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Allelopathic effects of *Eucalyptus urophylla* on Legume-Rhizobium symbiosis

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ABSTRACT

We assessed the effects of allelochemicals present in in *Eucalyptus urophylla* S.T. Blake on two legume species: *Albizia julibrissin* Durazz. and *Acacia mearn* De Wilde. and their symbiotic relationship with rhizobia. Addition of *E. urophylla* leaves or eucalypt soil increased the total soil phenolics and soil N, and the total soil phenolics were negatively correlated with root nodulation of these two legumes. The test phenolic compounds and those found in *Eucalyptus* plant significantly reduced the number of root nodules in *A. julibrissin*. However, these phenolic compounds had no effect on the rhizobia growth, but significantly inhibited the legume-rhizobium symbiosis by reducing the seedling root hairs deformation.

Key words: *Albizia julibrissin*, *Acacia mearn,* allelochemicals, *Eucalyptus urophylla*, legume-rhizobium symbiosis, legume species, nodulation, rhizobium symbiosis, root nodules, soil phenolic compounds, total soil phenolics

INTRODUCTION

In past century, fast-growing exotic tree species (e.g., pine, eucalyptus and acacia) introduced as forest plantations for faster restoration of degraded lands, proved detrimental to the soil characteristics and biodiversity (57,68). The introduction of such exotic tree species drastically changes the soil microbial community and ecosystem functions (26,29,43). Some exotic species can invade successfully due to their chemical agents that disrupt plant-microbial mutualism (12,61). For example, Brassicaceae species produce phytochemicals detrimental to the interactions between plants and beneficial soil microbes (3,40,45).

Among the soil microbial communities, the nitrogen-fixing rhizobium bacteria in legume roots are beneficial to soil bacteria (16). Rhizobia infects legume roots (usually via root hairs), form nodules and fix nitrogen (38). The legume-rhizobium symbiosis is important for legumes to grow especially in nitrogen deficient conditions. The symbiotic interactions between the legumes and rhizobia improves the plant productivity and plant community structure (28,59,62). The legume-rhizobium symbiosis is disrupted by some secondary chemical compounds from other plants (4,40).

Phenolic compounds are plant secondary chemicals released into the environment through root exudates, foliar leachates and residue decomposition (32,34). The reported effects of phenolic compounds on legume-rhizobium symbiosis are mixed. Phenolic compounds from the leguminous plants such as flavonoids, act as signalling molecules in the initiation of legume-rhizobium symbiosis (17). Mandal *et al* (35) reported that some endogenous phenolic acids present in the root nodules of *Vigna mungo* stimulated the

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efficiency of IAA production by its symbionts (*Rhizobium sp.*) and regulate nodule morphogenesis. However, some other phenolic compounds (p-coumaric, ferulic and vanillic acid) restricts the growth of certain strains of rhizobia (47). Batish *et al.* (4) found that some phenolic acids (protocatechuic, ferulic, p-coumaric and syringic acid), released by *Chenopodium murale* affected the nodulation of chickpea and pea.

Eucalyptus spp., have been widely introduced into many tropical and subtropical regions as afforestation or reforestation species. These were introduced to China in late 19th century and its plantation area in subtropical China reached 1.7 million ha (65). However, the large *Eucalyptus* plantations in southern China are allelopathic (15,19,41,57,68) and have produced many problems for the local environment, such as biodiversity loss, soil erosion and soil fertility decline (55,56,66). Numerous experiments have shown that *Eucalyptus* have direct allelopathic effects on seed germination and early growth of many plant species (9,20,24). Negative allelopathic effects of *Eucalyptus* on local microbial community composition and early growth of many plant species are well documented (6,14,48,63). Allelopathic effects of *Eucalyptus* are widely reported and the negative influence of *Eucalyptus* on local microbial community composition and function are well documented. However, the potential allelopathic effects of *Eucalyptus* on key soil microbial community such as the root symbiotic bacteria have not well been assessed.

Mixed plantation of *Eucalyptus* and N-fixing trees is an important strategy to mitigate the soil nutrients deficiency and soil degradation, increase soil nitrogen supply and improves the soil nitrogen availability (7,10,25,27,37). However, *Eucalyptus* litter significantly reduces the mycorrhizal colonization and nodulation of roots in leguminous plants (51,52). The phenolic compounds released from the litter fall and litter leachates are the main secondary substances from the *Eucalyptus* plants (2,13,22,50). However, no study has been done on the effects of phenolic acids from *Eucalyptus* on legume-rhizobium symbiosis. This study aimed to examine the allelopathic effects of *E. urophylla* foliage decomposition, leaf leachates, soil from *E. urophylla* plantation and pure phenolic compounds on the growth of legumes and their symbiotic relationship with rhizobia.

MATERIALS AND METHODS

Soil and plant material collection

Soil for these experiments was collected from a 15-year-old *E. urophylla* plantation in March 2016 from the Shuilian Mountains, Guangdong Province, China (22° 58′ N, 113° 42′ E, and 250-300 m above sea level). The climate of this region is subtropical marine monsoon with a mean annual precipitation of 1780 mm and rainy season from April to September. The average annual temperature is 22.2 °C, with a maximum monthly mean temperature of 28.5 °C in July and a minimum of 14.1 °C in January. Thirty cms of surface soil in *E. urophylla* plantation and control were collected from five adult trees within 2 m of tree base in three directions. The collected soil was thoroughly mixed and used as eucalypt soil. While, a field adjacent to the *E. urophylla* plantation was of typical subtropical forest and no *Eucalyptus* litter present in this area was selected as control field soil.

One Kg fresh leaves from *E. urophylla* plantation were collected, thoroughly mixed and divided into two parts. One half used as litter was cut to 2 mm × 2 mm pieces. And the other half (500 g) was soaked in 12 L distilled water for 3 days to prepare leaf leachate (39). A portion of the leaf leachate was diluted (1:1) with water to 0.5-fold concentrations. To simulate the field, we added the microbes associated with this substrate and their products, thus we did not sterilize the leaf leachates before addition. The legumes used in this experiment were two nitrogen-fixing plants, *Albizia julibrissin* Durazz. and *Acacia mearnsi* De Wilde. The seeds of two legumes were collected from Kunming Botanical Garden, Yunnan Province, China.

Eucalyptus allelopathy experiment

Seeds of *A. julibrissin* and *A. mearnsii* were surface-sterilized [(1 min in 70 % ethanol, and 3 min in 5 % sodium hypochlorite (NaClO)] before sown in control field soil. Two weeks after germination, the germinated seedlings were randomly selected and transplanted into pots (8 cm dia. and 10 cm depth) filled with 300 g field soil. For litter treatments, the 2 mm × 2 mm pieces of *E. urophylla* leaves were separately mixed with field soil in two doses: 5 % (Fresh wt of litter/w of soil) and 2.5 % (w/w). The mixture of soil and litter was put in to the pot and placed for 1 week before transplanting the seedlings. In leaf leachates treatments, the seedlings were treated every 2-3 days with 20 mL of 2.1 mg mL^{-1} and 4.2 mg mL^{-1} leaf leachates. In eucalypt soil treatment, one seedling that grown for 2-weeks was transplanted per pot, which contained fresh eucalypt soil. The experimental treatments comprised of 2 legume species × 6 treatments (control, eucalypt soil, 2.5 % leaves and 5 % leaves, 1-fold leachate and 0.5-fold leachate) and were replicated 20 times. All pots were placed randomly in growth chamber (28 °C, 70 % relative humidity, and 12 h of daylight). The experiment was carried out from April 2016 to October 2016.

After 6 months of culture, plants were uprooted, their root system was gently washed and nodulation status of two legumes was recorded. Shoot and root dry weights (1 week at 60 °C) were also determined. Soil nitrogen (diffusion method) was estimated using the methods described by Lu (33). Phenolic compounds were extracted by soaking 5 g of each soil sample in 20 mL of acetonitrile: methanol: acetone (25:70:5) mixed solution. The extraction was done on shaker (160 rpm) for 2 h. Then the extract was centrifuged for 10 min 4000 rpm and evaporated to 2 mL at 37 °C with nitrogen evaporators (Flyde MD 200). The phenolic content was determined as per the Folin–Ciocalteu method (49) and using gallic acid as standard.

Effects of individual phenolic acids on nodulation

Based on the pre-experiment in this study, we have found that nodulation of *A. julibrissin* was more susceptible to *Eucalyptus* allelopathic treatments (eucalypt soil, leaves and leaf leachates). Hence, we exposed *A. julibrissin* seedlings to phenolic acids commonly found in *Eucalyptus* plants [benzoic acid, *p*-hydroxybenzoic acid, vanillic acid] (13) and a mixture of these three acids (1:1:1) with 15 replications. We found that the concentration of total phenolics in eucalypt soil was about 50 mg Kg^{-1} of gallic acid equivalents and addition of *E. urophylla* leave litter increased the concentration (100 mg Kg^{-1}). However, the concentration of total phenolics in eucalypt soil (50 mg Kg^{-1}) was closer to natural conditions (51). Thus conc. of 50 mg Kg^{-1} was chosen as test concentration for each phenolic acid and three mixed acids (concentration of each phenolic acid was about 16.7 mg Kg^{-1}).

A. julibrissin seedlings were grown in pots (8 cm dia. and 10 cm depth) filled with 300 g of 1:1 field soil and trap soil to ensure a high rhizobia colonization potential. The trap soil was prepared by growing *A. julibrissin* in 1:1 mixture of sand and fresh field soil for 3 months to increase the rhizobium community in field soil, thereafter the roots were separated from the soil and the soil was used alone for the experiments**.** The *A. julibrissin* seeds were surface-sterilized. Three seeds were sown per pot and seedlings were thinned to one per pot after two weeks. The plants were treated with 20 mL phenolic solution (0.75 mg mL^{-1}) every week and irrigated every 2-3 days for 2 months. All pots were placed randomly in the growth chamber (28 °C, 70% relative humidity, and 12 h daylight).

Effects of phenolics on growth of rhizobia

To test the effects of phenolics on rhizobial growth, two rhizobium strains (strain1 and strain2) were isolated from the nodules of *A. julibrissin* grown in field soil, and exposed them to natural concentrations of phenolic acids. To isolate rhizobia, root nodules from *A. julibrissin* were removed, surface-sterilized with 75 % alcohol and 5 % NaClO for 3 min, and then rinsed them thrice in deionized water. The nodules were crushed and the contents were spread onto YMA (Yeast extract Mannitol agar) in Petri dishes (60) supplemented with Congo red. After purification by several sub-cultures, isolates were grown on fresh YMA agar slants and stored at 4 °C. The authenticity of these as rhizobia was established by isolating total genomic DNA of each isolate using bacterial DNA Kit (Tiangen Bio-tek), and then amplified by primer of P1: 5´-CGGGATCCAGAGTTGATCCTGGCTCAGAACGA ACGCT-3´ and P6: 5´- CGGGATCCTACGGCTACCTTGT TAC GACTT CACCCC-3´ (70). PCR amplification was done with a Thermal Cycler in 25 µL total volume. The reaction mixture contained premix (12.5 µL), primers (1 µL each), genomic DNA (1 µL) and 9.5 µL ddH2O. The PCR protocol was as under: initial denaturation (95 °C for 5 min); 35 denaturation cycles (30 s at 94 °C), annealing (30 s at 54 °C), and extension (1 min 30 s at 72 °C), and final extension (10 min 72 °C). Amplification products were visualized on 1 % agarose TAE gel stained with ethidium bromide. Synthesis of primers and sequencing were carried out with Sangon (Sangon, Guangzhou, China). Phylogenetic analyses were conducted with neighbour-joining tree method in MEGA package version 5. Two isolates shared 100% identity to rhizobia were used in following study.

To determine the effects of phenolics on rhizobial growth, 3 ml yeast mannitol broth containing 1 mL of 0.2 mg mL^{-1} phenolic acids (benzoic, *p*-hydroxybenzoic, and vanillic acids) in plastic tubes were inoculated with cells from fresh YMA slant and incubated at 25 °C on a rotary shaker (160 rpm). The optical density was determined at 600 nm using a spectrophotometer every 2 h for 40 h, and the maximum growth rate (μ_{max}) was calculated for each rhizobium strain in each treatment from the slope of growth curve in the exponential growth phase using a modified Gompertz equation (40). Each treatment had 3 replicates.

Root hair deformation assay

For this experiment, *A. julibrissin* seeds were surface-sterilized with 5 % NaClO for 3 min and 70 % alcohol for 5 min. Then thoroughly washed with sterilized water and germinated in a 150 mL sterilized conical flask containing 50 mL of 3 % agar medium with 20 seeds per flask. Germinated seeds with a root length of 4-5 cm was then transferred to a new tube containing 5 mL of solid agar medium and 10 mL of liquid Fåhraeus medium (5) with 30 µL of suspension of fresh rhizobium strains in TY medium (7) and 1 mL of phenolic acids (0.5 mg mL^{-1}) (31). The seedlings were grown at 25 °C with 12 h light per day. After 5 days, roots were microscopically examined and the number of deformed root hairs were determined based on 60 root hairs in each plant. Four plants were used per phenolic acid treatment.

Statistical analysis

All growth data was subjected to a one-way analysis of variance and means were compared with Tukey post hoc tests ($P < 0.05$). A penalized estimate of biomass and nodule number was used for these samples by using the equation $0.5 \times T_{\text{biom/nodule}}$, where $T_{\text{biom/nodule}}$ is the total biomass or nodule number of the smallest living plant in the experimental treatment (44). Mixed effects logistic regression (glmer, lme4 Package in R) was used to test whether the root nodulation (nodule rate) correlated with soil nitrogen or soil phenolic compounds (11). The number of nodules was analysed by generalized linear models (GLM), and the log-link function was a Poisson distribution. All analyses were carried out using R (Version 3.3.3, 2017).

RESULTS AND DISCUSSION

Legumes root nodulation

Eucalyptus treatments including eucalypt soil, 2.5 % and 5 % leaves, 1-fold and 0.5-fold leachates significantly reduced the nodule number in *A. julibrissin* and *A. mearnsii* (Table 1). *A. julibrissin* was not nodulated at 2.5 % and 5 % doses of *E. urophylla* leaves amendment. The *E. urophylla* leaves at 5 % level of amendment significantly reduced the number of nodules in *A. mearnsii*. The effect on legume shoot and root growth were not affected significantly in soils amended with *E. urophylla* leaves and eucalyptus soil compared with control soils (Table 1). However, only addition of *E. urophylla* leaf leachates decreased the growth of both legumes.

Table 1. Effects of *E. urophylla* leaves, leaf leachates, and eucalypt soil on shoot and root dry weight of *A. julibrissin* and *A. mearn* and soil properties

Treatment	Total dry weight (mg)	Shoot dry biomass (mg)	Root dry biomass (mg)	Nodule number (per plant)	Soil N (mg kg^{-1})	Total soil phenolics (mg kg^{-1})
			A. julibrissin			
Control	5.63±0.56ab	2.22±0.23b	3.41±0.35ab	3.55±0.96a	1.15±0.08c	25.92±6.39c
EU soil	6.27±0.94ab	2.79±0.42b	3.49±0.54a	0.10±0.06c	1.03±0.05c	56.91±8.92b
L0.5	4.15±0.38b	1.89±0.20bc	2.25±0.21ab	2.50±1.66a	1.12±0.03c	21.81±2.42c
L1.0	3.76±0.60b	1.82±0.29bc	1.94±0.32b	0.70±0.30b	1.08±0.02c	22.24±1.44c
D2.5	7.43±0.56a	4.39±0.39a	3.05±0.25ab	0.00±0.00c	1.48±0.05b	62.19±3.13b
D5.0	6.29±1.01ab	3.60±0.56ab	2.70±0.49ab	0.00±0.00c	1.68±0.08a	79.73±8.16a
			A. mearn			
Control	8.02±1.19a	4.63±0.68a	3.39±0.63a	13.2±3.8a	1.25±0.15b	34.51±6.37bc
EU soil	7.72±0.89a	4.88±0.56a	2.84±0.36ab	0.00±0.00	1.02±0.07c	50.83±3.77b
L0.5	2.62±0.38b	1.67±0.23b	0.95±0.17c	5.45±1.89c	1.19±0.05bc	24.69±3.71c
L1.0	2.64±0.45b	1.14±0.28b	1.51±0.21bc	7.30±2.64b	1.16±0.06bc	21.01±1.84c
D2.5	5.02±0.94ab	3.13±0.58a	1.89±0.40ab	5.25±1.78c	1.44±0.05ab	67.73±17.23ab
D5.0	6.95±1.42a	4.88±1.00a	2.07±0.45ab	1.65±0.59d	1.68±0.07a	91.76±6.44a

EU soil: Eucalypt soil; L 0.5, 0.5-folds (2.1 mg mL^{-1}) leaf leachate; L 1.0, 1-folds (4.2 mg mL^{-1}) leaf leachate; D 2.5, 2.5% addition of *E. urophylla* leaves; D 5.0, 5% addition of *E. urophylla* leaves. A. *julibrissi*:: *Albizzia julibrissin, A. mearn: Acacia mearn*

Different letters show significant differences at $P < 0.05$

The soil nitrogen and soil phenolics content increased significantly at 2.5 % and 5 % doses of *E. urophylla* leaves amendment with both legumes (Table 1). The eucalypt soil treatment had a higher content of soil phenolics but lower soil nitrogen than control. However, the leaf leachates treatment did not affect the content of soil nitrogen and total soil phenolics. The nodulation in two legumes was negatively correlated with the concentration of soil phenolics (Table 2). There was no uniform influence of soil nitrogen on nodulation. *A. mearn* nodulation was positively correlated with soil nitrogen, while there was no significant correlation between *A. julibrissin* root nodulation and soil nitrogen.

Table 2. Logistic regression results between nodulation and soil properties

Test legumes	Soil N			Total Soil phenolics		
	Slope	*Z*	*P*	Slope	*Z*	*P*
A. julibrissin	-0.236	-0.342	0.732	-1.138	-1.791	0.073
A. mearn	2.657	3.428	<0.001	-1.749	-2.614	<0.01

Effects of pure phenolic acids on legumes and nodulation

The effects of phenolics on nodulation showed that each tested phenolic acid significantly reduced the number of root nodules in *A. julibrissin* than water control (Table 3). However, these phenolic acids did not significantly decrease the plant biomass compared to water control. Of all treatments, addition of benzoic acid was most effective and reduced the number of nodules maximally.

Effects of phenolic acids on the growth of rhizobia and deformation of root hairs

The growth of rhizobium strains in the presence of phenolics was variable (Fig. 1). Mixture of phenolic acids significantly reduced the μ_{max} of strain 1, but the other phenolic treatments had no effect on the maximum growth rate of this strain. In contrast, vanillic acid and mixed acids treatments significantly improved μ_{max} of strain 2.

Table 3. Effects of phenolic acids or water on the growth of *A. julibrissin*

Treatment	Total dry weight (mg)	Shoot dry weight (mg)	Root dry weight (mg)	Nodule number (per plant)
Control	2.44±0.52	1.32±0.27	1.12±0.27	45.20±9.05a
P-hydroxybenzoic acid	1.07±0.26	0.59±0.11	0.48±0.15	10.00±2.09b
Vanillic acid	1.87±0.47	0.93±0.21	0.94±0.27	15.03±3.60b
Benzoic acid	1.35±0.32	0.96±0.20	0.39±0.13	3.60±1.20c
Mixture of acids	1.26±0.32	0.68±0.17	0.58±0.15	11.67±2.89b

Content of Phenolics: (50 mg kg^{-1}). Values are means +/- standard error

In root-hair deformation experiment, all phenolic acids significantly reduced the root hairs deformation in *A. julibrissin*, 5 days after inoculation with both rhizobia (Fig. 2). The vanillic acid and P-hydroxybenzoic acid at 50 mg L^{-1} concentration, deformed 18 % and 16.7 % root hairs of *A. julibrissin*, respectively. Only 10 % root hairs were deformed by the mixture of phenolic acids and benzoic acid, whereas 32 % root hairs were deformed in control with sterilized water and inoculation.

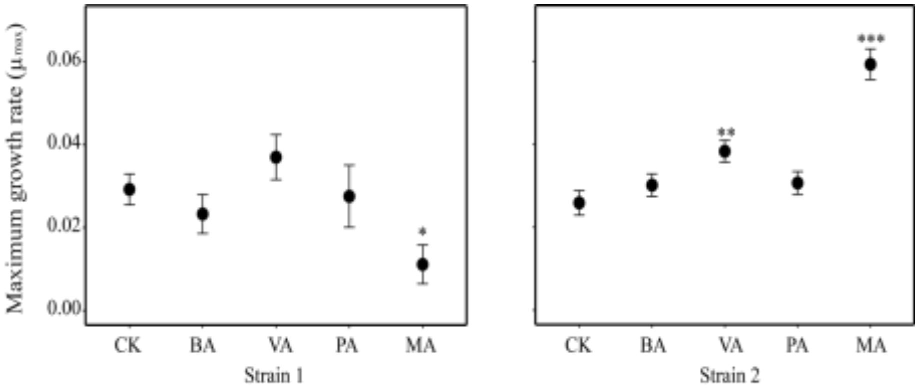

Figure 1. Effects of phenolic acid treatments on maximum growth rate of two rhizobium strains
Strain 1, *Rhizobium sp*. (KJ200402); Strain 2, *Rhizobium sp*. (JQ697689); CK, control; PA, P-hydroxybenzoic acid; VA, vanillic acid; BA, benzoic acid; MA, mixture of acids. Concentration 50 mg L^{-1}. Error bars are standard error of means. Asterisks indicate statistical difference between control and each of the chemical treatments ($P < 0.05$)

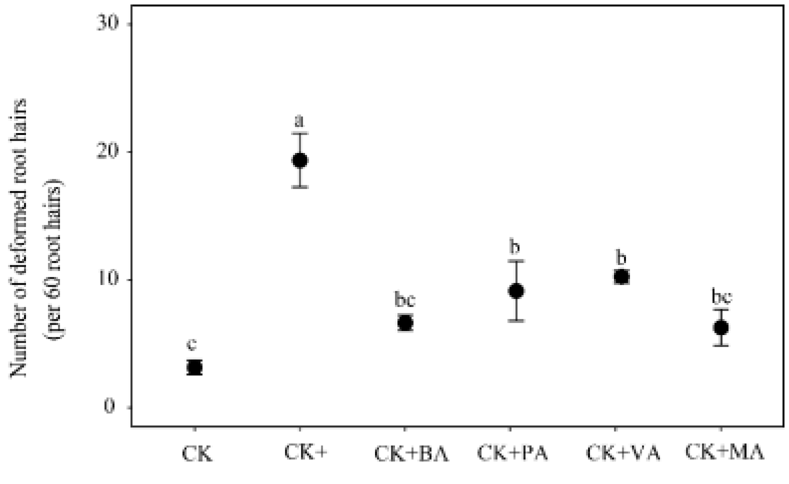

Figure 2. Effects of phenolic acids on *A. julibrissin* root hair deformation after five days of inoculation with two rhizobium strains
Values with different letters are significantly different ($P<0.05$). Error bars are standard error of means (n=4). CK-, control (sterilized water) without inoculation; CK+, control (sterilized water) with inoculation; CK+PA, P-hydroxybenzoic acid with inoculation; CK+VA, vanillic acid with inoculation; CK+BA, benzoic acid with inoculation, CK+MA, mixture of acids with inoculation

Very little is known about the influence of *Eucalyptus* on the soil nitrogen-fixing bacterial community, especially the symbiotic bacteria. The allelochemicals produced by *Eucalyptus* affects other plants and soil microorganisms (18,20) directly or indirectly induces undesirable soil biochemical changes (66,69). The present study demonstrated that

E. urophylla plantation soil, leaf leachates and leaves inhibited the legume root nodulation (Table 1). The root nodulation is important criterion for symbiosis between legumes and nodule bacteria (36).

Addition of *E. urophylla* leaves increased the soil nitrogen and phenolics content compared to control field soil. Soil nitrogen is required for optimal plant growth; but a large amount of available nitrogen in soil negatively affects the legume nodulation (1). In our research, suppression of root nodulation was not likely the result of increased soil nitrogen. With *A. mearn*, the soil nitrogen had a positive correlation with root nodulation (Table 2).

Phenolic compounds are most common plant secondary chemicals in nature (32) and many plants and legumes produce them (35,64). In this study we found that the phenolic content of the control soil varies with the legume grown (Table 1). This suggests that the legume plant itself releases phenolic acids and high concentrations of phenolic acids could inhibit the plant growth (46,50). However, except with treatment receiving the *E. urophylla* leaf leachates, the additions of *E. urophylla* leaves, phenolic compounds or eucalypt soil did not significantly alter the plant growth of *A. julibrissin*. Hence, reduced root nodulation is not due to loss of plant productivity. The *E. urophylla* leaf leachates reduced the two legumes biomass and this may be due to the presence of other allelochemicals in *E. urophylla* leaf leachates, such as terpenes, which may have decreased the plant growth (24).

High levels of the soil phenolics could decrease the soil microbial biomass, its activity and diversity (42,54,58). *E. urophylla* plantation soil and addition of *E. urophylla* leaves led to an increased the soil phenolics (Table 1). This finding is consistent with many previous studies which have found that *Eucalyptus* can modify soil properties by accumulation of antimicrobial compounds including phenolics (53). Our results showed that soluble phenolics present in *Eucalyptus* plant reduced the number of nodules (Table 3). It is likely that these compounds in *E. urophylla* leaves or eucalypt soil are responsible for the reduced root nodules and that nodule formation could be inhibited by these phenolics. The inhibition of *A. julibrissin* root nodulation was highest with only benzoic acid than mixture of phenolic acids. This may be due to a lower content of benzoic acid in mixture.

Phenolic acids from *Eucalyptus* could reduce the legumes root nodulation through several mechanisms. For example, either by reducing host plant and microbe performance, affecting the resources available for interrupting the mutualist’s communication (23). In our study, treatment with pure phenolic compounds did not significantly reduce the seedling biomass or rhizobial growth. The population μ_{max} of the two strains of rhizobia depended on the rhizobium species. This was similar to the results of Seneviratne and Jayasinghearachchi (47), who found that different rhizobium species vary in their growth response to phenolic acids. We find that phenolic compounds could disrupt the symbiosis by decreasing the root hairs deformation. All pure phenolic acids in this study significantly reduced the *A. julibrissin* root hairs deformation. Root hairs deformation is an early response of plants to rhizobium strains that penetrate into root cortical cells (67). Root hair curling and deformation facilitates subsequent nodulation events. Initiation and continuation of the nodulation process depends on constant exchange of chemical signals between the host legume and the bacteria (30). However, some chemicals in the soil that mimic or interfere with phytochemical signalling may confound recognition crucial to symbiosis. For example, plant phenolic compounds with a free hydroxyl groups can interfere with legume-rhizobium flavonoid signalling, and alter the symbiotic process (21). The root hair deformation reduction in presence of phenolics was seen this study, whether they interrupt the legume-rhizobium symbiosis signalling, needs further study.

CONCLUSIONS

This study showed that *Eucalyptus* plantations could have negative allelopathic effects on legume-rhizobium symbiosis. Allelochemicals, such as soluble phenolic compounds found in *Eucalyptus* leaves, or their accumulation in soil, are the main factors that affect the legume root nodulation. The mechanisms which affected the nodule formation were caused by the reduced root hairs deformation in presence of phenolic acids of *Eucalyptus*. Thus, it is important to consider the allelopathic effect of *Eucalyptu*s, before the legumes are introduced into *Eucalyptus* plantations.

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