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Allelopathic effects of leachates from two alien mangrove species, *Sonneratia apetala* and *Laguncularia racemosa* on seed germination, seedling growth and antioxidative activity of native mangrove species *Sonneratia caseolaris*

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ABSTRACT

To evaluate the impacts of two alien mangrove species *Laguncularia racemosa* and *Sonneratia apetala* on the native mangrove plants on Hainan Island, we chose a native mangrove species, *Sonneratia caseolaris*, because it has similar ecological requirements to the two alien species and they are usually planted close to it. The effects of aqueous leachates from the two alien species on *S. caseolaris* were analysed by measuring the germination rate of seeds and antioxidative activity of seedlings. The activities of SOD, POD and APX in the seedlings of *S. caseolaris* were inhibited by 0.2 and 0.4 g/mL concentration of leachates from the two exotic mangrove species, but the CAT activity increased, when the concentration of leachates increased. The leachates from *S. apetala*, increased the activities of SOD, POD, APX and CAT. However, the activities of SOD and CAT increased, while POD and APX were inhibited < 0.1g/mL concentration of the leachates from *L. racemosa*. The changes in the antioxidative activity may be an adaptive regulatory strategy in *S. caseolaris* seedlings in response to the allelochemicals of *L. racemosa* and *S. apetala*. Furthermore, the chemical components in aqueous leachates from mixed fresh branches, leaves and fruits of *L. racemosa* and *S. apetala* were characterized by GC-MS. Octadecanoic acid, 3-[(1-oxododecyl)oxy]-1,2-propanediyl ester was found at relatively high contents in every leachate of both of the exotic species, which may play an important allelopathic role in the leachates.

Key words: Allelochemicals, allelopathic effects, antioxidative activity, aqueous leachates, *Laguncularia racemosa*, *Sonneratia apetala*, enzymes, germination, *Sonneratia caseolaris*

INTRODUCTION

Biological invasions are one of the most important threats in the world because they can affect the biodiversity, species composition and the structure and functions of ecosystems, and have caused significant global economic losses (25,27,32). Strong allelopathic effects can be found from invasive plants (3,23), which can be harmful to other plants (43). Leachates are one of the ways to release the plant allelochemicals. Furthermore, these allelochemicals may affect the seed germination and growth of other plant species (7).

Mangrove wetlands receive considerable attention because they provide ecosystem

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services for millions of people by providing storm protection, food and fodder (13). Alien mangrove species are used to achieve rapid mangrove forest establishment (6,10). In China, an increase in area of > 80 % has been contributed by alien mangroves species since 2013 (10). However, there are few native mangroves that can live in the areas afforested by alien mangroves (42). Furthermore, some endangered native mangroves are facing the risk of habitat occupation by alien mangroves, even in mangrove natural reserves and wetland parks (8). How do alien mangrove plants defend the competition of native mangroves? Allelopathy has been suggested to be one of the major driving forces regulating mangrove forest succession (5). Some exotic mangrove plants have allelopathic effects on their neighbours (11). Do their allelochemicals play some role in the rapid expansion of exotic mangroves?

Laguncularia racemosa (Combretaceae) and *Sonneratia apetala* (Sonneratiaceae) are two alien mangrove species widely used for mangrove restoration projects in China, especially on Hainan Island. *L. racemosa* is native to the coasts of western Africa and the western Atlantic and eastern Pacific coasts of the Americas (14,29). It was first introduced to Dongzhai Harbour, Hainan, China from La Paz, Mexico (22). *S. apetala* is the other introduced mangrove species and was first planted in Dongzhai Harbour from Bangladesh in 1985 (28). Both alien species showed fast growth and high tolerance of environment stresses and were used as the pioneer species for mangrove restoration in estuarine and coastal areas in China (20,21,23). *L. racemosa* has high seed dispersal ability by sea water, which is an important characteristic of invasive plants, and is considered not suitable for planting in mangrove natural reserves (42,37). Unfortunately, due to the ease of germination, it was found in all natural mangrove reserves in China. The invasiveness of *S. apetala* and its potential to replace native mangrove species have been subjects of debate in China, and there are conflicts on whether *S. apetala* should be planted (20,21). Some researchers suggested that the competitive advantages, such as high productivity (21,41), high rates of seed dispersal and germination (33), and the invasive potential of *S. apetala*, should not be neglected (9). The invasion potential of both alien mangroves should be assessed in detail. Allelopathic effects of the two alien plants on the native mangroves *Sonneratia caseolaris* (19), *Bruguiera gymnorrhiza* (36), *Avicennia marina*, *Aegiceras corniculatum* and *Kandelia candel* (19) have been discussed either together or separately. However, the allelochemicals have not been illuminated yet.

This study aimed to determine the allelopathic effects of the two invasive mangrove plants, *L. racemosa* and *S. apetala* on the native mangrove *S. caseolaris*, which has similar ecological requirements to the alien species, and these species are usually planted together. The allelochemicals of the two alien mangroves were characterized by GC-MS analysis of aqueous leachates. Furthermore, the allelopathic effects of the aqueous leachates of two alien mangroves on the seed germination and seedling growth and antioxidant enzyme activities of *S. caseolaris* were analysed to explore the competitive ability of the two alien mangrove species on the native mangrove plants on Hainan Island, China.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

One kg of fresh leaves, branches and fruits of *L. racemosa* and *S. apetala* were separately collected from the coast of Wanluyuan Garden, Haikou, China (110°31'E, 20°03'N), in June 2017. They were washed with distilled water, air- dried in shade and cut into pieces (< 2cm) in a plastic drum. Then, the samples were soaked in 4 L of distilled water for 2 days with 15 min stirring in each 12 h period at 25°C (19). The leachates were filtered through 4 sheets of sterile cotton gauze. The aqueous leachates were rotary evaporated to reach the mass concentration of 0.4 g/mL and kept in a refrigerator at 4°C. Seeds of *S. caseolaris* were also collected from the coast of Wanluyuan Garden, Haikou, China in June 2017. The viable and healthy seeds were sterilised in 0.5% $KMnO_4$ for 2 h before sowing.

GC-MS Analysis

The quarter leachates of the two alien mangroves were freeze-dried and extracted with methanol (38), ethyl ether (34) and n-hexane (39). Then, the leachate contents were analysed using a Thermo Quest TRACE GC/MS system (Trace 2000, Thermo Finnigan) equipped with a programmable split injector (port temperature of 250°C throughout the run) and Xcalibur analysis software. The sample injection volume was 1 µL. The Thermo Quest TRACE mass spectrometer was equipped with an ion source (EI+, 70 eV) and operated in full scan mode (59.60 - 480.40 atomic mass units) (43). The detailed methods can be found in a previous study (38).

Laboratory Bioassay

Thirty *S. caseolaris* seeds were planted in Petri dishes (11 cm dia) in a dispersive arrangement under two sheets of filter paper. The remaining leachates of the two alien mangrove plants were respectively diluted with distilled water to obtain the 0.1 g/mL, 0.2 g/mL and 0.4 g/mL concentrations. The distilled water was used as control. Then, 15 mL leachates of *L. racemosa* and *S. apetala* (0, 0.1, 0.2 and 0.4 g/mL) were separately added to each treatment and treatments were replicated thrice. The humidity of the greenhouse was 80 %, the germination temperature was 25^{o}C and the illumination time was 12 h. The germination rate was recorded daily, and the root and hypocotyl lengths of seedlings were measured 15 days after sowing (10). The following germination parameters were determined:

$$\text{Germination Index (GI)} = \Sigma\ (Gt/Dt),$$

Where, Gt : Number of seeds that germinated t days after planting, and Dt : Days after germination.

The magnitude of inhibition and stimulation was denoted by the response index (RI) and calculated as under:

$$RI = T/C-1,$$

Where, T : Treatment data and C : Control data. RI > 0 indicates stimulation, and RI < 0 indicates inhibition (10).

Nursery Substrate Culture

Twenty seeds of *S. caseolaris* were sown on June 10, 2017 in Petri plates (10 cms dia and lined with 3-filter papers). Ten mL distilled water or leachates (0, 0.1, 0.2 and 0.4 g/mL concentrations) were added per petri plate, every 3-days to keep the filter papers moist till seeds germinated. These were kept in greenhouse (25 ^{0}C and 12 h light). On July 10, 2017, two weeks old 3-seedlings of *S. caseolaris* were transplanted per pot (2.5 cms dia and 5 cms depth). Twenty-five mL distilled water or leachates (0, 0.1, 0.2 and 0.4 g/mL concentrations) were added daily per pot to keep the culture medium moist till the seedlings reached 2 true leaves stage. Then the protective enzymes, proline and malondialdehyde were determined.

Activity of Protective Enzymes, Proline and Malondialdehyde (MDA)

The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were determined as per the operation manual of the Activity Assay Kit for these enzymes (Jianchen, Nanjing, China). The content of proline was analysed by the Proline Assay Kit (Solarbio, Beijing, China) and the content of MDA was determined following the protocol of the Malondialdehyde Assay Kit (Oxis International, Inc. OXIS).

Statistical Analysis

The data were processed by Microsoft Excel 2010. The analysis was done in 3 replications. Statistical significance was determined by SPSS (16.0) and LSD (the least significant difference method) tests. Differences were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

GC-MS Analysis of mixed branch, leaf and fruit leachates from *L. racemosa* and *S. apetala*

Leachates from mixed branches, leaves and fruits of *L. racemosa* and *S. apetala* were analysed by GC-MS by using methanol, ethylether and n-hexane treatments. The representative chemical components with relatively high contents in each extract are listed in Table 1 and Table 2. The GC-MS analysis showed that 26 chemical compounds were found in *L. racemosa* leachates and 21 chemical compounds were found in *S. apetala*. The main constituents of *L. racemosa* leachates were octadecanoic acid, 3-[(1-oxododecyl) oxy]-1,2-propanediyl ester, octadecanoic acid, 3-[(1-oxohexadecyl) oxy]-2-[(1-oxotetradecyl)oxy]propyl ester, octadecanoic acid, 2-[(1-oxododecyl)oxy]-1,3-propanediyl ester, propanoic acid, 2-hydroxy-, butyl ester and 4-pyrimidine carboxylic acid, 2,6-bis[(tert-butyldimethylsilyl) oxy]-, and tert- butyldimethylsilyl ester. The main constituents of *S. apetala* leachates were muramic acid, octadecanoic acid, 3-[(1-oxohexadecyl)oxy]-2-[(1-oxotetradecyl)oxy] propyl ester, ethanol, 2-methoxy-, carbonate (2:1) and silane, dimethyl(2-ethylhexyloxy)octadecyloxy-. Among the chemical compounds of *L. racemosa* leachates, there was one named octadecanoic acid 3-[(1-oxohexadecyl) oxy]-2-[(1-oxotetradecyl)oxy]propyl ester. This can be extracted by three chemical reagents:

methanol, ethylether and n-hexane and had the highest relative content, but in ethylether, it was the third most abundant compound. Interestingly, the same chemical compounds in *S. apetala* extracts, could also be found with the high relative content except in compounds extracted in ethylether. Another chemical compound, muramic acid, was only found at high relative levels in *S. apetala* extracts when extracted with methanol and ethylether. Muramic acid is an amino acid sugar. In terms of chemical composition, it is the ether of lactic acid and glucosamine. It occurs naturally as N-acetyl muramic acid in peptidoglycan, whose primary function is as a structural component of many typical bacterial cell walls (30). Muramic acid has also been used as a measure of microbial biomass in estuarine and marine samples (12). Ethanol, 2-methoxy-, carbonate (2:1) was found only in *S. apetala* methanol extracts at a relative level of 37.65%. A previous study has discussed the allelopathy of the two alien mangrove species to assess the risk and benefits of aqueous leachates of different organs on seed germination or seedling growth of the native mangroves or of the alien species themselves (19,20,23,36), but the allelochemicals of two alien mangrove species

Table 1. GC-MS analysis of mixed fresh branches, leaves and fruits aqueous leachates of *Sonneratia apetala*

No	Compound name	Relative content (%)		
		Methanol	Ethylether	n-hexane
1	Oleyl alcohol, trifluoroacetate	1.31	-	-
2	Pentafluoropropionic acid, octadecyl ester	0.43	-	-
3	Decanoic acid, silver(1+) salt	0.42	-	-
4	n-Hexadecanoic acid	0.16	-	
5	Octadecanoic acid, 3-[(1-oxododecyl)oxy]-1,2-propanediyl ester	9.37	-	43.93
6	Octadecanoic acid, 3-[(1-oxohexadecyl) oxy]-2-[(1-oxotetradecyl)oxy]propyl ester	62.52	13.65	48.05
7	Dodecanoic acid, 1,2,3-propanetriyl ester	4.64	-	-
8	l-Alanine, n-propargyloxycarbonyl-, octadecyl ester	0.20	-	-
9	Ethanol, 2-methoxy-, carbonate (2:1)	4.80	-	-
10	Octadecanoic acid, 2-[(1-oxododecyl)oxy]-1,3-propanediyl ester	18.10	-	2.40
11	Hexadecanoic acid, 2-[(1-oxotetradecyl)oxy]-1,3-propanediyl ester	0.03	-	0.15
12	Nickel(II), bis(.eta.-3(Ni),.eta.-1(Sn)-syn-butenediyl)bis[bis (trimethylsilyl) methyl]-tin(IV)-(trimethylphosphine), (Z)	0.01	-	-
13	Ethanol, 2,2'-[oxybis(2,1-ethanediyloxy)]bis-	0.30	-	-
14	Sulfurous acid, 2-ethylhexyl heptadecyl ester	-	0.91	0.12
15	Succinic acid, di(3,5-dimethylphenyl) ester	-	1.21	-
16	Propanoic acid, 2-hydroxy-, butyl ester	-	40.24	-
17	Pentacosane	-	1.09	0.09
18	Oxalic acid, isobutyl heptadecyl ester	-	6.98	-
19	Octadecanoic acid, 2-[(1-oxohexadecyl)oxy]-1-[[(1-oxohexadecyl oxy]methyl]ethyl ester	-	6.69	2.40
20	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	-	4.22	-
21	Octadecane, 1-iodo-	-	0.83	0.10
22	Hexacosane	-	1.60	-
23	Hentriacontane	-	1.54	0.08
24	9-Octadecenoic acid (Z)-, 2-hydroxy-1,3-propanediyl ester	-	0.41	-
25	4-Pyrimidinecarboxylic acid, 2,6-bis [(tert-butyldimethylsilyl)oxy]-, tert-butyldimethylsilylester	-	20.85	-
26	Cobalt, [1,1',1'',1'''-[(1,2,3,4-.eta.)- 1,3-cyclohexadiene- 1,2, 3,[4-tetrayl]tetrakis[benzene]](.eta.5-2,4-cyclopentadien-1-yl)-	-	-	1.21

remain unidentified. The leachate contents of two alien mangrove plants were analysed in this paper. The chemical compounds octadecanoic acid, ethyl ester and muramic acids that have high relative contents may play a major role in allelopathic effects of the two exotic mangrove species, which should be extracted and analysed for their allelopathic effects. Some polyphenol compounds were identified in five mangroves including *S. apetala*, which proved to have the allelopathic effects but were not found in this study (28).

Table 2. GC-MS analysis of mixed fresh branches, leaves and fruits aqueous leachates of *Laguncularia racemosa*

No	Compound	Relative content (%)		
		Methanol	Ethylether	n-hexane
1	3-Methoxymethoxy-2-methyl-non-1-ene	0.71	-	-
2	Ethanol, 2-methoxy-, carbonate (2:1)	37.65	-	-
3	Fumaric acid, 2-methylcyclohex-1-enylmethyl pentadecyl ester	0.59	-	-
4	Hexadecanoic acid, 2-[(1-oxotetradecyl)oxy]-1,3-propanediyl ester	1.61	0.63	-
5	Indeno[3a,4-b]oxiren-2-ol, octahydro-4a-methyl-5-[(tetrahydro-2H-pyran-2-yl)oxy]-, (1a.alpha.,2.beta.,4a.beta.,5.beta.,7aS*)-	0.59	-	-
6	Muramic acid	47.00	25.02	-
7	N,N-Dimethylformamide diisopropyl acetal	0.80	-	-
8	Octadecanoic acid, 3-[(1-oxododecyl)oxy]-1,2-propanediyl ester	0.70	7.33	-
9	Octadecanoic acid, 3-[(1-oxohexadecyl)oxy]-2-[(1-oxotetradecyl)oxy]propyl ester	12.97	-	83.33
10	2-Tetradecanol	-	0.23	-
11	2-Tridecanol	-	0.51	-
12	Ether, 1-hexadecenyl methyl	-	0.55	-
13	Hentriacontane	-	0.35	-
14	l-Norvaline, N-(2-methoxyethoxycarbonyl)-, hexadecyl ester	-	0.34	-
15	Octacosane	-	0.22	-
16	Octatriacontylpentafluoropropionate	-	1.22	-
17	Silane, dimethyl(2-ethylhexyloxy)octadecyloxy-	-	5.10	15.21
18	Succinic acid, di(2,3-dimethylphenyl) ester	-	0.40	-
19	Sulfurous acid, hexyl nonyl ester	-	0.38	-
20	Tetrapentacontane, 1,54-dibromo-	-	1.80	-
21	Octadecane, 1-iodo-	-	-	1.46

Seed germination and seedling growth

S. caseolaris have the same environmental adaptation as *L. racemosa* and *S. apetala* on Hainan Island, and all three species were chosen for mangrove reforestation projects in China (40). *S. caseolaris*, native mangrove on Hainan Island, have higher specific leaf area and a lower leaf development cost than *S. apetala* (9). In this study, *S. caseolaris* was chosen from native mangrove species to analyse the leachates of the two alien mangrove species, *L.*

racemosa and *S. apetala*. Our results showed that leachates of mixed fresh leaves, branches and fruits of two alien mangrove species inhibited the seed germination of *S. caseolaris* than control (Figure 1, Table 3). This is consistent with previous study, in which *S. apetala* leachates inhibited its own seed germination and that of *S. caseolaris* (23). Compared with *L. racemosa, S. apetala* was more inhibitory to seed germination of *S. caseolaris*. We also observed that root length and hypocotyl length of *S. caseolaris* increased at the lower concentration of leachates (0.1 g/mL), while, they decreased at the higher concentrations (0.2 and 0.4 g/mL) (Table 3). The allelopathic effects of *L. racemosa* on the growth of *B. gymnorrhiza* seedlings and those of *S. apetala* on the growth of seedlings of the indigenous mangroves, *Avicennia marina*, *Aegiceras corniculatum*, *B. gymnorrhiza* and *Kandelia candel*, have also been observed previously (18,36).

Table 3. Effects of leachates of *L. racemosa* and *S. apetala* on the seed germination of *S. caseolaris*

Leachate concentration (g/ml)	RI					
	Germination Index		Root length		Hypocotyl length	
	Lr	Sa	Lr	Sa	Lr	Sa
0.1	-0.012	-0.011	0.050a	-0.037a	0.063a	0.058a
0.2	-0.020	-0.035	-0.029b	-0.048a	-0.039b	-0.043b
0.4	-0.032	-0.046	-0.047b	-0.103b	-0.047b	-0.063b

The same letter in rows is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test
Lr : *L. racemosa*; Sa: *S. apetala*

Activities of protective enzymes of *S. caseolaris*

Allelopathy is a common biological phenomenon by which one organism produces biochemicals that influence the growth, survival, development, and reproduction of other organisms. These biochemicals are known as allelochemicals and have beneficial or detrimental effects on target organisms (6,16). Under environmental stress such allelochemicals, activates the chemical defence signals to start-up antioxidant enzyme systems, including many reactions of other signals (24). Among them oxidative stress (OS) in plants can easily emerge under these environmental stresses. SOD, POD and CAT are components of antioxidant enzyme systems, and cell wall-bound SOD and POD participate in the generation of ROS (2). These enzymes are important enzymes for clearing free radicals and maintaining the plant membrane system (12). Prior research has reported the phenolic compounds and their anti-oxidative properties of the mangrove plant *L. racemosa* (31). The activities of SOD, POD and APX of *S. caseolaris* were inhibited in the 0.2 and 0.4 g/mL concentrations of leachates from the two exotic mangrove species, but the CAT activity increased with these two concentrations of the leachates (Figure 1). At the lower leachate concentration (0.1 g/mL), the activities of SOD, POD, APX and CAT increased, when treated with the leachates from *S. apetala*. However, when treated with the leachates from *L. racemosa*, the activities of SOD and CAT increased, while those of POD and APX were inhibited. Previous studies indicated that the CAT activity was reduced because of the allelopathic effects (12,43). The depletion of CAT activity may be associated with the biosynthesis of salicylic acid (SA), one of the key components in the signal transduction pathway leading to plant resistance to abiotic and biotic stresses. Previous studies also showed that allelopathic effects can increase the CAT activity (12,43), as was also observed in this study. The activities of APX were inhibited, except for the treatment of 0.1g/mL concentration of leachates from *S. apetala and L. racemosa* (Figure 1). The repression of APX activity in the leachate-treated seedlings coincided with enhanced ROS production (7, 9).

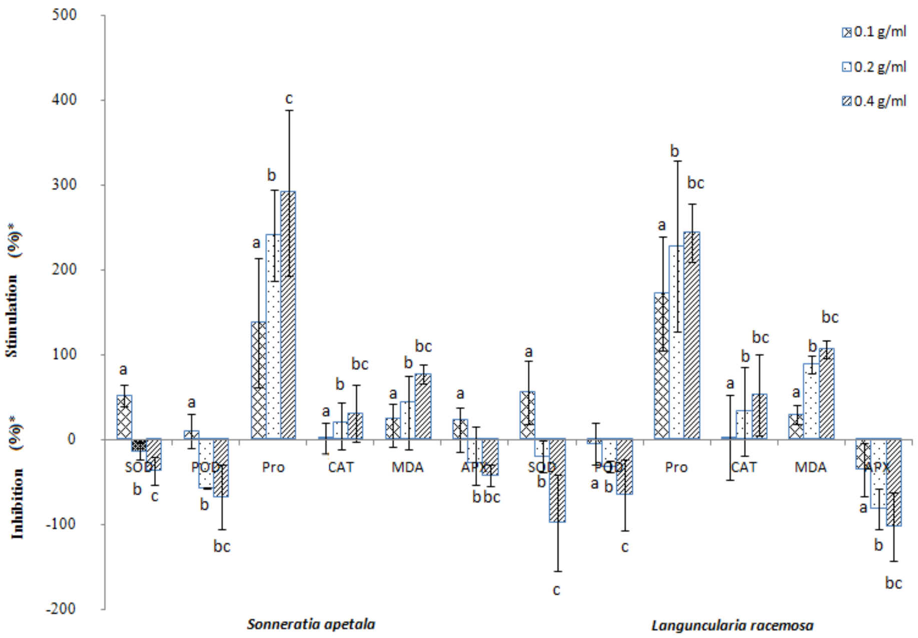

Figure 1. Effects of leachates of *S. apetala and L. racemosa* on the activity of antioxidant enzymes of *S. apetala.* *: % inhibition/stimulation over control. MDA: malondialdehyde; Pro: proline; SOD: superoxide dismutase; POD: peroxidase; APX: ascorbate peroxidase; and CAT: catalase. The same letters mean that the values are not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test.

Cell membrane peroxidation of *S. caseolaris*

The loss of membrane stability in the leachate-treated leaf discs can be attributed to the phytotoxic chemical constituents in the leachates (4). Maintenance of plant membrane functions is an energy-demanding process. MDA is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for the damage to cell membranes (28,29). An increase in the amount of MDA occurred in *S. caseolaris* seedlings in all leachate concentrations examined, and higher MDA was observed in the higher concentrations of leachates from both *S. apetala and L. racemosa* (Figure 1). The MDA contents of each treatment were > 40% higher than control. The same results were also found in our previous studies that showed that allelochemicals caused an accumulation of MDA (33). For the proline analysis of the *S. caseolaris* seedlings, the same tendency with MDA could be found in all leachate treatments of the two exotic mangrove plants. In plants, as an osmolyte and reservoir of carbon and nitrogen, proline to protects the plants against free radical- induced damage. The accumulation is linked with the quenching of singlet oxygen (37). The activities of enzymes involved in proline metabolism (glutamate dehydrogenase, pyrroline-5-carboxylate synthetase, pyrroline5-carboxylate reductase and ornithine-d-amino transferase) were significantly enhanced in the leaves under allelopathic stress with and without a water deficit, while the activity of proline dehydrogenase decreased with an increase in stress (1). Then, the metabolites became unbalanced due to the increase of active oxygen and free radicals (26). Furthermore, the peroxidation of cell membranes damaged the structure and function of the cell membranes. A significant effect can be found on the contents of MDA and proline of *S. caseolaris* seedlings from the leachates of two alien mangroves. The results mean that the two alien mangroves affected the seedling growth of native mangrove plants by influencing the cell membrane peroxidation of the seedlings. Those effects may be caused by the main constituents in leachates such as octadecanoic acid, ethyl ester and muramic acid. In *Casuarina equisetifolia* woodlands, the octadecanoic acid, ethyl ester was found in special fungal metabolites with allelopathic potential (35).

CONCLUSIONS

The leachates from *S. apetala* and *L. racemosa* inhibited the seed germination and seedling growth of *S. caseolaris* at higher concentrations (0.2 and 0.4 g/mL) and were stimulatory at low concentrations (0.1 g/mL). The leachates at higher concentration inhibited the activities of antioxidant enzymes (SOD, POD, and APX). The content of MDA and proline significantly increased with the increase in the leachate concentrations. The CAT activity increased compared with the increase in the leachate concentrations in both mangroves. Using the GC-MS, 26 chemical compounds were detected in *L. racemosa* and 21 in *S. apetala* and Octadecanoic acid and muramic acid contents were highest in both alien mangroves. These changes may be an adaptive regulation of *S. caseolaris* seedlings in response to the allelochemicals of *S. apetala* and *L. racemosa.*

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