

Establishment of a homogenized method for environmental biosafety assessments of transgenic plants

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Abstract The development of transgenic plants may help alleviate both environmental and food problems. In Japan, transgenic plants cannot be planted in the field without undergoing an environmental biosafety assessment under confined and semi-confined conditions. The main objectives of environmental biosafety assessment studies are to define the properties of the host plant and to evaluate its influence on other organisms. To appraise the influence of a transgenic plant on other plants, the transgenic plant is examined for new compounds that influence plant growth by measuring germinating seeds. Previously, we assessed the allelopathic activity of several transgenic plants using the sandwich method to assay the allelopathic activity of leachate from dried leaf samples. However, because *Eucalyptus* leaves are difficult to dry, the sandwich method does not allow the evaluation of multiple samples at the same time. Here, we report a new “homogenized method” that relies on homogenizing fleshy leaf samples, instead of drying them. The allelopathic activity of a non-transgenic plant and an *Antisense-lim* transgenic plant were evaluated using both the sandwich method and the homogenized method to determine whether the homogenized method was available for in biosafety assessments of transgenic plants. The homogenized method may be an effective and useful tool for evaluating differences between non-transgenic and transgenic plants using multiple, concurrent samples.

Key words: Allelopathic activity, environmental biosafety assessment, *Eucalyptus*, homogenized method, transgenic plants.

The development of transgenic plants could potentially mitigate many environmental and food problems. Although some transgenic plants have been approved for general use (Kikuchi et al. 2006, 2008), precautionary environmental biosafety assessments of transgenic plants must be conducted. The Cartagena Protocol on Biosafety promulgated guidelines for evaluating the biosafety of Living Modified Organisms (<http://www.biodiv.org/biosafety/>).

In Japan, transgenic plants cannot be planted in the field without an environmental biosafety assessment conducted under both confined and semi-confined conditions. The evaluation of transgenic plants is carried out using a step-by-step precautionary approach (Watanabe et al. 2005). Cultivation on commercial plantations or environmental release can be permitted after evaluation in a growth room, special netted-house, and isolated field (Teutonico 2006). The main public concerns and objectives of environmental biosafety assessments are to define property of the host plant and assess the influence of the plant on other organisms. To

evaluate the former, host plant characters, such as weediness, competition with local natural vegetation, and mating properties, are examined (Teutonico 2006). To assess the latter, activities of the transgenic plant that influence other organisms, such as allelopathic activities affecting other plants and soil microbe populations, are investigated. When the transgenic plant shows no conspicuous differences from non-transgenic plants lacking the properties conferred by the transgene, the evaluation of the transgenic plant shifts to the next step, e.g., transferring it from the special netted-house to the isolation field.

Among the methods used to evaluate the allelopathic activity of plants are the sandwich, soil mix (SUKIKOMI), plant box, and dish pack methods (Shiomi et al. 1992; Yamaguchi et al. 1994; Sekine et al. 2007). The sandwich method, which is among the most used, assays the allelopathic activity of leachates from dried leaves on seed germination and growth receptor plants such as lettuce (Fujii et al. 2003, 2004). This method has been used to compare the allelopathic activity of non-

Abbreviations: *Anti-lim*, antisense construction of *Nlim* driven by the 35S promoter; GRH, relative growth rates of the hypocotyls; GRR, relative growth rates of the roots; SGR, seed germination rate; *Nlim*, a transcriptional factor isolated from tobacco involved in lignin biosynthesis.

This article can be found at <http://www.jspcmb.jp/>

transgenic plants with that of transgenic plants (<http://www.niaes.affrc.go.jp/magazine/066/mgzn06605.html>), and we have evaluated several transgenic *Eucalyptus* lines using this method (Kikuchi et al. 2006, J-BCH 2005, 2007). However, this method may not be appropriate for evaluating multiple samples of *Eucalyptus* concurrently, as *Eucalyptus* leaves retain moisture comparatively well during droughts (Grunwald and Karschon 1982), and drying numerous leaves at the same time is difficult.

To evaluate numerous samples of a transgenic *Eucalyptus* concurrently, we established a new method that avoided the leaf-drying step and assayed the growth regulation activity of leaf extracts on the growth of germinating seeds. Here, we demonstrate the effectiveness of this method by assessing transgenic *Eucalyptus globulus*.

Materials and methods

Plant materials

Eucalyptus globulus was used as the plant material, with non-transgenic line (NT) serving as the control. Transgenic *Eucalyptus* carries a gene that suppresses lignin biosynthesis involving the *Ntlim* gene, which is a transcriptional factor isolated from tobacco. Transgenic tobacco with an antisense construction of *Ntlim* driven by the 35S promoter (*Anti-lim*) shows low lignin contents compared to non-transgenic plants (Kawaoka et al. 2000). This antisense construction was introduced into *E. globulus*, mediated by *Agrobacterium* (data not shown). Four lines (90-14, 99-8, 187-8, 188-2) were used as *Anti-lim* plants. *Anti-lim* transgenic and non-transgenic plantlets were propagated via tissue culture. However, *Anti-lim* was difficult to propagate, and each *Anti-lim* line generated only one plantlet. The existences of transgene were confirmed by southern blot analysis (data not shown), but physiological properties of them were not evaluated yet. The plantlets (ca. 10 cm in height) were transplanted into pots with soil and transferred to the special netted-house. For the experiment, we used plants from the special netted-house that were 2–3 m in height. Leaf samples were picked from each tree at a height of 1.7 m from the soil surface.

Sandwich method

Allelopathic activity was evaluated according to the previously described sandwich method (Fujii et al. 2003, 2004). A dried leaf was placed in each well of a 6-well multi-dish plastic plate ($\phi 35$ mm), and 5 mL of a low-melting agar (0.5% w/v) solution was poured on top. After solidification, another 5 mL of low-melting agar (0.5% w/v) was added on top of the first layer. After solidification, five lettuce seeds (*Lactuca sativa* L. var. *capitata*: Great Lakes 366 variety; Takii Seed, Kyoto, Japan) were placed in each well. Each plate was sealed with parafilm and incubated for 72 h at 25°C under dark conditions. The plates were then frozen at -20°C for 1 day to stop growth. After defrosting the agar, the germination rate and the lengths of roots and hypocotyls were recorded. Each treatment was repeated three times.

Homogenized method

Frozen leaves were ground to a fine powder in liquid nitrogen. The powder was ground again in distilled water using a mortar and pestle. The ground material was transferred to a 50-mL centrifuge tube and filled with distilled water to 20 mL, followed by centrifugation ($1800\times g$, 20 min). The supernatant was filtered using a sterile filter ($\phi 0.45\ \mu\text{m}$, Sterile Millex; Millipore Corp., Billerica, MA, USA). The same amount of low-melting agar (1.0% w/v) was added to the filtered solution and mixed gently. We then poured 5 mL of the mixture into each of the six multi-dish plastic plate wells ($\phi 35$ mm). After solidification, another 5 mL of agar (0.5% w/v) was added on top of the first layer. After solidification, we placed five assay seeds in each well and followed the procedure described above for the sandwich method.

Results and discussion

Germination growth assay of lettuce seeds using the homogenized method

To determine the optimum amount of *Eucalyptus globulus* leaves per 6-well plate for the homogenized method, we prepared test plates using various amounts (0.1, 0.25, 0.5, 0.6, and 0.75 g/plate) of leaves from four individuals of NT. The experiment was repeated three times for each plate. The plant growth regulation activity of each amount of leaves was evaluated by comparing the seed germination rate (SGR) and the relative growth rates of the roots (GRR) and hypocotyls (GRH) against those of control plate (without leaf sample) seeds. The 0.1 and 0.25 g/plate samples showed little promotion activity, whereas those plates with >0.5 g/plate displayed inhibitory activity (Figure 1). The 0.5 g/plates showed 50% inhibition of both GRR and GRH. This amount was used for further analyses.

Comparison between non-transgenic and transgenic plants of their plant growth regulation activity using the homogenized method

The plant growth regulation activity of NT and *Anti-lim* transgenic plants was evaluated using the homogenized method. Leaf samples were taken from four NT plants and plants from four different *Anti-lim* lines (90-14, 99-8, 187-8, and 188-2). The experiment was repeated three times per plate. Figure 2 summarizes the GRR, GRH, and SGR of the NT and *Anti-lim* lines. All of the parameters of the *Anti-lim* lines were significantly less than those of the NT plants (Figure 2), as shown by an analysis of variance (ANOVA; Table 1: GGR: $P < 0.01$, GRF: $P < 0.05$, SGR: $P < 0.05$). To evaluate differences between NT and the transgenic lines, we performed a Tukey HSD multiple-comparison test (asterisk, Figure 2). The GGR of all of the *Anti-lim* lines except 187-8 differed significantly (90-14: $P < 0.05$; 99-8: $P < 0.01$; 188-2: $P < 0.05$) from that of NT. The GRH of 90-14 and 99-8 was significantly different ($P < 0.05$) compared to

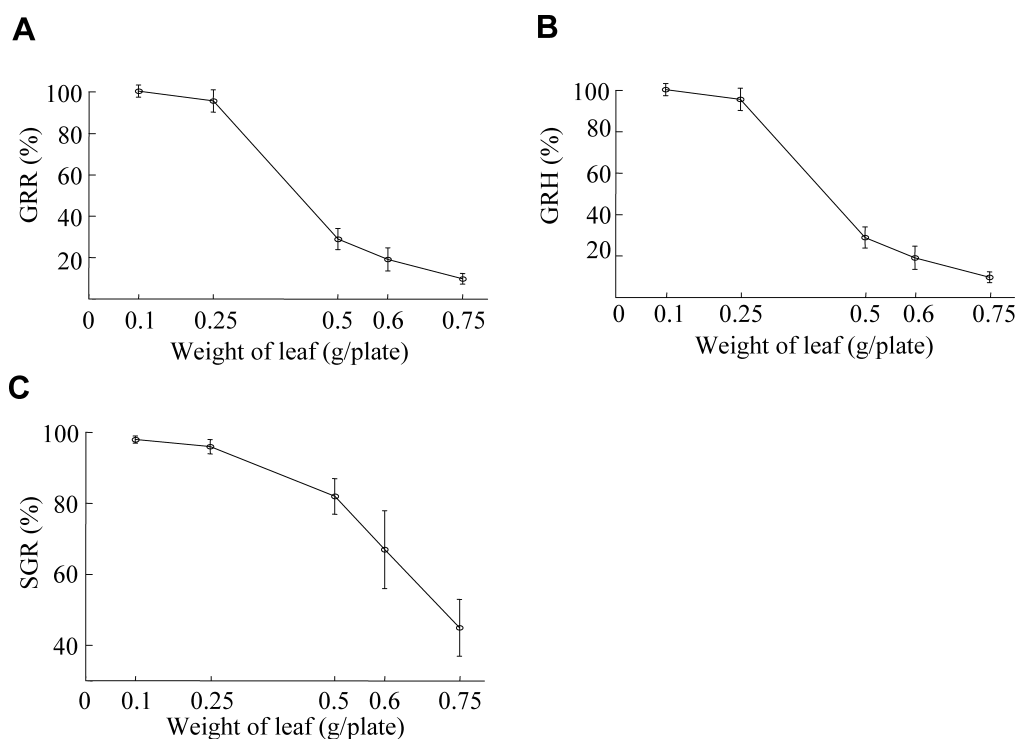


Figure 1. Effect of various amounts of leaf sample on germination growth using the homogenized method. Leaf samples were taken from four individuals of a non-transgenic line (NT). The bar for each genotype is compared to the average of the (A) relative growth rate of the root (GRR), (B) relative growth rate of hypocotyls (GRH) of control plate (without leaf sample) seeds, and (C) the seed germination rate (SGR). The error bars indicate the standard error.

that of NT. On average, the *Anti-lim* transgenic lines showed strong activity in retarding seed germination growth.

Comparison between non-transgenic and transgenic plants of their allelopathic activity using the sandwich method

The allelopathic activity of NT and the four *Anti-lim* plants was evaluated using the sandwich method. Test plates were prepared using various amounts (10, 20, or 50 mg/well) of dried leaves. The experiment was repeated three times per plate. The SGR was less than 5% in the 50 mg sample/well plates, and no allelopathic activity was detected in the 10 mg sample/well plates (data not shown). Figure 3 summarizes the 20 mg/well results. Among the transgenics, *Anti-lim* line 187-8 showed slightly stronger retardation of all the parameters, although ANOVA did not show the differences to be significant ($P > 0.25$; Table 2).

Environmental biosafety assessment of the transgenic plant using the homogenized method

The results of the sandwich and homogenized methods using the same *Eucalyptus* plants differed. In the homogenized method, the *Anti-lim* transgenics retarded growth more so than the non-transgenic plants (Table 1, Figure 2). However, the sandwich method (Table 2,

Figure 3) resulted in no significant differences. A major difference between the two methods is that the sandwich method assesses the allelopathic activity of leachates from dried leaves, whereas the homogenized method evaluates the plant growth regulation activity of all soluble compounds in the leaf, including some processed by homogenizing. Some compounds could also be changed by the process of drying or homogenizing the leaves.

The influence of a transgenic plant on other plants is assessed by comparing the non-transgenic plant (host plant) to the transgenic plant. We evaluated whether transgenic plants contain new compounds that influence the growth of other plants by measuring the growth of germinating seeds. The influence of a transgenic plant on other plants is assessed by comparing the non-transgenic plant (host plant) to the transgenic plant. We evaluated whether transgenic plants contain new compounds that influence the growth of other plants by measuring the growth of germinating seeds. In our experiments, the impacts of transgenic plants were different in sandwich method and homogenized method. It would indicate that chemical changes (modification or processing of some compounds) occurred during the process of homogenization which leads enhancement of the effect of the transgenic plant on the growth of germinating seeds. Even if the chemical changes that influence on

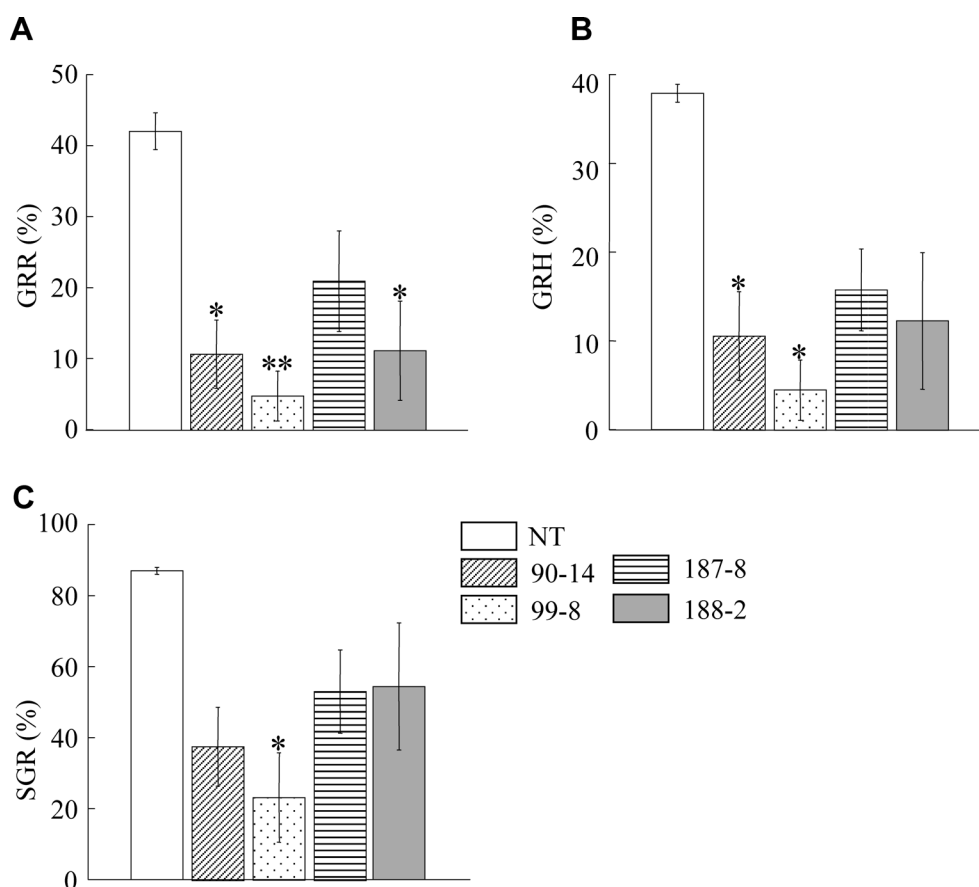


Figure 2. Evaluation of plant growth regulation activity using the homogenized method. Leaf samples were taken from four individuals of a non-transgenic line (NT) and four different lines of *Anti-lim* transgenic (90-14, 99-8, 187-8, and 188-2) plants. The bars indicating the NT and *Anti-lim* transgenic lines are compared to the average of the relative growth rates of the (A) roots (GRR) and (B) hypocotyls (GRH) and (C) the seed germination rate (SGR) of the control plate (without leaf samples) seeds. The error bars indicate the standard error. Asterisks denote a significant difference from NT at the 5% (single) and 1% (double) significance levels using the Tukey HSD multiple comparisons test.

Table 1. ANOVA result for the homogenized method

Analysis of variance of Source		Sum of Squares	df	Mean Square	F-ratio	P
GRR	Lines	2586.44	4	646.66	7.08	0.006
	Error	913.04	10	91.30		
GRH	Lines	1262.44	4	490.66	5.36	0.014
	Error	913.04	10	91.30		
SGR	Lines	6751.10	4	1687.77	3.59	0.046
	Error	4698.01	10	469.80		

growth of germinating seeds were induced during sample preparation (drying or homogenization process), it would be thought to reflect the chemical differences between the non-transgenic plants and the transgenic plants. Thus, both the sandwich method and homogenized method would be able to use for the evaluation.

The sandwich method is more labor-intensive, as it requires drying the sample and weighing it six times to make one plate. In contrast, the homogenized method needs no drying step and only one weighing per plate. Thus, the homogenized method may be of greater utility in evaluating differences between non-transgenic and transgenic plants in multiple samples at the same time.

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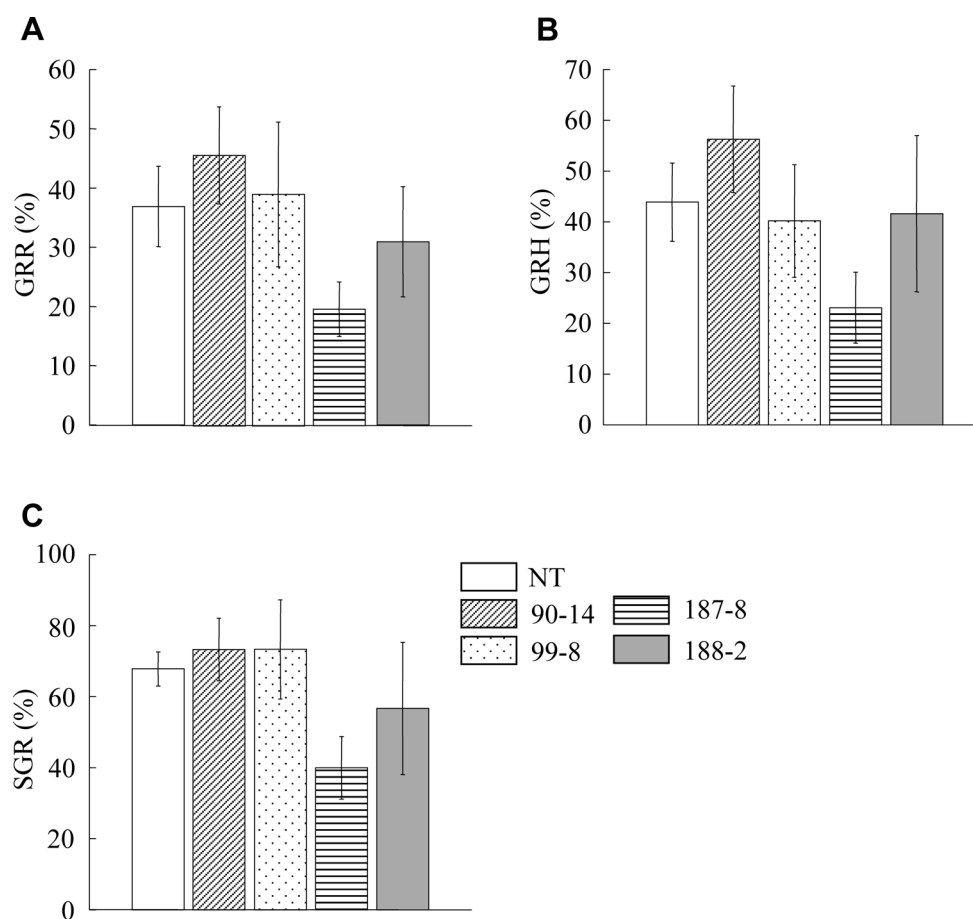


Figure 3. Evaluation of allelopathic activity using the sandwich method. The leaf samples were taken from four individuals of a non-transgenic line (NT) and four different lines of *Anti-lim* transgenics (90-14, 99-8, 187-8, and 188-2). The bars indicating the NT and *Anti-lim* transgenic lines are compared to the average of the relative growth rates of the (A) roots (GRR) and (B) hypocotyls (GRH) and (C) the seed germination rate (SGR). The error bars indicate the standard error.

Table 2. ANOVA result for the homogenized method

Analysis of variance of Source		Sum of Squares	df	Mean Square	F-ratio	P
GRR	Lines	1181.77	4	295.44	1.36	0.315
	Error	2172.50	10	217.25		
GRH	Lines	1661.38	4	415.35	1.23	0.358
	Error	3375.29	10	337.53		
SGR	Lines	2425.04	4	606.26	1.43	0.294
	Error	4241.37	10	424.14		

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