マツ葉微生物に及ぼすアワユキセンダングサ抽出液の影響

Effect of *Bidens pilosa* L. var. *radiata* extract on phyllosphere microflora of pine needle

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Abstract

Extract of *Bidens pilosa* var. *radiata* distributed in Okinawa are known to have high insecticidal and repellent activity against plant parasitic nematodes, agricultural pests, and pine wilt disease-related organisms. The effects of this plant extract on crops and soil microorganisms have been studied, but the effects on microorganisms of pine needles have not been investigated. Therefore, we surveyed the influence of the plant extract on pine seedling (*Pinus luchuensis* Mayr) and its phyllosphere microbes of pine needle. It became clear that the harmful effect on the pine seedling and phyllosphere microbial flora were not observed. Thus, *B. pilosa* var. *radiata* extract is judged to be an environmentally friendly control material from the viewpoint of environmental assessment.

Keywords: *Bidens pilosa* var. *radiata,* pine, phyiiosphere, microorganism, environmentally friendly control

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1. Introduction

Pine wilt disease is caused by pine wood nematode (*Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle) transmitted by the Japanese pine sawyer (*Monochamus alternatus* Hope) [1]. Most of the pine trees native to North America are resistant, but major Japanese species such as *Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl. as well as *P. luchuensis* Mayr, *P. pumila* (Pall.) Regel and *P. koraiensis* Siebold & Zucc. are known to be susceptible to this disease[2]. This is considered to be the cause of the rapid spread of pine wilt disease in Japan, and it is said that about 240 million ha of damage was caused annually at the peak of the 1970s, and it still occurs in all prefectures except Hokkaido. Furthermore, this disease has become widely spread around the world and is now found in Japan, China, Korea, USA, Canada, and Europe, and has become an international problem[3-5].

Although there are several methods for controlling pine wilt disease, such as spraying, fumigating and injection of chemicals, crushing and incineration of dead wood and breeding resistant varieties[6], biological control methods have recently attracted attention, and a control method using *Beauveria bassiana* (Balsamo) Vuillemin[7] which is a natural enemy microorganism. Moreover, regarding pine wood nematode, a study to reduce nematode density in pine wood using *Trichoderma* spp.[8, 9] and nematode endo-parasitic fungus [10] is also performed.

Taba et al.[11] clarified that extract of *Bidens pilosa* L. var. *radiata*, an Asteraceae weed, have high repellent and insecticidal activity against pine wilt-related organisms such as pine wood nematode and Japanese pine sawyer. Because the plant extracts are ingredients of natural origin, they have the image of being safe for people and the

environment. However, since even if it is a natural ingredient, and is one of the chemical substances, which is considered particularly important to evaluate their impact on the environment and human. The effects of *B. pilosa* var. *rariata* extract on major agricultural crops and soil microorganisms including free-living nematode have already been evaluated and it has been cleared that there is almost no effect [12, 13]. However, there are no reports regarding the effects of the plant extract on pine needle phyllosphere organisms. In this study, we report that effect on the phyllosphere microflora of *P. luchuensis.*

2. Materials and methods

2.1. Effects on microbial flora inhabiting pine needles

1）Isolation of microorganisms from the leaf surface

One-year old seedlings of *P. luchuensis* received from Okinawa Prefectural Forest Resources Research Center were used for this test. Stock or 10× diluted solution of plant extract was sprayed onto the pine seedlings using a manual trigger-sprayer and cultured for 2 months (August 27 to October 29, 2013) in a simple greenhouse covered with a net (2 mm mesh) in the open air (Fig.1a). Next, 10 g of each leaf subjected to each treatment were collected. Distilled water was used as control. The leaves collected from the pine seedlings were placed in a 500-mL Erlenmeyer flask containing 90 mL of sterile distilled water and a surface-active agent (Tween 20, Nacalai Tesque, Kyoto, Japan) to achieve a 5,000× solution. The solution was agitated for 10 min using a stirrer, and then diluted to 100,000 ×. Microorganisms were isolated according to Moromizato et al.[14] using the diluted solution. Martin medium (partially modified: 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5.0 g peptone, 10.0 g glucose, 15.0 g agar, 3.3 mL 1% rose bengal, and 1 L of distilled water), and egg-albumin medium (0.25 g egg albumin, 1.0 g glucose, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, trace $Fe(SO_4)_3$, 15 g agar, and 1 L of distilled water) were used as culture media.

2）Isolation of endophytes

The plant extract (stock solution and 10× dilution) was prepared as in the experiment with phyllosphere microorganisms and sprayed on the pine seedlings. After culturing for about 4 months (August 27 to December 21, 2013) in in a simple greenhouse covered with a net (2 mm mesh) in the open air., 10 g of leaf from each treatment were collected and used to isolate the endophytes. Pine leaves were cut into 5 mm pieces (Fig. 1b), immersed in 70% ethanol for 30 s, then immersed in 3% sodium hypochlorite for 90 to 120 s, and finally washed twice with sterile distilled water; after blotting on a sterilized filter paper on a clean bench, they were placed in PDA medium and cultured at 25 °C. The frequency of appearance of isolated fungi was calculated and 50 strains were isolated.

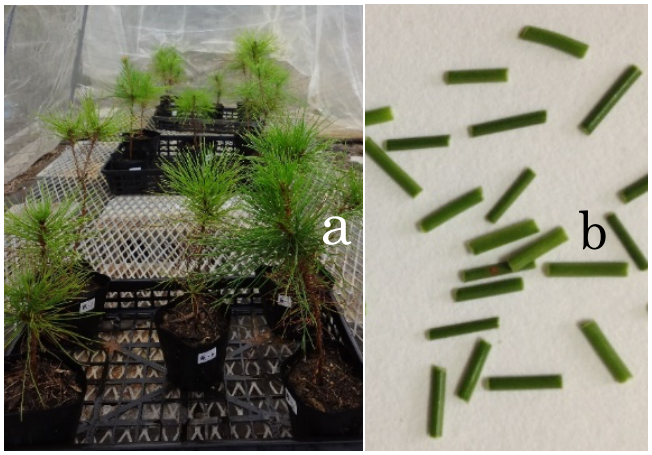

Fig. 1. Pine seedlings sprayed with *Bidens pilosa* var. *radiata* extract (a) and pine needles cut into small pieces (b) for endophytic isolation.

3）Identification of phyllosphere microorganisms

For bacteria, a discrimination test was conducted using the 3% KOH method (Tsuchiya 1993, partially modified)[15]. One droplet of 3% KOH solution was dropped onto parafilm using a Pasteur pipette. The bacteria colony was cultured on potato sucrose agar medium (300 g potato, 20 g sucrose, 15 g agar, and 1 L distilled water) for 4 days. The colony was scratched using a sterile toothpick and dipped in the

3% KOH solution. Gram-negative bacteria were confirmed when a viscous thread was observed after mixing the toothpick with the solution for 30 s, while gram-positive bacteria were confirmed when no thread was visible. *Bacillus subtilis* (Ehrenberg) Cohn was used as an indicator of gram-positive bacteria, and *Ralstonia solanacearum* (Smith) was used to indicate gram-negative bacteria (both were stock isolates from the Phytopathology Laboratory at the University of the Ryukyus). Identification of fungi was performed through morphological observations under a light microscope (ECLIPSE E200, Nikon, Tokyo, Japan) in combination with genetic analysis (internal transcribed spacer 1 and 4 domains)[16]. Each treatment comprised 45–50 isolated strains.

3. Results

3.1. Effects on microbial flora inhabiting pine needles

1) Microorganisms from the leaf surface

Although there was no significant difference between the 10× dilution and the control treatment, the number of microorganisms was significantly higher in the stock solution (Table 1).

2) Bacterial microflora

Gram-positive bacteria were dominant in all treatments and comprised 100% of bacteria found in the stock solution (Table 2).

3) Fungal microflora

Fungi isolated from leaf surfaces belonged to 15 genera. In the control treatment, *Pseudocercospora* species accounted for 36% of all fungi detected, showing the highest abundance; in the stock solution, *Cladosporium* species comprised 52% of all fungi. In the 10× dilution, *Pseudocercospora* spp., *Pestalotiopsis* spp., and *Phoma* spp. were the dominant fungi, each accounting for 22–26% of all fungi. Moreover, five endophyte fungi genera were isolated, with *Nigrospora* showing the highest abundance and accounting for 73.3–87.2% of all fungi (Table 3).

Table 1. Number of microorganisms inhabiting the phyllosphre of pine needles.

Microorganism	Number of microorganisms (CFU/g)		
	Stock solution	10 × dilution	Control (sterile distilled water)
Bacteria	4.7×10^5 bB[a]	4.4×10^4 aA	6.6×10^4 aA
Fungi	2.0×10^6 bB	4.9×10^4 aA	5.4×10^4 aA

[a]Mean±standard deviation: Different lowercase letters within columns and different capital letters within rows indicate significant differences (Tukey's HSD multiple comparison test, $P < 0.05$). CFU: colony forming units.

Table 2. Bacterial flora on pine needles[a].

Type of bacteria	Treatment		
	Stock solution	10 × dilution	Control (sterile distilled water)
Gram-positive	100.0 aA[b]	92.0 aA	94.0 aA
Gram-negative	0.0 bB	6.0 aA	2.0 aA
Gram -variable	0.0 bB	2.0 aA	4.0 aA

[a]Mean (%) per 50 isolated cultures.

[b]Different lowercase letters within columns and different capital letters within rows indicate significant differences (χ^2 test, $P<0.05$).

Table 3. Abundance of fungal flora on pine needles of *Pinus luchuensis*.

Fungi genera	Epiphyte (%)[a]			Endophyte (%)[b]		
	Stock solution	10 × dilution	Control (sterile distilled water)	Stock solution	10 × dilution	Cotrol (sterile distilled water)
Acremonium	0.0 cB[c]	0.0 dB	2.0 cA	0.0 cB	0.0 cB	0.0 cB
Alternaria	14.0 aA	4.0 bB	12.0 aA	0.0 cC	0.0 cC	0.0 cC
Arthrobotrys	0.0 cB	0.0 dB	2.0 cA	0.0 cB	0.0 cB	0.0 cB
Aureobasidium	0.0 cB	0.0 dB	0.0 dB	0.0 cB	2.2 bA	0.0 cB
Aspergillus	0.0 cB	4.0 bA	0.0 dB	0.0 cB	0.0 cB	0.0 cB
Chaetomium	0.0 cB	2.0 cA	0.0 dB	0.0 cB	0.0 cB	0.0 cB
Cladosporium	52.0 aA	2.0 cB	2.0 cB	2.2 bB	0.0 cC	0.0 cC
Fusarium	0.0 cB	0.0 dB	2.0 cA	0.0 cB	0.0 cB	0.0 cB
Humicola	0.0 cB	2.0 cA	0.0 dB	0.0 cB	0.0 cB	0.0 cB
Nigrospora	0.0 cC	4.0 bB	4.0 bB	87.2 aA	82.2 aA	73.3 aA
Pestalotiopsis	20.0 aA	22.0 aA	18.0 aA	0.0 cC	8.9 bB	0.0 cC
Penicillium	4.0 bA	2.0 cB	2.0 cB	0.0 cC	0.0 cC	0.0 cC
Phoma	0.0 cB	26.0 aA	0.0 dB	0.0 cB	0.0 cB	0.0 cB
Pseudocercospora	0.0 cB	24.0 aA	36.0 aA	0.0 cB	0.0 cB	0.0 cB
Rhizoctonia like	0.0 cC	2.0 cB	14.0 aA	8.5 bA	15.6 aA	24.4 aA
Trichoderma	6.0 bA	6.0 bA	0.0 dB	0.0 cB	0.0 cB	0.0 cB
Unknown	4.0 bA	0.0 dB	6.0 bA	2.1 bA	2.2 bA	2.2 bA

[a, b]Mean per 45-50 isolated cultures.

[c]Different lowercase letters within columns anddifferent capital letter within rows column indicate significant differences (χ^2 test, $P<0.05$).

4. Discussion

In this study, we investigated pine needle epiphyte and endophyte of one-year old seedling *P. luchuensis* and found that epiphyte of 10 and endophyte 2 genera excluding unknown species (control) (Table 3). Osono[17] reported that an endophyte of 24 genera including genus *Acremonium* and an epiphyte of 22 genera were detected in a survey on 28 species of phyllosphere fungi in trees in Japan. Of these, a total of 9 genera (4 endophyte and 5 epiphyte) were consistent with the results of this investigation. It is thought that Okinawa-specific species are distributed in addition to species common to the mainland. On the other hand, Magan et al.[18] investigated the effects of SO_2 and O_3 on the phyllosphere of conifer needles in Europe. SO_2 decreased *Aureobasidium pullulans* (de Bary) Arnau of Sitka spruce (*Picea sitchensis* L.), and O_3 treatment increased *Epicoccum nigrum* Link and *Cladosporium* spp. Even when *B. pilosa* var. *radiata* extract was treated to pine needles (*P. luchuensis*), the epiphyte *Cladosporium* spp. increased, and the endophyte *Rhizoctonia* like fungi were decreased, but no significant changes in microbial flora or an increase in plant pathogens were observed (Table 1-3). When a tree is treated with some substances in this way, it is thought to affect the microflora of phyllosphere. the Forestry Agency[6] and Fushiwaki and Fujimori[19] have evaluated the effect of spraying insecticides by aircraft on the natural environment, and have emphasized their importance, but it is considered to be valuable information as there is little knowledge about the effects of the plant extract on phyllosphere microorganisms as in this study.

In recent years, the adverse effects of chemical control for pests have been discussed, particularly the effect of drift and damage or influence on the surrounding environment[20]. *B. pilosa* var. *radiata* is used as a drink, in cosmetics, and in foods[21], it is also being studied for its potential application as a specific medicine for human diseases[22, 23]. There seems to be no harmful influence on human health or agricultural scene. Therefore, it is possible to use the plant extracts directly for pest control.

Moreover, environmentally friendly methods of pest control that utilize organisms including natural enemy insects and useful plants have been studied from the food-safety viewpoint[24, 25]. However, it is also a chemical even if it is natural substance; thus, the possibility of an environmental impact cannot be ignored. When selecting a control method, high effectiveness is most important, but impact on crops and environmental assessment of agricultural environment are also significant. Hayashi et al.[26] reported the general evaluation frame in Japan is not enough, and the influence on common land and soil organisms should be investigated. In short, it is desirable to consider all chemical substances that might be used for pest control when conducting a risk assessment for the ecosystem.

Taba et al.[12, 13] evaluated the effect of *B. pilosa* var. *radiata* extract on the growth of major crops such as Leguminosae, Solanaceae and Cucurbitaceae, and soil microorganisms (bacteria, actinomycetes, fungi and free-living nematodes), it cleared that they had only slightly affected. Ajitomi et al.[27] reported that the number of trap organs increased in nematode-trapping fungi, which are the natural enemies of plant parasitic nematodes by the plant extract. In addition, it has been revealed that *B. pilosa* var. *radiata* extract exhibits high repellent and lethal activity not only for pine wilt-related organisms[11] but also for several plant parasitic nematodes[28] and agricultural pests such as tobacco cutworm and aphids[29]. Therefore, *B. pilosa* var. *radiata* is widely applicable to the environmentally friendly control agent of agricultural noxious insects.

This time, we surveyed only the effect of *B. pilosa* var *radiata* extract on the phyllosphere microflora of pine needles. However, phyllosphere microorganisms are known to have various biological functions such as plant disease control[30], fungal toxin decomposing ability[31] and generation of ultraviolet A wave (UVA) absorbing component[32]. In the future, it will be important to study the interaction of phyllosphere microorganisms with plants and microorganisms.

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マツ葉微生物に及ぼすアワユキセンダングサ抽出液の影響

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要 約

沖縄に分布するアワユキセンダングサの抽出液は，植物寄生性線虫，一般農業害虫およびマツ材線虫病関連生物に対して高い殺虫・忌避活性を有することが知られている．農作物や土壌微生物に対する本植物抽出液の影響は調査されているが，マツやマツ葉微生物に対する影響は調査されていない．そこで，リュウキュウマツとマツ葉針微生物に及ぼす影響を調べた結果，悪影響は認められないことが明らかになった．従って，アワユキセンダングサ抽出液は環境評価の観点から，環境に優しい防除資材であると判断された．