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Table: 1, Figs : 2

Allelopathic effects of volatile compounds from *Eucalyptus grandis* on *Vigna radiata*, *Raphanus sativus* and *Lactuca sativa*

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ABSTRACT

The allelopathic effects of 3-relatively abundant volatile compounds [n-octane, 2,4- di-tert-butyl phenol and 2,2'- methylene bis (6-tert-butyl-4-methyl phenol)] present in the soil of *Eucalyptus grandis* plantation (1-10 years old) were studied on the seed germination, seedling growth and two physiological indicators [(seedling's malondialdehyde (MDA) and roots' vitality (by triphenyl tetrazolium chloride (TTC)] of *Vigna radiata*, *Raphanus sativus* and *Lactuca sativa*. The inhibitory effects of 2, 4- di-tert-butyl phenol was strongest on the target plants. The three volatile compounds at high concentrations [n-octane，1%; 2,4- di-tert-butyl phenol, 10 mmol/L; 2,2'- methylene bis (6-tert-butyl-4-methyl phenol)], 10 mmol/L) inhibited the seed germination, root length, seedling height and the seedling / root fresh weight ratio of *Vigna radiata*, *Raphanus sativus* and *Lactuca sativa*, but low concentrations were less inhibitory or stimulatory. All three volatile compounds at low concentrations were slightly stimulatory to seedling's MDA content and were less inhibitory to seedlings roots' vitality TTC. At low concentrations, the n-octane and 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) were less stimulatory to the seedlings root's vitality. However, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) even at lower concentrations inhibited the *V. radiata* seedling's MDA content

Key words: Allelopathic effects, *Eucalyptus grandis*, *Lactuca sativa,* lettuce, malondialdchyde (MDA), mungbean, radish, *Raphanus sativus*, roots vitality, seed germination seedling growth, *Vigna radiata*, volatile compounds.

INTRODUCTION

The plantation forests area has increased rapidly worldwide, with total area > 50 million hm^2 (10). Plantation forests, reduces the dependence on natural forests, slows the increase in CO_2 concentration in atmosphere and brings economic benefits, however, they also cause soil degradation and decreases the biodiversity. Allelopathic effects are one of the mechanisms causing these problems (22,26,27). To study the plants allelochemicals, their volatility and water solubility should be considered, however, most of the present allelopathic research in forest plantations, focuses on the effects of water-soluble phenolic acids on target plants (5,12) and few studies have been done on the volatile substances.

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Hence, the allelopathic effects of plantation forests have not been sudied comprehensively. The leaves, bark, roots and other organs of *E. grandis* produces certain volatile chemicals (4,23,27), which have direct allelopathic effects on other plants (i).Via the air and (ii). Enter the soil by leaching in rain or fog or directly absorbed and thereby influences the surrounding plants and soil microbed (1,6,9,12,13). In our previous research, we found 38 different volatile allelopathic substances in the rhizosphere of 1-10 years old *E. grandis* plantation (31). Hence in present research, we selected three volatile substances [n-octane, 2, 4- di-tert-butyl phenol and 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) with higher content in the soil of *E. grandis* plantation], to study their allelopathic effects on the seed germination, seedling growth and physiological indices of *V. radiata, R. sativus* and *L. sativa*. These volatile compounds have not received much attention, due to their low water-solubility. Some volatile allelochemicals are soluble in water and affects other plants (15,20,30), thus research on the allelopathic effects of volatile allelochemicals on other plants is important.

Eucalyptus plantations cover an area of 14 million hm^2 worldwide (1). Identification of allelopathic effects of eucalyptus plantations has been controversial because the volatile allelochemicals exist in eucalyptus tissues and in rhizosphere (4,21,27). We did research on *Eucalyptus grandis* plantation, converted from cropland in Sichuan Province, China. We had already reported 38 types of volatile allelochemicals in the rhizosphere of *E. grandis* plantation forest (26), hence, study of allelopathic effects of these volatile allelochemicals in the *E. grandis* plantation is of great significance. This study aimed to determine the effects of major volatile allelochemicals in soil [n-octane, 2, 4- di-tert-butyl phenol and 2, 2’-methylene bis (6-tert-butyl-4-methyl phenol)] of *E. grandis* on the seed germination, seedling growth and physiological indexes of lettuce, mungbean and radish to discover the mechanism of allelopathy through the exogenous addition of their various concentrations.

MATERIALS AND METHODS

Study Site

The sampling for identification of volatile chemicals in *Eucalyptus grandis* plantations (1-10 years) was done in Danling (102°57'-103° 04' E, 29 55-29 59 N, 570-593 m a.s.l), western Sichuan Province, southwestern China. The study site has subtropical climate and its mean annual temperature, precipitation and relative humidity are : 17.5 ℃, 1.397 mm and 82 %, respectively. More volatile compounds were found in the rhizosphere soil and roots of younger *E. grandis* plantation (about 4 years old) (27). Therefore, we selected three major compounds amongst the volatile chemicals to evaluate their potential allelopathic effects.

Materials preparation

Three volatile substances: (i). n-octane, (ii). 2, 4-di-tert-butyl phenol and (iii). 2, 2'-methylene bis (6- tert-butyl-4- methyl phenol) were applied to the seeds of 3-target plants: Mung bean (*Vigna radiata* L.), radish (*Raphanus sativus* L.) and lettuce (*Lactuca sativa* L.). Stock solutions of 2, 4-di-tert-butyl phenol and 2, 2'-methylene bis (6-tert-butyl-4- methyl phenol) powders were made by dissolving in acetone at 1 mol/L and stored at 4^{o}C. Five concs (C0,C1,C2,C3,C4) were prepared by diluting the stock solutions with sterile deionized water. The exerimental treatments consisted of two factors: (i). Volatile compounds concentrations: 4 (C1, C2, C3, C4) (Table 1) and (ii). Test plant spp.: 3 (Lettuce, mungbean, radish). The laboratory biassays were done in Petri plates. These solutions quantity added to Petri dishes was as per Table 1, containing of target plants, thus allowing sufficient volatilization. The treatments were replicated thrice in completley randomized design. As only little acetone were added to 2, 4- di-tert-butyl phenol and 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) treatments, we used water + acetone as control. Water was used as control for n-octane treatment.

Table 1. Details of concentrations of 3-volatile allelochemicals from *Eucalyptus grandis* in various treatments

Concentration	Octane (V/V)	2,4-2 tertiary butyl phenol (mmol/L)	2, 2'-methylene bis (6-tert-butyl-4-methyl phenol) (mmol/L)
C1 (0.05 ml)	0.05 % (0.05ml)	0.5 (0.05ml)	0.5 (0.05ml)
C2 (0.1ml)	0.1 % (0.1ml)	1 (0.1ml)	1 (0.1ml)
C3 (0.5 ml)	0.5 % (0.5ml)	5 (0.5ml)	5 (0.5ml)
C4 (1.0 ml)	1 % (1ml)	10 (1ml)	10 (1ml)

Note: a, the quantity added to each Petri late

Laboratory bioassay

Seeds of lettuce, mungbean and radish were disinfected with 0.15 % potassium permanganate solution for 2 h and rinsed with deionized water (24). In each petri plate, 50 seeds of mung bean and 100 of lettuce and radish, were placed equidistant on triple layer of filter paper in sterilized Petri dishes (10 cm dia.). The Petri dishes were sealed with sealing tape to prevent volatilization of compounds and placed in an incubator [Day/night temperatures : 25/20 ^{o}C; 14h/10h; relative humidity 80 %]. The experiment was continued for 5-7 days. Seeds were considered germinated, when the 3 mm radicle had emerged from the seed coat. The number of germinated seeds were counted; the lengths of radicals and shoots were measured and fresh weight were recorded and ratio of shoot/radical freshe weight ratio was calculated

Malondialdehyde content and seedling root activity

A malondialdehyde (MDA) test kit from Nanjing Jiancheng Biological Engineering Institute was used to determine the MDA content of seedlings. Leaf sample (0.2 g) was placed in a mortar with 1.8 ml sterile deionized water and small amounts of quartz sand and grounded to homogenized content (0.1 ml), it was transferred to a centrifuge tube and reagents were added according to the Manufacturer's Instructions. Centrifuge tubes with anhydrous ethanol and standard solution were used as control. The samples in the centrifuge tubes were mixed and boiled for 40 min in boiling water bath and then quickly cooled in ice bath. The samples were centrifuged for 10 min at 4000 rpm. The supernatants were by filtered through Whatman filter paper and the optical densities were determined at 532 nm. Then MDA contents were calculated as per the Manufacturer's Manual.

Root activities were determined by triphenyl tetrazolium chloride (TTC) reduction method (29) and expressed as amount of TTC reducted per unit of fresh roots per unit time (mg g^{-1} h^{-1}). The formula used was as under :

$$\text{Root activity} = C / (W*h),$$

Where, C: TTC reduction amount, W: Root weight and h: Time.

Allelopathy index (RI): It was used to measure the allelopathic strength of three chemical substances on the germination and seedling growth of the target plants as under:

$$RI = 1 - C/T \ (T \geq C), \text{ or } RI = T/C-1 \ (T < C)$$

Where, C: Control value, T: Treatment value. When RI > 0: indicates stimulation and RI < 0 indicates inhibitory effect.

Statistical analyses: Statistical analysis was done separately for each parameter. Differences in germination (%) and seedling growth of target plants were determined using a one-way ANOVA. Normality and homogeneity of variances were examined. Data were log-transformed to satisfy the requirement of variance homogeneity. If the data failed the equal variance tests, the homogeneity of variances was determined with the Levene test. Post-hoc means comparisons were conducted using the Tukey test. The statistically significant level used through this work was 0.05. All of the statistical analyses were performed using the SPSS 19.0 software package.

RESULTS AND DISCUSSION

Germination and growth

The increasing concentration of n-octane had inhibitory or stimultory effects on all target plants (Fig. 1). In *V. radiata*, it increased the inhibitory effects on seed germination rate, radicle length and shoot length. The C2 concentration, rather stimulated the root length.The fresh weight of radicle was stimulated at C1-C2 level but was inhibited with increasing concentrations. For *R. sativus,* C1-C3 concentrations increased the seed germination but C4 was inhibitory. The highest concentration (C4) inhibited the radicle growth, but lower concentrations (C2-C4) were stimulatory.

v-octane: The increasing concentration of n-octane stimulated the fresh weight of roots. The C3 and C4 were inhibitory to fresh weight of shoot but C2 was stimulatory. The C2 to C4 were inhibitory to radicle length of *L. sativa* but C1 level was stimulatory. The fresh weight of root and shoot were inhibited. There were no significant effects of n-octane on the seed germination rate and shoot length of *L. sativa*.

2, 4-di-tert-butyl phenol : *V. radiata* seed germination was completely inhibited at C3 and C4 concentrations (Fig. 1). The C3 concentration was less inhibitory to germination, root length and seedling height. The C1 and C2 were inhibitory to freshweight of radicle and shoot of *V. radiata*. In *R. sativus*, the seed germination, radicle length and shoot length were significantly inhibited. The C1 concentration stimulated the seed germination of

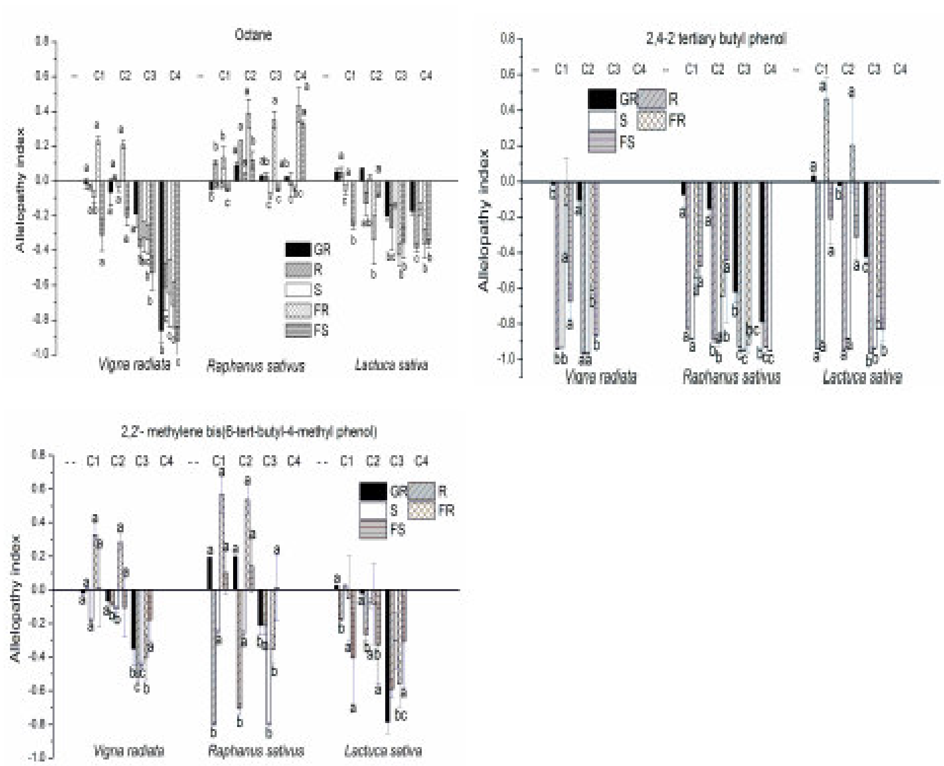

Figure 1. Effects of varying concentrations (C1, C2, C3, C4) of three volatile chemicals [Octane, 2,4-2 tertiary butyl phenol and 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol)] from *Eucalyptus grandis* on germination and initial seedling growth of three target plants. GR: Germination rate, R: Root length; S: Dhoot length; FR: Root fresh weight; FS: Dhoot freshe weight. C1: Octane 1%, 2,4-2 tertiary butyl phenol 10 mmol/L, 2, 2'-methylene bis (6- tert-butyl -4- methyl phenol) 10 mmol/L; C2: Octane 0.5%, 2,4-2 tertiary butyl phenol 5 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 5 mmol/L; C3: Octane 0.1%, 2,4-2 tertiary butyl phenol 1 mmol/L, 2, 2'- methylene bis (6-tert-butyl -4- methyl phenol) 1 mmol/L; C4: Octane 0.05%, 2,4-2 tertiary butyl phenol 0.5 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 0.5 mmol/L. S/R FW, fresh weight ratio of shoot and radicle.

L. sativa. With increased concentrations, the allelopathic inhibitory effects on seed germination, seedling height and radicle length were significantly strengthened. As for the root fresh weight, the C1-C2 concentration were little stimulatory. At C4 levels, the germination of *L. Sativa* seedc was completely inhibited.

2,2'-methylene bis (6-tert-butyl-4-methyl phenol: The seed germination of all target plants was completely inhibited at C4 concentration (Fig. 1). The inhibitory effects on germination, shoot length. radicle length of *V. radiata* were significantly more when the concentrations were increased. The fresh weight of root was stimulated at C1-C2 but was inhibited at C3. The seed germination, radicle length and height were decreased. The fresh weight of root and shoot was stimulated at C1-C2 but was inhibited at C3. In *L. sativa*, the C4 level completely inhibited the germination, the germination was stimulated at C1 and become inhibitory at C2,C3, C4 showed that the inhibitory effectes were strengthened at higher concentrations. The root length was inhibited significantly at higher concentrations, but there were no significant change in the shoot length. The roots fresh weight was inhibited and the inhibitory effects became more when the concentrations were increased. The inhibitory effects on shoot fresh weight did not change with the increase in concentrations.

The alkane-type allelochemicals are present in *E. grandis* leaves, roots and rhizosphere (4,10,28,31), but, there was little research on the mechanisms of action of allelochemicals. In this study, octane inhibited the seed germination and seedling growth of three target plants. The effects were inhibitory at high concentrations but stimulatory or ineffective at low concentration. *V. radiata* was most affected, followed by *L. sativa* and *R. sativus*. The octane significantly inhibited the seed germination and root length of three target plants. This might be because the radicles comes first in contact with the allelopathic substances, which might inhibit the radicle activity and growth (18,19). At the test concentrations, the inhibitory effects of 2, 4-di-tert-butylphenol was strongest of all three test volatile substances, and the inhibitory effects were concentration dependent. Phenols are the main products of secondary metabolism and these are the main substance in rain leachates of *E. grandis* leaves (9,12,23). Phenols inhibits the germination of plants through the inhibition of key enzymes in seed germination.

Our previous studies found that relative concentration of 2,2'-methylene bis(6-tert-butyl-4-methyl phenol) in the soils of *E. grandis* plantation was relatively high. Till now no studies have been done on the allelopathic effect of 2,2'-methylene bis(6-tert-butyl-4-methyl phenol) on plants. This research found that its allelopathic inhibitory effects on seed germination and seedling growth of *R. sativus* were stronger. In *V. radiata* and *R. sativus*, the seed germination and seedling growth were inhibited at high concentrations. The lower concentrations were stimulatory. This concentration dependent behavior is consistent with already reported allelopathic effects (13,20,32). However the C3 stimulated the fresh weight of radicle and shoot, perhaps due to different inhibitory impacts of allelochemicals on the shoot length and radicle length.

Plant physiological indices

In *V. radiata*, the effects of n-octane on MDA contents were inhibitory at all concentrations (Fig. 2). For the root activity allelopathic index, the inhibitory effects became stronger as the concentration increased. In *R. sativus,* the MDA contents in seeds was increased at C1-C2 levels but decreased at C3 and C4 concentrations. The root activity was stimulated at C1-C3 concentration, but was inhibited at C4. In *L. sativa,* C1 level was inhibitory and the MDA increased at C2-C4 concentrations of n-octane. The root activity was stimulated at C1 level, but was inhibited at C2-C4 concentration and the inhibitory effects became stronger with the increase in concentrations.

At high concentrations of 2,4-di tert-butyl (C3 and C4), *V. radiata* seed germination was completely inhibited. MDA content of *V. radiata* seedlings did not show significant allelopathic changes at C3-C4 level. With the increasing concentration, the

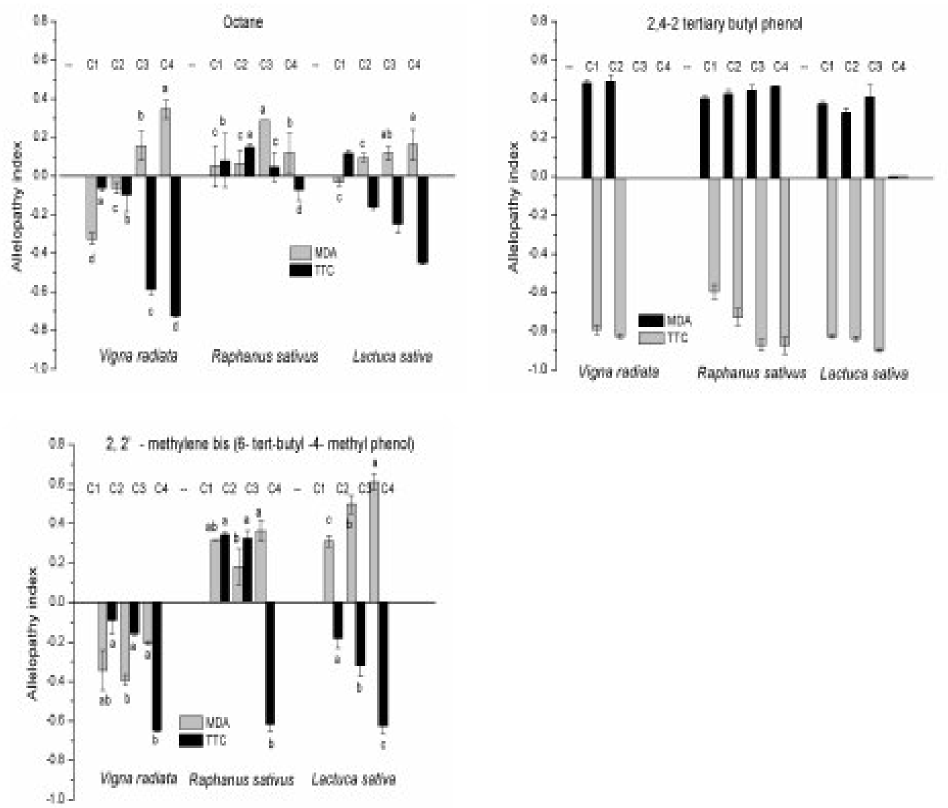

Figure 2. Index of allelopathic effects of varying concentrations (C1, C2, C3, C4) of three volatile chemicals [Octane, 2,4-2 tertiary butyl phenol and 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol)] from *Eucalyptus grandis* on physiological indexes (malondialdehyde (MDA) and triphenyl tetrazolium chloride (TTC)) of initial seedling growth for three target plants. C1: Octane 1%, 2,4-2 tertiary butyl phenol 10 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 10 mmol/L; C2: Octane 0.5%, 2,4-2 tertiary butyl phenol 5 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 5 mmol/L; C3: Octane 0.1%, 2,4-2 tertiary butyl phenol 1 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 1 mmol/L; C4: Octane 0.05%, 2,4-2 tertiary butyl phenol 0.5 mmol/L, 2, 2'- methylene bis (6- tert-butyl -4- methyl phenol) 0.5 mmol/L.

effects on the *R. sativus* MDA contents were slightly stimulatory. In *R. sativus* root activity, the allelopathic inhibitory effects were increased. At C4 concentration, the germination of *L. sativa* seeds was completely inhibited. As the concentrations increased there were stimulatory effects on the MDA contents of seedlings, but there were non-significant differences. The allelopathic effects on root activities for *V. radiata* were inhibitory but did not show significant changes. However, the inhibitory effects on *V. radiata* were not significantly different at C1-C2 level and increased at C3 level.

At the highest concentration (C4) of 2,2'-methylenebis(6-tert-butyl-4-methyl phenol), the seed germination of all three target plants was completely inhibited (Fig. 2). In *V. radiata* seedlings, the MDA content was inhibited and the effects were decreased with the substance concentration. For *R. sativus* the stimulatory effects on the seedlings' MDA contents were decreased at first (C1 to C2) and then increased with C2 to C3 levels. Regarding the root activities, the effects were stimulatory at C1 and C2 and were inhibitory at C3 to C4 levels. The allelopathic inhibitory effects on the root activities for *V. radiata* and *L. sativa* were significantly increased with the increment in the concentration of test substance (Fig. 4-c).

The root activity represents the strength of metabolic activities of roots (14) and it indicates the roots development and absorption functions. The octane inhibited the root activities (TTC) of three target plants at high concentrations but was stimulatory at low concentrations. The allelochemicals inhibited the cell division and elongation and also affects the key enzyme activities that are required for seed germination, thus, reducing seed vitality and seed germination (13,20,28). Initially the cell membranes were damaged due to plant allelopathy, which allows a stress signal to be transported into the cells. It affects the hormone production, ion absorption and changes the photosynthesis and cell division i.e. inhibitory to plants (2,14). MDA indicates the membrane lipid peroxidation level and is harmful substance. It can react strongly with various components within the cell, thereby causing serious damage to the membranes and enzymes leading to destruction of cell membrane structure and the physiological integrity (20,32). The octane increased the MDA contents in the seedlings of three target plants at low concentration and decreased at the high concentration. Thus octane could effectively contribute to the membrane lipid oxidation and inhibit the germination and seedling growth of target plants. Previous studies found that there were alkane-type allelochemicals in *E. grandis* leaves, roots and rhizosphere (4,10,28,31). However, little research has been done on the mechanisms of action of allelochemicals. In this study, octane significantly inhibited the seed germination and seedling growth of target plants. The effects were stimulatory or ineffective at low concentration and became inhibitory at high concentrations. The allelopathic effects of octane on the seed germination and root length of three target plants were significant, perhaps, on the contacts the allelopathic substances firstcomes in contact with roots, which might inhibit the radicle activity and growth (18,19). *V. radiata* was affected the most, followed by *L. sativa* and *R. sativus*.

Phenols also affect the seedling growth through interfering with the physiological features of seedlings i.e. photosynthetic characteristics and growth (12,14). Moreover, they inhibit the seedling growth by interfering with the photosynthesis and transpiration rates etc. (12,16). In this study, 2,4-di-tert-butylphenol at different concentrations stimulated the MDA contents of seedlings of target plants and also affects the seedling root activities. The low concentrations of 2,2'-methylene bis (6-tert-butyl-4-methyl phenol) were inhibitory to MDA content in *V. radiata* seedlings and the inhibitory effects weakened with the increase in concentrations. Perhaps at low concentrations of phenolic compounds, the plants have certain defensive mechanisms for protection against the effects of allelochemical on *V. radiata* seedlings. Under unfavourable conditions they can release H^+, to resist the membrane lipid peroxidation (12,14,17). Simultaneously this substance might also influence the enzymes, needed for the seed germination and thus inhibited the activities of root system to affect the germination and growth of *V. radiata* seeds.

CONCLUSIONS

The test volatile compunds [n-octane, 2,4- di-tert-butyl phenol and 2,2'-methylene bis (6-tert-butyl-4-methyl phenol)] were inhibitory to test plants (*Vigna radiata*, *Raphanus sativus* and *Lactuca sativa*) at high concentrations and stimulatory at low concentrations. The three volatile substances might enhance the MDA production and thereby suppress the root activities of seedlings to influence the germination and growth of target seedlings at high concentrations. The effects on the root activities were more obvious. The *E. grandis* plantation area used in this study has plenty of rainfall, which may leach the water soluble chemical compounds, thereby, reducing the allelopathic substances concentrations. The increasing concentrations of allelopathic substances in soil, including the test volatile substances in this research and other allelochemicals, may reduce the diversity in the *E. grandis* plantations.

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