Title

Dark aerobic methane emission associated to leaf factors of two *Acacia* and five *Eucalyptus* species

Author names and affiliations

MAKOTO WATANABE[1, 2], YOKO WATANABE[3], YONGSUK KIM[1, 2, 4] and TAKAYOSHI KOIKE[2*]

[1] JSPS Research Fellow

[2] Silviculture and Forest Ecological Studies, Hokkaido University, Sapporo, Hokkaido 060–8589, Japan

[3] Forest Research Station, Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060–0809, Japan

[4] Present Address: Division of Forest Water and Soil Conservation, Korea Forest Research Institute, Seoul 130-712, Republic of Korea

Corresponding author

Takayoshi Koike,

Tel: +81–11–706–3854, Fax: +81–11–706–2517

E-mail: tkoike@for.agr.hokudai.ac.jp

Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Highlights

> Factors explaining variations between species in leaf methane emission were studied.

> We measured the rate of methane emission by detached leaves under dark conditions.

> There was a negative correlation between LMA and the methane emission rate.

> Leaf structure is an important in variations of methane emission rate between species.

Abstract

We sought the biological factors determining variations in the methane emission rates from leaves of different plant species under aerobic conditions. Accordingly, we studied relations between the methane emission rate and leaf traits of two *Acacia* and five *Eucalyptus* species. We grew seedlings of each species in a glasshouse and measured the methane emission rate of the detached leaves under dark conditions at 30 ºC. At the same time we measured the leaf mass per area (LMA), water content, and concentrations of carbon and nitrogen. There was no correlation between the leaf nitrogen concentration and the methane emission rate. This is consistent with previous findings that enzymatic processes do not influence methane emission. We found a significant negative correlation between LMA and the methane emission rate. Our results suggest that leaf structure is primarily responsible for differences in the rates of aerobic methane emission from leaves of different species.

Key words:

aerobic methane emission; correlation analysis; evergreen tree species; greenhouse gas; leaf mass per area; leaf structural trait

1. Introduction

Methane is the second most important anthropogenic greenhouse gas in the atmosphere after carbon dioxide. The radiative forcing effect of methane is considered to be 25 times greater than that of CO_2 (Forster et al., 2007). Methane

is produced under anaerobic conditions through microbial metabolism, and is released into the atmosphere. The largest source of methane production is estimated to be wetlands, with relatively high emissions from rice paddies and ruminant animals (Denman et al., 2007). As well, non-microbial emissions occur as a result of fossil fuel usage and biomass burning. The total global methane emission from all these sources ranges from 500 to 600 Tg/year.

Keppler et al. (2006) found that terrestrial plants may also emit methane under aerobic conditions. The mechanism is not yet clear, but these emissions may contribute substantially to the global methane budget. There are many uncertainties in scaling up from laboratory experiments to the global methane budget (Kirschbaum et al., 2006; Parsons et al., 2006), but the resulting estimate of methane emission by terrestrial plants (62–236 Tg $year^{-1}$) can be substantial (Keppler et al., 2006; Schiermeier, 2006).

Some further studies (Dueck et al., 2007; Kirschbaum and Walcroft, 2008; Beerling et al., 2008) did not confirm the initial findings of Keppler et al. (2006). On the other hand, there are several studies on the mechanisms of methane emission from plants under aerobic conditions. Brüggemann et al. (2009) used isotope pulse labelling experiments to verify non-microbial aerobic methane emission from poplar shoot cultures under sterile conditions. Keppler et al. (2008) reported the possibility of methane emission deriving from the methoxyl groups of pectin present in the cell wall. Pectin methyl esterases substantially reduce the methane emission rate due to pectin (Bruhn et al., 2009). Vigano et al. (2008) and McLeod et al. (2008) found that aerobic methane emissions from detached fresh

and dry plant material, and from pectin, increased under UV radiation and with higher temperature. McLeod et al. (2008), Messenger et al. (2009) and Wang et al. (2011b) demonstrated the role of reactive oxygen species (ROS) in methane formation in pectin and plant leaves. Wang et al. (2009; 2011a) reported that physical wounding enhanced the emission of methane from detached plant samples and asserted the significance of ROS in methane emission. Wishkerman et al. (2011) found a site-specific disturbance of the electron transport chain at cytochrome *c* oxidase in mitochondria of plant cells cultures causes methane formation.

Despite various studies of the mechanism of aerobic methane emission, the reason for large variations in the methane emission rates with species is unclear (Keppler et al., 2006; Kitaoka et al., 2007; Qaderi and Reid, 2009; Wang et al., 2008). Below, we report the relations between the rate of aerobic methane emission from leaves and basic leaf traits of two *Acacia* and five *Eucalyptus* species, chosen as significant for afforestation worldwide because of their fast growth traits and their acclimation capacity to various growth conditions. Our aim is to determine the biological factors that explain the variations in the aerobic methane emission rates from leaves of different species.

2. Materials and methods

2.1 Plant materials

We studied seedlings of two *Acacia* species (*A. mangium* and *A. auriculiformis*) and five *Eucalyptus* species (*E. camaldulensis*, *E. urophylla*, *E.*

grandis, *E. globulus*, *E. camaldulensis* × *E. deglupta*). All seedlings were cultivated in 7-L pots containing a 1:1 (v/v) mixture of Kanuma pumice soil and clay soil. We grew three seedlings of each species in a non-sterile glasshouse (50 m^2 of growth space and 4m in height) located in the experimental field of Hokkaido University (Sapporo, Hokkaido, Japan), from April 2008 to May 2009. Liquid fertilizer (N:P:K = 6:10:5; HYPONeX Japan Co., Japan) was supplied at a rate of 18 mg N per pot per week. The average temperature in the glass house during the experiment was 24.9 ºC.

2.2 Measurement of methane emission

We examined the rate of methane emission using detached leaves under dark conditions. The incubation experiments were repeated three times. In 2009, on 5 March, 30 April, and 28 May, we harvested mature and non-shaded young leaves, weighing approximately 600 mg, between 10:00 and 12:00 hours. The leaf area was determined using a scanner (CanoScan LiDE 600F; Canon, Tokyo, Japan) and image analysis software (LIA32 for Win32; Yamamoto, 2004). The leaves were rolled and placed in 60 mL sample vial for gas chromatography (SVG-50; Nichiden–rika Glass Co. Ltd., Kobe, Hyogo, Japan) containing ambient air, which were sealed with butyl rubber caps. The methane concentration in the ambient air was about 1.9 μmol mol^{-1}. These procedures were completed not more than 1 hour after harvesting. The vials were incubated at 30 ºC for 24 h in an incubator (IN600; Yamato Scientific Co. Ltd., Tokyo, Japan). We observed slight dew condensation on the inside wall of the incubation vials, because the sample

leaf emitted water by transpiration and/or evaporation during incubation. We measured the fresh weight of leaves immediately after incubation. The rate of water loss of leaves during incubation was calculated from the weight of the leaves before and after incubation. The leaves were then dried at 80 ºC for 5 days and weighed. We calculated the water content from the fresh weight before incubation, the dry weight, and the leaf mass per unit area (LMA).

We collected the air in the head space of the vials before and after incubation. The methane concentration in the sample air was determined using a gas chromatograph with a flame ionization detector (GC–14B; Shimadzu, Kyoto, Japan). Three blank measurements were conducted on each day of measurement in the absence of any leaf sample. Since the methane concentrations in blank vials increased slightly during incubation, we calculated the rate of methane emission by the sample leaf as the increase of methane concentration in the sample vial minus that in a blank vial. We do not know certain reason for the increasing methane concentration in blank vials. The increase rates of methane were rather constant among the blank vials, about 10% of mean increase rate in sample vials. The rate of methane emission is expressed in nanograms per gram dry weight (DW) per hour (ng g^{-1} DW h^{-1}).

2.3 Measurement of carbon and nitrogen concentrations

The dried leaf sample used for the incubation experiment was ground to a fine powder. The concentrations of C and N in the leaves were determined with an NC analyser (NC-1000; Sumika Chemical Analysis Service, Osaka, Japan).

2.4 Anatomical analysis

On 28 May 2009 we collected leaf samples for anatomical analysis from the same leaves for which the methane emission rate had been measured. The samples were fixed in 4% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2). These samples were washed and then cut into small pieces, dehydrated through a graded alcohol series, and embedded in epoxy resin (Epok 812; Oken, Japan). Transverse sections, 1 μm thick, were cut using an ultramicrotome (Ultracut N; Reichert-Nissei, Germany), and were stained with toluidine blue (1% w/v) for 10 min. We photographed the sections using a light microscope (Axioskop 2 plus; Zeiss, Germany) equipped with a digital camera (Nikon Digital Sight; Nikon, Tokyo, Japan).

2.5 Statistical analysis

Statistical analyses were performed with the SPSS software (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA) was used to test the significance of differences among species and experiments in the methane emission rates and traits of leaves.

3. Results

Methane emission was observed from leaves of all *Acacia* and *Eucalyptus* species (Table 1). The average methane emission rates in each species ranged from 0.2 to 0.7 ng g^{-1} DW h^{-1}. The methane emission rates differed

significantly between species and between experiments. The concentrations of C and of N, the C/N ratio, the LMA and the water content all differed significantly between the species and between experiments. No significant interaction was found between the species and the experiments for any parameter.

Figure 1 shows relations between the methane emission rates and leaf traits of two *Acacia* and five *Eucalyptus* species. The methane emission rate is significantly correlated with the LMA (Fig. 1d) and the leaf water content (Fig. 1e); these two parameters showed opposite trends. We did not find any significant correlation between methane emission rates and the C concentration, N concentration, C/N ratio, or rate of water loss (Fig. 1a, b, c, and f). When we analysed the relation of methane emission rate with LMA and water content using the average values for each species, only LMA showed significant correlation with the methane emission rate (Fig. 1 g and h).

4. Discussion

The methane emission rates in the present study of *Acacia* and *Eucalyptus* species are similar than those of the detached tree leaves under dark condition in the previous studies although methane emission rate in the previous studies have a big variation among the species from no detectable to 6.2 ng g^{-1} DW h^{-1} (Fig. 2). Average of methane emission rate in the present study (0.49 ng g^{-1} DW h^{-1}) was almost median in the results of the previous studies. Therefore, *Acacia* and *Eucalyptus* would not be considered as typical emitters of methane.

In general, N content is a good indicator for protein content (e.g.

Takashima et al. 2004). No significant correlation was observed between methane emission and the N concentrations (Fig. 1b). Aerobic methane emission from fresh and dry detached leaves is considered to be a non-enzymatic process (Keppler et al., 2006). Our finding of no correlation of methane emission with the N concentration in leaves, is consistent with non-enzymatic process for aerobic methane emission (Keppler et al. 2006).

We found significant negative correlations between LMA and methane emission rates in the two *Acacia* and five *Eucalyptus* species (Fig. 1d and g). As the variations in LMA are due mainly to leaf structural traits (Poorter et al., 2009), we observed the transverse section of the leaves that typically show two trends: high methane emission rate correlated with low LMA (*E. garandis*), and low methane emission rate correlated with high LMA (*E. camaldulensis*) (Fig. 3). The leaves with high LMA had a more complex structure and a larger cell wall surface area than leaves with low LMA. Unfortunately, it is hard to specify the precise role of leaf structure on the rate of methane emission because the mechanism of methane emission under aerobic conditions is not known. However, leaf structure might be primarily responsible for differences in the rates of aerobic methane emission from leaves of different species

We also observed a significant correlation between the methane emission rate and the leaf water content (Fig. 1e). Although methane is a non-polar molecule, it is sparingly soluble in water (Lide, 2004). It is therefore possible that methane is emitted from the water present in leaves. Nisbet et al. (2009) reported that plants absorb and transpire water containing dissolved methane; this process

might explain the observed methane emission rates from plants. The correlation between methane emission rate and the leaf water content was not as clear as that between methane emission and LMA, however (Fig. 1d and e), and the significance vanished when we analysed their relationship using average values for each species (Fig. 1h). Also, there was no significant correlation between the methane emission rate and the rate of water loss (Fig. 1f). According to Henry's law, the partial pressure for 2.0 μl CH_4 μmol mol^{-1} in the atmosphere would result in an equilibrium CH_4 concentration of approximately 48 ng l^{-1} in water. The amount of water emitted during incubation in our experiments ranged from 12 to 47 ml (data not shown), so that 0.57 - 2.24 pg of CH_4 can be emitted with water. The actual CH_4 emissions from leaves were about 1000 times greater than these values (nano gram order). We found a significant negative correlation between water content and LMA (Fig. 4). LMA is associated with the water status of leaves (Poorter et al., 2009). In the present study, although the seedlings were supplied with adequate water, the variations in temperature and solar radiation might have changed the water status in the leaves, causing variations in LMA values. We conclude that the leaf water content does not directly affect the emission rate of methane.

On extrapolating the relationship between the methane emission rate and LMA, we found that methane is not emitted from leaves with a LMA exceeding 100 g m^{-2} (Fig. 1d). Wang et al. (2008) reported that 80% of plant species growing in the Inner Mongolia steppe could not be detected methane under aerobic conditions. This region has a relatively dry climate with a mean annual

precipitation of about 350 mm (Wang et al., 2008). In such conditions the plant will have high LMA (above 100 g m^{-2}) (Poorter et al., 2009). This structural trait of plants growing in the Inner Mongolia steppe may therefore be responsible for the absence of methane emission.

Our study of *Acacia* and *Eucalyptus* species suggests that leaf structure is a factor explaining variations in dark aerobic methane emission rates from leaves of different species, and that LMA is a good indicator of methane emission. Since environmental conditions have a major effect on LMA, it would be necessary to determine the methane emission rates under various climatic and soil conditions in order to obtain an accurate estimate methane emission by terrestrial plants. For example, an increase in atmospheric CO_2 concentration might lead to an increase in the amount of leaves (Ainsworth & Long, 2005; Eguchi et al., 2008), thereby increasing methane emission; elevated CO_2 concentration also tends to increase the LMA (Poorter et al., 2009). An increase in methane emission due to the large amount of leaves might therefore be counterbalanced by lower methane emission rates of leaves with high LMA. Further studies on the response of plants to environmental changes such as drought, light condition, nutrient availability and elevated CO_2 are needed in regard to methane emission, as also proposed by Qaderi and Reid (2009) and Keppler et al. (2009).

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Figure captions

Fig. 1 Relations between the methane emission rate and C concentration (a), N concentration (b), C/N ratio (c), leaf mass per area (LMA) (d, g), leaf water content (e, h) and rate of water loss during incubation (f) of samples of two *Acacia* and five *Eucalyptus* species. Original values, obtained from three incubation experiments with three seedlings per species (total, 63 plots), are shown from (a) to (f). As LMA (d) and water content (e) are significantly correlated with the methane emission rate, the relations are shown in (g) and (h) using average values for each species (7 plots). Significance according to Pearson's correlation test: ** $P < 0.01$; *** $P < 0.001$.

Fig. 2 Number of observations in each range of methane emission rate of the detached tree leaves under dark condition in the previous studies (Keppler et al., 2006; Ishizuka and Takahashi, 2006; Wang et al., 2008; Wang et al., 2011). One observation indicates the result of one species in a study. The incubation temperatures were ranged from 20 to 40 ºC.

Fig. 3 Transverse section of leaves of *Eucalyptus grandis* (a) and *E. camaldulensis* (b). The methane emission rate and leaf mass per area are respectively 0.74 ng g^{-1} DW h^{-1} and 27 g m^{-2} for *E. grandis*, and 0.05 ng g^{-1} DW h^{-1} and 85 g m^{-2} for *E. camaldulensis*. Scale bars: 30 μm.

Fig. 4 Relation between water content and leaf mass per area (LMA) of two *Acacia* and five *Eucalyptus* species. The dataset is the same as that in Fig. 1. Significance according to Pearson's correlation test: *** $P < 0.001$ (n = 63).

Figure 1

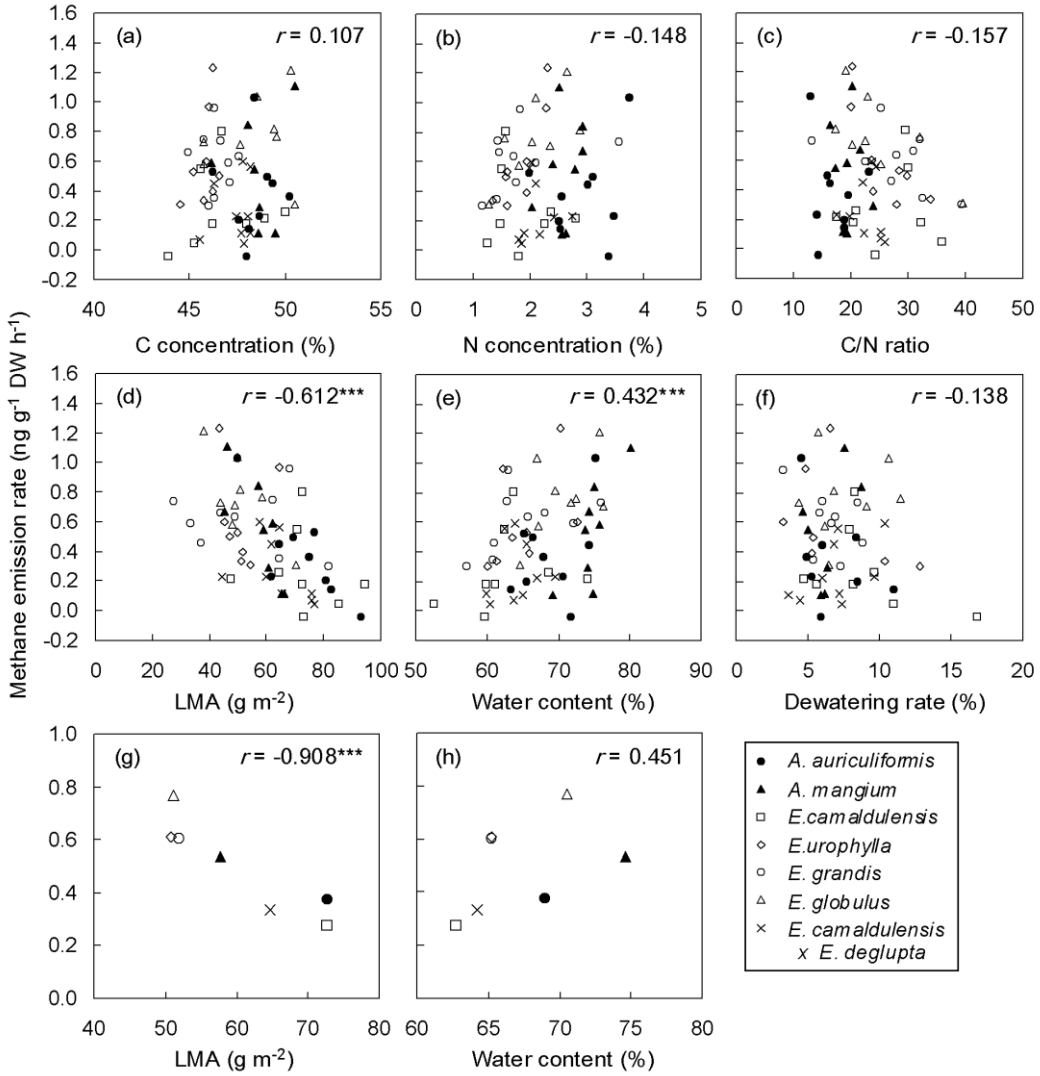
(a) r = 0.107
(b) r = -0.148
(c) r = -0.157
(d) r = -0.612***
(e) r = 0.432***
(f) r = -0.138
(g) r = -0.908***
(h) r = 0.451
Methane emission rate (ng g-1 DW h-1)
C concentration (%)
N concentration (%)
C/N ratio
LMA (g m-2)
Water content (%)
Dewatering rate (%)
LMA (g m-2)
Water content (%)
A. auriculiformis
A. mangium
E.camaldulensis
E.urophylla
E. grandis
E. globulus
E. camaldulensis x E. deglupta

Figure 2

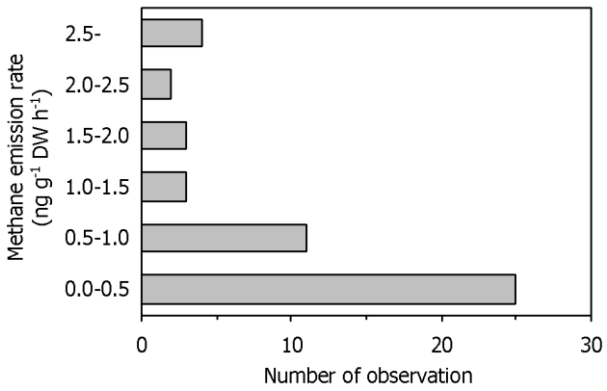
Methane emission rate
(ng g-1 DW h-1)
2.5-
2.0-2.5
1.5-2.0
1.0-1.5
0.5-1.0
0.0-0.5
0
10
20
30
Number of observation

Figure 3

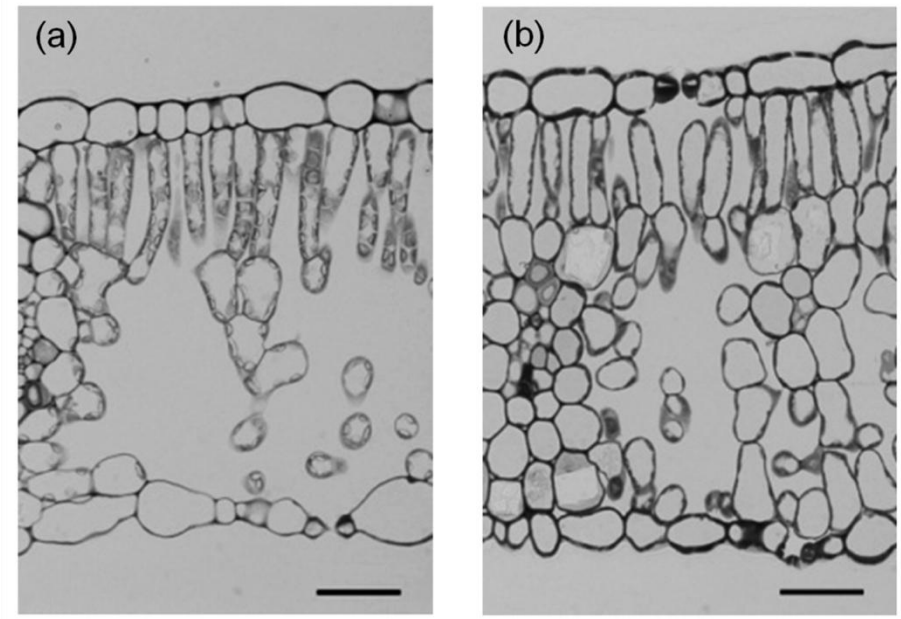

Figure 4

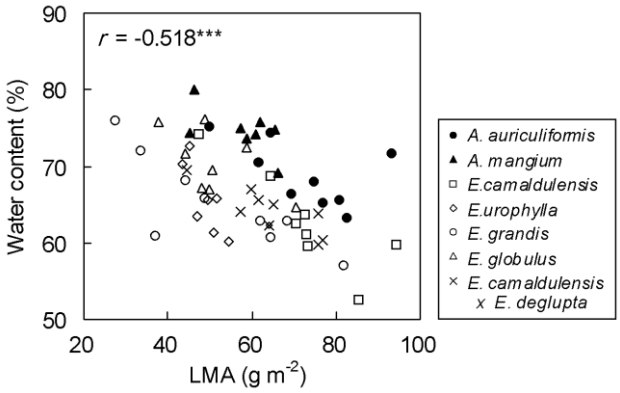
r = -0.518***
Water content (%)
LMA (g m-2)
90
80
70
60
50
20
40
60
80
100
A. auriculiformis
A. mangium
E.camaldulensis
E.urophylla
E. grandis
E. globulus
E. camaldulensis x E. deglupta

Table 1 Methane emission rates and leaf traits of two *Acacia* and five *Eucalyptus* species.

Species	Methane emission rate ($ng\ g^{-1}\ DW\ h^{-1}$)	C conc. (%)	N conc. (%)	C/N ratio	LMA ($g\ m^{-2}$)	Water content (%)	Dewatering rate (%)
(a) 5-Mar.							
A. auriculiformis	0.59 (0.41)	48.7 (0.3)	3.4 (0.3)	14.2 (1.4)	60.3 (9.9)	70.8 (4.4)	6.0 (2.0)
A. mangium	0.50 (0.37)	48.3 (0.3)	2.8 (0.2)	17.4 (1.1)	60.5 (4.3)	74.5 (0.7)	6.7 (1.9)
E. camaldulensis	0.30 (0.21)	46.6 (1.2)	1.7 (0.4)	27.6 (6.3)	79.3 (13.2)	61.2 (1.3)	7.2 (1.4)
E. urophylla	0.45 (0.05)	46.4 (0.2)	1.8 (0.2)	26.8 (2.9)	49.3 (2.3)	64.7 (1.2)	5.3 (0.0)
E. grandis	0.68 (0.31)	46.1 (0.3)	1.6 (0.2)	30.0 (4.0)	64.9 (3.1)	62.2 (1.2)	4.9 (1.4)
E. globulus	0.71 (0.12)	47.0 (2.1)	2.2 (0.6)	21.7 (4.1)	47.6 (3.3)	69.4 (2.3)	5.8 (1.3)
E. camaldulensis × *E. deglupta*	0.66 (0.14)	47.8 (0.4)	2.3 (0.4)	21.8 (3.8)	55.3 (10.1)	65.3 (3.8)	9.0 (1.7)
(b) 30-Apr.							
A. auriculiformis	0.26 (0.26)	49.2 (1.1)	3.0 (0.4)	16.7 (2.7)	77.5 (14.5)	71.3 (3.2)	5.6 (0.6)
A. mangium	0.63 (0.50)	54.5 (7.9)	2.7 (0.2)	20.4 (1.2)	52.5 (11.9)	74.5 (5.5)	6.0 (1.5)
E. camaldulensis	0.43 (0.33)	48.5 (1.7)	2.3 (0.6)	22.7 (6.3)	61.5 (12.8)	68.9 (5.2)	7.5 (2.6)
E. urophylla	0.94 (0.32)	46.0 (0.2)	2.2 (0.2)	21.3 (2.1)	51.0 (11.5)	68.4 (5.5)	4.9 (1.6)
E. grandis	0.56 (0.09)	47.2 (0.3)	1.8 (0.2)	25.8 (2.9)	39.8 (8.1)	66.2 (5.5)	7.5 (1.2)
E. globulus	0.99 (0.25)	48.8 (1.4)	2.4 (0.3)	20.8 (2.1)	45.6 (6.7)	73.0 (5.2)	8.5 (2.5)
E. camaldulensis × *E. deglupta*	0.15 (0.06)	48.0 (0.3)	2.2 (0.3)	22.5 (2.7)	67.0 (8.2)	63.9 (3.7)	5.6 (1.8)
(c) 28-May							
A. auriculiformis	0.29 (0.21)	47.3 (1.0)	2.4 (0.3)	20.3 (2.5)	80.1 (2.9)	64.7 (1.3)	3.1 (11.7)
A. mangium	0.44 (0.15)	47.4 (1.3)	2.2 (0.2)	21.6 (2.3)	61.4 (0.6)	74.9 (0.8)	2.6 (3.7)
E. camaldulensis	0.00 (0.04)	44.6 (0.7)	1.5 (0.3)	30.1 (5.9)	79.3 (6.1)	56.1 (3.5)	13.9 (2.9)
E. urophylla	0.39 (0.12)	45.1 (0.6)	1.5 (0.1)	30.1 (3.3)	51.7 (2.5)	62.3 (2.9)	4.1 (13.1)
E. grandis	0.57 (0.23)	45.8 (0.9)	2.1 (1.3)	27.8 (13.4)	51.0 (27.9)	67.1 (9.5)	7.2 (1.3)
E. globulus	0.54 (0.23)	50.0 (0.5)	1.4 (0.1)	35.7 (3.8)	64.5 (5.9)	68.5 (3.9)	9.0 (2.5)
E. camaldulensis × *E. deglupta*	0.19 (0.22)	46.5 (1.2)	1.9 (0.2)	24.4 (2.1)	71.5 (8.7)	63.2 (2.6)	6.2 (1.5)
ANOVA							
Species	**	**	***	***	***	***	n.s.
Exp.	*	**	**	**	*	*	n.s.
Species × Exp.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

The experiments were repeated three times (5 March, 30 April, and 28 May 2009). We used same three seedlings of each species for all the experiments.
Standard deviation is shown in parentheses (n = 3).
Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s.: not significant.