Pak. J. Bot., 49(1): 345-351, 2017.

DIFFERENCE BETWEEN NON-TRANSGENIC AND SALT TOLERANT TRANSGENIC *EUCALYPTUS CAMALDULENSIS* FOR DIVERSITY AND ALLELOPATHIC EFFECTS OF ESSENTIAL OILS

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Abstract

Eucalyptus camaldulensis is one of the most widely planted eucalypts in the world that has importance in agricultural, ecological, cultural and recreational areas. Allelopathic effects of essential oils extracted from non-transgenic and transgenic (salt tolerant conferring full *mangrin* gene and core *mangrin* gene) *E. camaldulensis* lines were studied on lettuce (*Lactuca sativa* L.) seed germination and early (72 h) growth of root and hypocotyl. Seed germination was significantly higher in most of the transgenic lines than non-transgenic lines. Low (1–3 µl) oil concentration stimulated root growth but was inhibitory for the hypocotyl. Higher concentrations (>20 µl) of these oils arrested growth of both the roots and hypocotyls of lettuce seeds completely. In general, all the results showed no significant inhibitory effects of transgenic and non-transgenic lines on lettuce seeds. GCMS analysis showed 18 chemical compounds with 1,8-cineole and α-pinene, as a major oil constituents of *E. camaldulensis* with no variation between transgenic and non-transgenic lines. However, the average quantity of α-pinene in non-transgenic lines was higher than the transgenic lines but in case of 1,8-cineole no difference was observed between transgenic and non-transgenic genotypes.

Key words: Allelopathy, Essential oils, Mangrin gene, Seedling growth, Transgenic *Eucalyptus camaldulensis*.

Introduction

Eucalyptus camaldulensis trees are being planted on a large scale especially in tropical regions of the world because it is a valuable and low cost commercial source of pulp and paper (Moraes, 2008). In Brazil, for example, Brazilian Paper and Pulp Association possesses 1.7 million hectare of land which is utilized for cultivating *Eucalyptus* trees to obtain pulp that is a major demand from India and China (Moraes, 2008).

Despite the plantation of trees for various commercial purposes on irrigated and suitable lands, this is also noteworthy that about 23% of the cultivated and 33% of the irrigated land in the world is facing the problem of salinity (Khan & Duke, 2001). According to some estimates, about 25 million hectares of agricultural land have gone out of cultivation and an additional two million hectares of land are becoming victim of this menace annually (Watanabe & Ebinuma, 2004). This unabated salinization of agricultural lands does not only have serious impact on global ecosystem but is also causing devastating diseases in plants (Kozlowski, 1997). Salinity is one of the main barriers for crop productivity (Shereen *et al*., 2015). Biochemical markers may be used to identify salinity tolerant varieities (Javed *et al*., 2014).

Salt tolerant *Eucalyptus* may be planted in saline areas as an ameliorative measure to remove excess amount of water and salts from the soil. Our group (Watanabe Lab, Gene Research Center University of Tsukuba, Tsukuba Japan) and Nippon Paper Company, Tokyo, Japan has developed transgenic plants through introgression of '*mangrin*' gene into *Eucalyptus camaldulensis* which can be used for this purpose. Previously, the same group has developed transgenic *Eucalyptus camaldulensis* conferring *codA* gene and conducted allelopathic tests for environmental biosafety assessments (Kikuchi *et al*., 2006, 2009). *Mangrin* gene, comprising of 70 amino acids, was isolated from Mangrove (*Bruguiera sexangula*) that expressed salt tolerance when introgressed into various organisms (Yamada *et al*., 2002) including *Eucalyptus* and thus may have potential of improving biomass yield. However, it is necessary to study the risk assessments of these transgenic plants in semi-confined conditions (special netted-house) before transferring into isolated fields and finally to commercial production (Yu *et al*., 2009).

Like other species of *Eucalyptus*, *E. camaldulensis* is rich in essential oils. Its major oil constituents are 1,8-cineole and α-pinene (Masamba *et al*., 2001) which are considered highly toxic (Moral & Muller, 1970). However, the amount falls within safe limits (<70%) for pharmaceutical and cosmetic industries (Lawrence, 1986; Zrira *et al*., 1992; Farah *et al*., 2002). Allelopathic effect of crude essential oils extracted from *E. globulus* and *E. citriodora* were previously reported in literature (Kohli & Singh, 1991). Both the plants species showed allelopathic activity while *E. citriodora* exhibited higher allelopathic effects compared to *E. globulus*. Essential oils of non-transgenic *E. camaldulensis* have also shown strong allelopathic effects due to the presence of 1,8-cineole and α-pinene (Moral & Muller, 1970). The review of the essential oil composition in *Artemisia absinthium* by GCMS analysis revealed 16 different chemo-types of *A. absinthium* based on collections from different countries in which even major compounds such as thujone were either present or absent (Gilani *et al*., 2012).

The aim of this study was to compare between transgenic and non-transgenic *E. camaldulensis* lines for their allelopathic effect and the qualitative and quantitative variation in major constituents of essential oils considering the substantial equivalence. A new method known as 'dish-pack method' (Sekine *et al*., 2007) was used for this purpose.

Materials and Methods

Plant collection: Fourteen months old transgenic *Eucalyptus* lines expressing full *mangrin* (No. 10, 15, 60, 65, 71) and core *mangrin* genes (No. 40, 56), respectively were maintained along with five non-transgenic *E. camaldulensis* lines under semi-confined conditions (special netted-house) (Table 1; Yu *et al*., 2013). These netted houses are designated as Type II use application under the Japanese law of environmental biosafety. Five trees were selected from each line of *E. camaldulensis* for this experiment (Table 1). The fresh leaves of seven transgenic lines (No. 10, 15, 60, 65, 71, 40, 56) and five non-transgenic lines (cam2, cam4, cam6, cam11, CML2) of *Eucalyptus camaldulensis* were collected for the allelopathic studies and oil extraction.

Table 1. List of salt tolerant transgenic (expressing *Mangrin* gene) and non-transgenic *Eucalyptus* trees which were used for the allelopathic studies.

S. No.	Lines	Transgene construct	Number of trees (clones)
		Non-transgenic lines	
1.	cam2	Nil	5
2.	cam4	Nil	5
3.	cam6	Nil	5
4.	cam11	Nil	5
5.	CML2	Nil	5
		Transgenic lines	
1.	10	PMC8-Full *Mangrin*	5
2.	15	PMC8-Full *Mangrin*	5
3.	60	PMC8-Full *Mangrin*	5
4.	65	PMC8-Full *Mangrin*	5
5.	71	PMC8-Full *Mangrin*	5
6.	40	PMC8-Core *Mangrin*	5
7.	56	PMC8-Core *Mangrin*	5

Essential oil extraction: Fresh leaves of transgenic and non-transgenic *Eucalyptus camaldulensis* were crushed into powder. Each line represented five trees as replications. The fresh leaves weighing 30-50 g were crushed and used for extraction of essential oils through Clevenger type distillation apparatus for 5 hours. Diethyl ether was added into the essential oils to dissolve oils and water vapors. It was then separated from the oil layer through separating funnel. Magnesium sulphate was added and the oil sample was left for 3 hours in the refrigerator. After three hours, the oil was separated from magnesium sulphate and stored at -20^{o}C till further use.

Allelopathic studies: Allelopathic studies of essential oils of *E. camaldulensis* were carried out using dish pack method (Sekine *et al*., 2007). A six well (35 mmØ/well) multi-dish plate (TPP/BME, Made in Europe/Switzerland) was used for bioassay analysis. From six well, one well contained a 0.25 ml polystyrene sample cup (φ11.0 x φ 13.5 x 16.3) while in rest wells, filter papers (33mm, ADVANTEC No. 1, Qualitative, Toyo Roshi Kaisha Ltd, Japan) were placed to make the seed bed. Sample cup were used for determine the allelopathic effect of various concentrations (1, 2, 3, 4, 5, 20 and 50 µl) of extracted oil on seeds germination and early seedling growth. In all wells except sample cup containing well, added 0.7 ml distilled water followed by placed seven lettuce seeds (variety Grate Lakes No. 366; Takii Seeds Corp., Japan) on filter papers. Lettuce (*Lactuca sativa*) seeds were around 41 mm, 58 mm, 82 mm and 92 mm far from the well containing sample cup with extracted oil as mentioned previously (Sekine *et al*., 2007). The multi-dish plates were sealed with plastic tape and incubated at 26^{o}C for 72 hours. Each treatment was repeated thrice. After 72 hours incubation, seed germination was recorded in each well. From the 7 seeds, the longest and shortest seeds were discarded while lengths of roots and hypocotyls of remaining 5 seeds were measured.

GCMS analysis: Five trees per lines were used as replication for essential oil analysis. 10 µl oil sample was added into 10 ml n-Hexane from which 100 ppm (v/v) of essential oil was injected into GC-MS QP-5050 (Shimadzu). EQUITY-5 (Supelco) column (5% Phenyl 95% - Dimethylpolysiloxane) of 30 m, 0.25 mm i.d., with film thickness of 0.25 µm was used. Operating conditions of GC-MS followed the time program of 50-150^{o}C with rise of 3^{o}C/min which was held for 10 minutes and then was increased to 280^{o}C with rise of 10^{o}C/min. The compounds were identified with mass spectra of NIST/NBS, Wiley libraries and literature (Taniguchi *et al*., 2008).

Data analysis: For statistical analysis, SYSTAT version 11 was used. Randomized complete block design (RCBD) was followed and 2-way ANOVA was performed for roots, hypocotyls and seed germination as dependent variables with two factors: transgenes (non-transgenic and transgenic) and oil concentrations (1, 2, 3, 4, and 5 µl). After analyzing major component of oil i.e., 1,8-cineole and α-pinene from transgenic and non-transgenic plants, regression analysis was also carried out to see the correlation between toxicity level of essential oils and root, hypocotyl and seed germination of lettuce seeds.

Results

Allelopathic effect of essential oils on lettuce seeds: Hypocotyl of lettuce seeds present at 41 mm showed more inhibition than 92 mm distance (Fig. 1A). Whereas, the distances (41, 58, 82 and 92 mm) of seeds from the sample oil did not show any uniform effect on root growth (Fig. 1B). The effect of transgenic and non-transgenic lines of *E. camaldulensis* on seedling of lettuce was almost similar (Fig. 1).

Seed germination showed significant differences between non-transgenic and full *mangrin* gene (P = 0.01; Table 2), which was because of low seed germination rate in some of the non-transgenic lines at 5 µl essential oil concentration as compared to the transgenic lines. In case of *E. camaldulensis*, >20 µl concentrations of essential oils from both the transgenic and non-transgenic plants were strong enough to inhibit the germination of lettuce seeds completely (Fig. 2A). No difference in allelopathic effect of essential oil extracted from transgenic and non-transgenic lines of *E. camaldulensis* was found on seed germination (Fig. 2A).

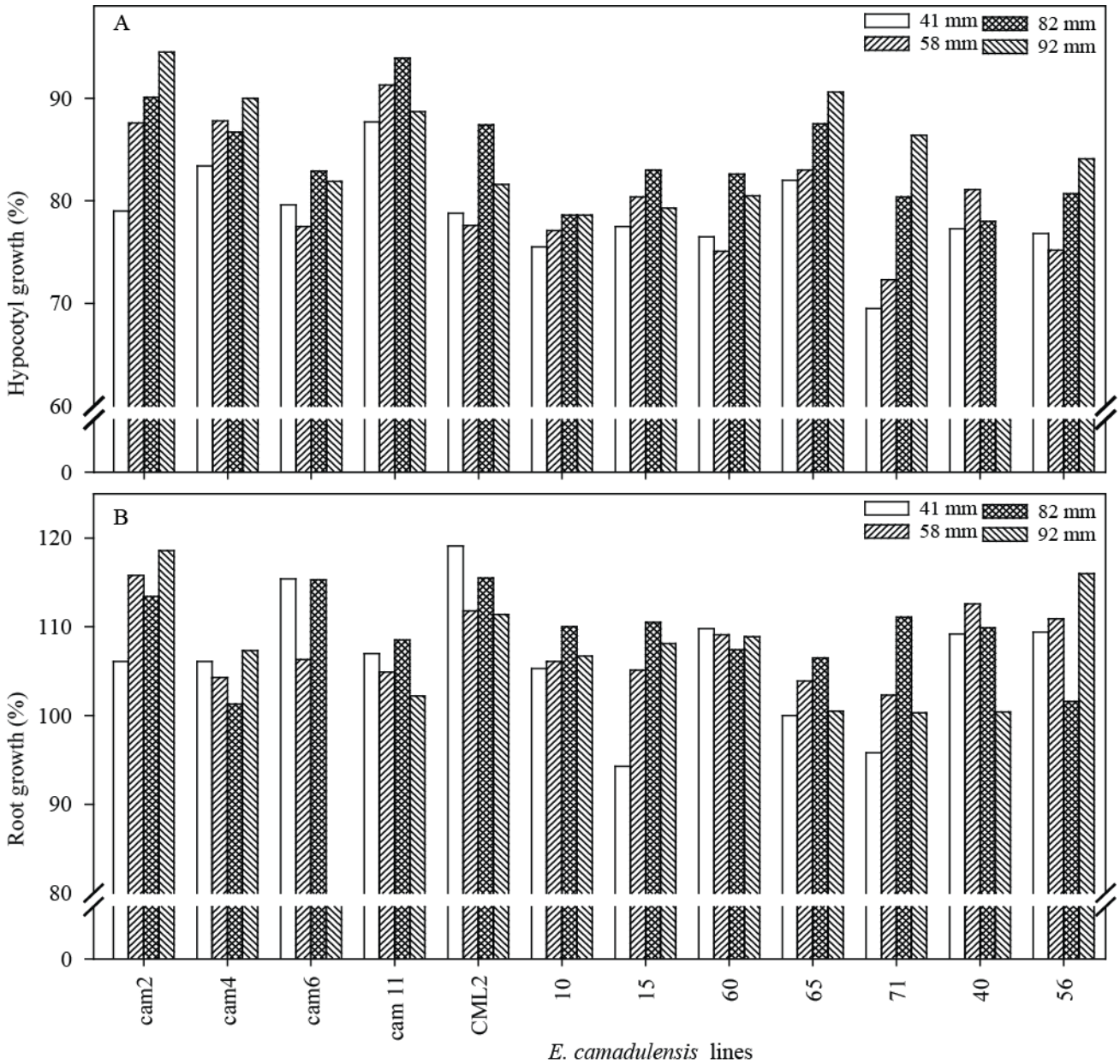

Fig. 1. Effects of essential oil (1 µl) extracted from transgenic and non-transgenic *Eucalyptus camaldulensis* on growth of lettuce seedling present at various distances.

Table 2. A two-way ANOVA of construct type (Cons. Type) and extracted oil concentration (Conc.) their interactions on germination and seedling growth of lettuce seeds.

	Cons. Type		Conc.		Cons. Type x Conc.	
	***F*-value**	***p*-value**	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Roots						
Full *Mangrin*	0.77	0.378	1587.31	<0.01	12.50	<0.01
Core *Mangrin*	37.18	<0.01*	934.61	<0.01	5.25	<0.01
Both full and core	10.55	<0.01	1889.10	<0.01	11.49	<0.01
Hypocotyl						
Full *Mangrin*	0.16	0.686	1229.77	<0.01	7.19	<0.01
Core *Mangrin*	1.70	0.193	726.88	<0.01	2.45	0.044
Both full and core	0.76	0.381	1560.45	<0.01	6.12	<0.01
Germination						
Full *Mangrin*	32.66	<0.01*	2030.32	<0.01	12.39	<0.01
Core *Mangrin*	14.45	<0.01*	2751.57	<0.01	3.19	0.013

*Significant differences occurred in ANOVA results that was due to variation in control non- transgenic lines with lower seed germinations as compared to transgenic lines

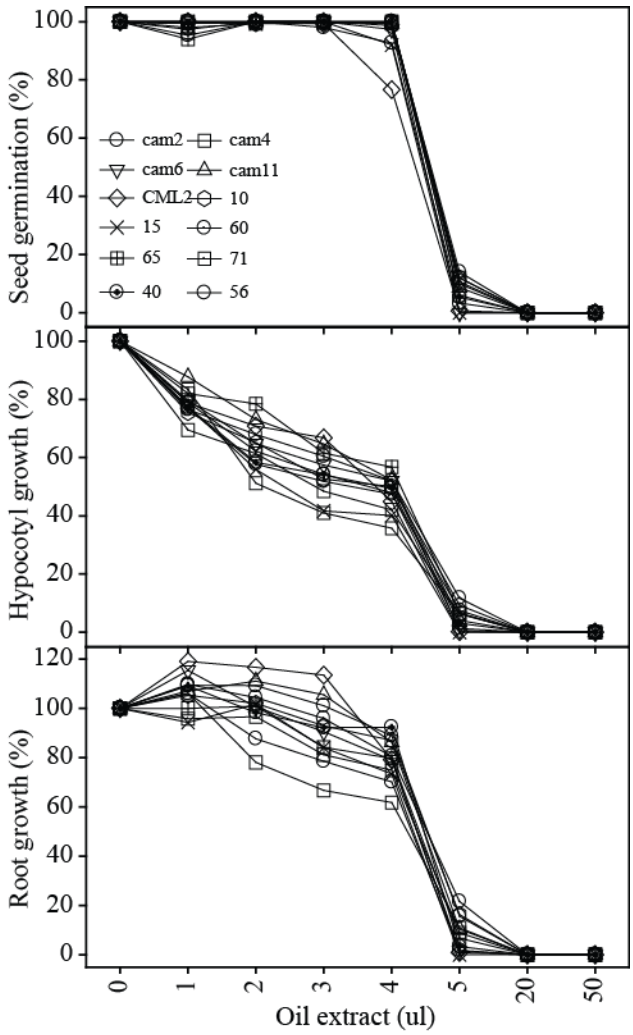

Fig. 2. Effects of different quantity of essential oil (0-50 µl) extracted from transgenic and non-transgenic *Eucalyptus camaldulensis* on lettuce seed germination (A) and seedling growth (B: hypocotyl; C: root).

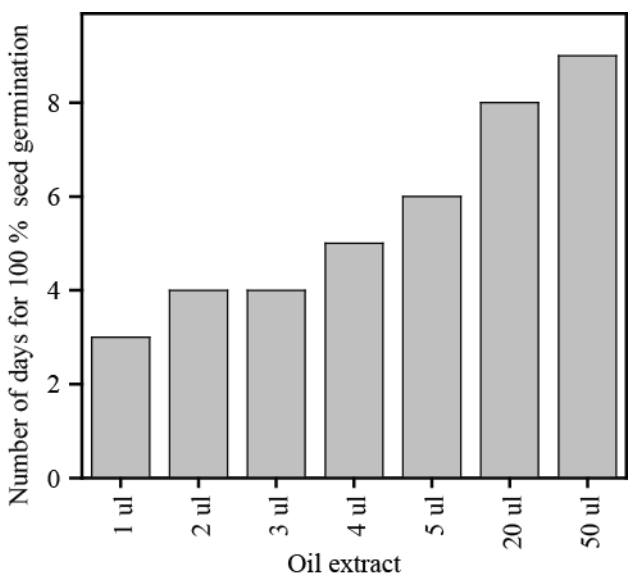

Fig. 3. Number of days that took to lose toxicity of various concentrations of essential oil both in transgenic and non-transgenic *Eucalyptus camaldulensis* and resultantly 100 % lettuce seed germinated.

The effect of essential oils on root and hypocotyl growth of lettuce seedlings were concentration dependent in all the *E. camaldulensis* lines (P = 0.01; Table 2). When the allelopathic effects of non-transgenic (cam2, cam4, cam6, cam11 and CML2) lines with transgenic full *mangrin* gene lines (No. 10, 15, 60, 65 and 71) were compared with each other, the roots (P = 0.378) and hypocotyl growths (P = 0.686; Table 2) showed no significant differences with each other. At 1 µl to 5 µl of essential oil, All the 5 non-transgenic lines showed stimulatory effects on root growths of lettuce seeds at 1 µl from 106.1 to 119.1% while in full *mangrin* gene lines, the stimulatory effects were observed from 100 to 109.8 in three lines No. 10, 60 and 65, only while in rest of the two lines No. 15 and 71, 94.3 and 95.8% inhibitory effect on root growth of lettuce seedlings was observed. In core *mangrin* gene lines (No. 40 and 56), stimulatory effects of 109% was observed on root growth of lettuce seedlings (Fig. 2C). In case of hypocotyle, the 5 non-transgenic lines exhibited inhibitory effects ranging from 78.8 to 87.7%; the full *mangrin* gene lines showed 79.5 to 82.0% inhibitory effects; and two lines of core *mangrin* gene showed 76.8 to 77.3% inhibitory effects (Fig. 2B).

The transgenic lines with core *mangrin* gene (No. 40 and 56) were compared with non-transgenic lines (cam2, cam4, cam6, cam11 and CML2), the hypocotyls lengths did not show significant differences (P = 0.193) but significant differences were observed for root lengths (P = 0.01) and seed germination (P = 0.01) (Table 2). The significant difference in root lengths was caused by the variation in root lengths of non-transgenic lines, cam2 and cam4 but not because of transgenic lines (data not shown).

The seeds in all the treatment of both transgenic and non-transgenic lines, fully germinated when left for a longer incubation periods and were dose dependent. Plates with lower concentration exhibited fast seed germination than higher concentrations. Germination of lettuce seed's was completed during 3, 6, 8 and 9 days when exposed with 1, 5, 20 and 50 µl concentration of essential oil, respectively (Fig. 3).

Qualitative and quantitative comparison of major oil constituents in transgenic and non-transgenic *E. camaldulensis*: GCMS analysis of essential oils of both transgenic and non-transgenic *Eucalyptus camaldulensis* showed 18 compounds (Table 3). Among these 18 compounds, the major constituents were 1,8-cineole and α-pinene and present in all transgenic and non-transgenic lines. α-pinene quantity in non-transgenic *E. camaldulensis* was 2.33–12.62%, in transgenic genotypes with full *mangrin* gene 1.48–8.71% and with core *mangrin* gene 3.89–4.46% (Fig. 4A). Similarly, the non-transgenic genotypes showed 63.08–86.58% of 1,8-cineole while transgenic genotypes with full *mangrin* gene 76.5–87.68% and transgenic genotypes expressing core *mangrin* gene had 78.92–83.18% (Fig. 4B).

In our studies, correlation analysis of 1,8-cineole concentration resulting from GCMS analysis was studied against the effects of 1 – 5 µl concentrations of the same oils on root, hypocotyl lengths and seed germination of lettuce seeds (Table 4). However, no significant correlation was observed among the 1,8-cineole and allelopathic properties of the same oils (Table 4) in any of the transgenic and non-transgenic *E. camaldulensis* lines. Similarly, α-pinene also did not show significant correlation with allelopathic effects (Table 4).

Table 3. Chemical profiling of essential oils of transgenic and non-transgenic *Eucalyptus camaldulensis* by GCMS analysis.

Chemical compound	RT	cam2	cam4	cam6	cam11	CML2	10	15	60	65	71	40	56
α-pinene	8	3.85	4.49	12.62	2.33	3.75	8.12	8.71	1.47	4.67	2.31	4.46	3.89
(-)-β-pinene	9	0.42	-	0.61	-	-	0.57	-	-	-	-	-	-
Myrcene	10	0.71	0.63	0.68	-	0.68	0.88	0.65	0.75	0.78	0.74	0.67	0.71
1-methyl-2-iso-propylbenzene	11	0.64	0.49	0.73	2.22	1.21	-	-	0.88	-	-	0.61	-
1,8-cineole	12	83.29	86.58	74.63	78.37	63.08	76.50	79.09	82.63	87.68	87.09	83.18	78.92
γ-terpinene	13	1.25	0.78	0.69	4.19	0.65	-	-	1.49	0.53	0.55	0.75	0.86
(-)-trans-pinocarveol	16	-	0.88	0.89	-	-	-	0.74	-	-	-	-	-
(-)-terpinen-ol	18	2.36	1.19	0.52	1.85	-	0.58	0.55	0.76	0.70	0.78	0.58	-
α-terpineol	19	2.24	1.57	1.37	0.85	0.87	1.54	1.02	1.34	1.10	1.84	1.63	1.53
Terpenyl acetate	26	-	-	3.58	4.38	4.14	-	3.78	3.64	-	-	1.40	-
α-gurjurene	29	0.40	-	1.93	-	1.45	0.90	0.56	0.82	4.48	-	0.73	1.62
Aromadendrene	30	2.04	2.06	3.16	2.65	9.54	3.67	2.76	2.31	1.21	2.54	3.34	5.88
Alloaromadendrene	31	1.01	1.09	1.20	1.06	2.80	1.47	0.91	1.30	0.52	1.08	1.46	2.54
Ledol	35	-	-	1.01	-	1.14	-	-	-	-	-	-	1.07
Globulol	37	1.64	1.89	2.51	2.04	7.12	3.13	2.10	2.24	0.91	2.11	2.66	3.63
Epiglobulol	37	0.59	0.70	1.74	0.58	1.64	0.80	-	0.73	-	0.57	0.68	1.26
β-eudesmol	38	-	-	0.61	-	0.71	-	-	-	-	-	-	-
γ-eudesmol	39	0.38	-	0.89	-	1.02	0.55	-	-	-	-	-	1.14

Note: Values represent average percentage compositions with standard deviations based on five trees data for each line

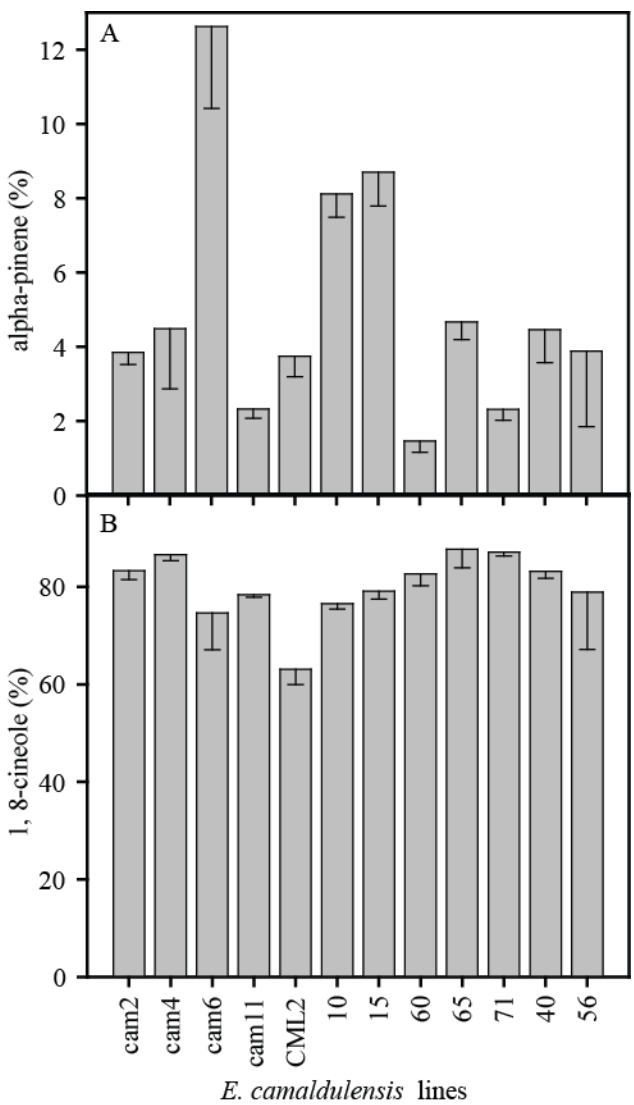

Fig. 4. Yield of α-pinene (A) and 1,8-cineole (B) in transgenic and non-transgenic *Eucalyptus camaldulensis*.

Table 4. Correlation analysis of major constituents (1,8-cineole and α-pinene) of transgenic and non-transgenic *Eucalyptus camaldulensis* against lettuce seedling growths at various concentrations of essential oils (R^2 value) of *Eucalyptus camaldulensis*.

Growth	1 µl	2 µl	3 µl	4 µl	5 µl
1,8-cineole					
Root	0.4573	0.3217	0.4791	0.0486	0.0220
Hypocotyl	0.0324	0.0682	0.2155	0.0157	0.0897
Seed germination	0.0688	6E-05	0.0297	0.6065	0.0768
α-pinene					
Root	0.0100	0.0136	0.0252	0.0030	0.0914
Hypocotyl	0.0092	0.0135	0.0453	0.0104	0.0929
Seed germination	0.0012	0.1665	0.0800	0.0059	0.1211

Discussion

Allelopathic effect of essential oils on lettuce seeds: The environmental effects of transgenic *E. camaldulensis* may be examined in terms of allelopathic studies with four methods, sandwich method, soil germination method, GC for qualitative analysis of volatile compounds (essential oils) and HPLC studies (Kikuchi *et al*., 2009). Besides these methods, the essential oils of *E. camaldulensis* may also be studied in detail. The allelopathic studies and the detailed quantitative GCMS analysis of major chemical constituents of transgenic *E. camaldulensis* may also be conducted. Recommended concentration essential oil is around 50 µl for allelopathic studies using dish pack method (Sekine *et al*., 2007; Gilani *et al*., 2012) but for *E. camaldulensis* more than 6 µl caused no germination in lettuce seeds. It shows that the concentration used for allelopathic studies will vary from species to species. It also confirmed the earlier reports on *E. camaldulensis* that toxicity level of essential oils of *E. camaldulensis* increased with increasing concentration (Kohli & Singh, 1991).

Using various concentrations of essential oils for allelopathic effects to evaluate the range of difference between transgenic and non-transgenic plants may be the first time which we used in case of transgenic *E.*

camaldulensis. Therefore, before performing experiment, various concentrations of essential oils may be applied. However, previously transgenic *E. camaldulensis*, genotypes *oda* 12-5B, *oda* 12-5C, and *oda* 20C were analyzed using sandwich method and did not find any difference in the toxicity levels of dried leaves of transgenic and non-transgenic lines (Kikuchi *et al*., 2009).

Line to line variation in roots and hypocotyl growth in current study in some cases was observed but when two-way ANOVA was performed it showed no significant differences among transgenic and non-transgenic *Eucalyptus* lines at all the concentrations. Similar kind of results were observed when performed one-way ANOVA while assessing transgenic *E. camaldulensis* with *oda* gene and poplars overexpressing xyloglucanase (*AaXEG2*) genes (Adams, 1995; Kikuchi *et al*., 2009).

Qualitative and quantitative comparison of major oil constituents in transgenic and non-transgenic *E. camaldulensis*: No change or absence of major oil constituents were observed in either of the lines which is in confirmation with the previous findings who studied the essential oils in three transgenic *E. camaldulensis* Dehnh (Kikuchi *et al*., 2006, 2009). Lines, *oda* 12-5C, *oda* 12-5B and *oda* 20-C, conferring salt tolerance. 1,8-cineole had completely inhibited the seed germination of *Phaseolus aureus* and *Lens esculentum* previously (Kohli & Singh, 1991). Fluctuations in quantities of essential oils are not uncommon in *E. camaldulensis* which have the properties of toxicity variation even within the same uniform environment (Moral & Muller, 1970). Wide ranges of concentrations of 1,8-cineole (9.8 to 35.5%) and α-pinene (1.2 to 8.7%), have already shown considerable quantitative variations in non-transgenic *E. camaldulensis* (Masamba *et al*., 2001). However, minor oil constituents were lacking or present both in transgenic and non-transgenic plants (Table 4) which is not uncommon in literature (Iqbal *et al*., 2008) and our review on several other reports from more than 10 countries revealed that even major constituents were lacking within *E. camaldulensis*, in addition to the minor oil compounds (data not shown). On the basis of our findings and reviewing the literature, it may be concluded that the presence or absence of essential oils is a common phenomenon.

There was no significant difference in the allelopathic effects of essential oils of transgenic and non-transgenic *E. camaldulensis*. Our results are in confirmation with the previous findings who studied the allelopathic assessments of salt-tolerant transgenic *E. camaldulensis* conferring bacterial coline oxidase (*oda*) gene in the designated special netted-house conditions (Kikuchi *et al*., 2006, 2007, 2009). They found no difference between three transgenic and a non-transgenic *E. camaldulensis* in allelopathic effects using sandwich method. The essential oils of these plants also remained the same qualitatively, when observed through gas chromatography (Kikuchi *et al*., 2006, 2007, 2009). Similarly, the toxicity of essential oils is also not a long lasting but will be decreased in transgenic and non-transgenic lines as the time passes because of their volatile nature. The quality and quantity of both the transgenic *E. camaldulensis* expressing full *mangrin* and core *mangrin* genes also did not change significantly. Range of variation in quantities of both the non-transgenic and transgenic genotypes is also not uncommon in nature. Therefore, it can be safely concluded from the current results that transgenic *Eucalyptus* did not show significant variations in toxicity levels and also quality and quantity of major essential oil constituents did not change in our study. It is also recommended that for comparative studies of effects of essential oils-rich leaves of any transgenic plant may be studied with dish pack method using various concentrations and enough replications to get reproducible data, as we did in our study.

Acknowledgements

The authors are thankful to Nippon Paper Co, LTD for providing plant materials and to Prof. Yoshihiro Ozeki and Assoc. Prof, Akiyo Yamada, Tokyo University of Agriculture and Technology for access to the facilities in their lab to evaluate the *mangrin* gene. This research was supported in part by Plant Transgenic Design (PTraD) Research Initiative, at Gene Research Renter, University of Tsukuba.

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(Received for publication 15 June 2015)