of constituents from organic matter are in progress. Hyphenated techniques, such as LC-PDA-MS/MS should be employed to unambiguously analyze and identify the ingredients from all matrices. It is thus, concluded from the present findings that the unique pattern of lacking understory species on the *Rhododendron* floor is highly possible due to the allelopathic agents, either additively or synergistically from *Rhododendron* plant part, nevertheless, further studies need to be conducted.

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