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# Allelopathy assessments for the environmental biosafety of the salt-tolerant transgenic *Eucalyptus camaldulensis*, genotypes *codA* 12-5B, *cod*A 12-5C and *cod*A 20C

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Key words: allelopathy, bioassay, environmental biosafety, *Eucalyptus camaldulensis,* salt tolerance

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Abstract

Allelopathy tests were conducted on the salt-tolerant transgenic eucalyptus trees conferring bacterial *codA* gene in the designated net-house conditions under the Type II use (contained use) of the Japanese law on environmental biosafety aiming for Type I (field use) application. Three transgenic and corresponding non-transgenic genotypes were employed for four different tests: 1) sandwich bioassay; 2) soil germination method, 3) gas chromatography for volatile substances from the plants; and 4) high performance liquid chromatography on phenolic compounds from fresh leaves which are the primary allelopathic substances on the species. The simple approaches, bioassays indicated no significance among transgenic and non-genetically modified genotypes. There was no qualitative difference between transgenic and non-transgenic ones on gas and liquid chromatography. Absence of the quantitative difference was suggested by repetitive examination and subsequent analysis of variance (ANOVA) assessments with the chromatographic methods and bioassays. Moreover, it was also indicated that bioassays may be the primary assessment method for allelopathy in considering the ease, promptness and low-cost performance as well as reproducibility. Overall, substantial equivalence was considered on the three transgenic genotypes with *codA* gene compared with the non-transgenic *Eucalyptus camaldulensis* lines. The experiments supported the application to the isolated field testing of the transgenic *Eucalyptus camaldulensis* genotypes as the first case and experience in Japanese regulatory approval processes Type I Use for the deliberate release to the environment.

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Introduction

Allelopathy tests on the host and transgenic plants are the key elements in the environment biosafety assessments. Allelopathy tests are aimed for evaluating the negative interactions from genetically modified (GM) organisms to native organisms surrounding the transgenic entities. The evaluation targets could be interactions with plants, soil microorganisms etc. The choice of the specific methodology for risk assessment is conditional and case by case. [1,2] And the employment of the specific technical methods is upon individual cases based on the Host Genotype x Transgene x Environment. It is also important to consider how the transgenic plants are deliberately released to the environment; e.g. small-scale field testing or commercial mass-production. Also it is cardinal to examine the components on the methodology on the risk assessments such as cost-efficiency, time, at ease on tests and preciseness and quantity of overall information.[1,3]

There are many reporting on the risk assessments of annual plant species at overseas, but yet scarce in Japan. These reported cases are mainly on the crops for agriculture fields which can be rather monitored easily for the environmental effects.[1] There is also some information available on tree species such as eucalypts and poplar in known data base such as OECD Biosafety BioTrack (http://www.oecd.org/department/0,2688,en_2649_34385_1_1_1_1_1,00.html) and Biosafety Clearing-House,[4] but yet the cases on the perennial plant species are meager compared with the examples on the annual crops, and case building is one of important challenge for the perennial species. In case of transgenic tree uses, except for the confined field testing for seedling stage without flowering, more elements and their modality of the measurements on the environmental effects shall be considered such as the allelopathic influence to the vegetation surrounding the transgenic entities.

Majority of eucalypts which have more than five hundred species in relatives,[5] are known to

have allelopathic influence in nature,[6] and specific attention should be made on the transgenic eucalypts which should not exceed the level of such negative effects compared with the non-transgenic ones.

Our group has been working on the development and testing of various eucalypts with transgene conferring abiotic stress tolerances for future application to major field production.[7] One of the steam-lines of such transgenic materials is with *codA* gene derived from *Arthrobacter globiformis*, which induces salt-tolerance by increasing the amount of the competitive solute, glycinebetaine in plant cell. [8] The *codA* gene is driven by a constitutive promoter CAMV35S, and the GM seedlings have shown relevant phenotypes associated with the salt and drought tolerances in the net-house evaluations.[10] By using the transgenic materials, we have tested and case recommendation of the allelopathic influence of the transgenic materials compared with the non-transgenic original ones.

Four different testing methods for allelopathy examination were applied in this experiment to examine whether substantial difference occurring on the transgenic and non-transgenic *Eucalyptus camaldulensis* seedling trees. They are : 1) sandwich bioassay; 2) soil germination method, 3) gas chromatography for volatile substances from the plants; and 4) High performance liquid chromatography (HPLC) on phenolic compounds from fresh leaves which are the primary allelopathic substances on the species. The four methods have been employed for providing the risk information to make decision making under Japanese regulatory system, but the choice of the methods has been optional upon the characteristics of the transgenic organisms with the targeted environment. And also it is depending on how the consequence of primary assessment: no visible risk or potential risk followed by the fine evaluation to examine further on the risk possibilities. We have consideration of drawing a recommendation to make a primary choice out of the four methods to make further systematic approach to use the specific allelopathy testing.

In addition to the case study of the allelopathy evaluation, this research was conducted to support regulatory application for relevant environmental biosafety information required by Japanese government for the deliberate release to the confined field as Type I Use (Field use, http://www.bch.biodic.go.jp/english/e_index.html),[4,9,10] especially by introducing the documents made in Japanese.

## Materials and Methods

The transgenic materials confer *codA* gene driven by CaMV 35S constitutive promoter. Transgenic and its original non-transgenic genotypes of *Eucalyptus camaldulensis* were grown in a net-house designated as Type II Use under the Japanese law( http://www.bch.biodic.go.jp/english/e_index.html ). The eighteen-month-old seedlings were used for the assessments, which were grown in potted soil with the amount of 30 litter. All materials were exposed to the salinity stress with 400 ml of 200 mM NaCl for four weeks by putting the solution onto the pot by every other day.

## Sandwich method

Growth measurements of hypocotyls and roots were made on germinated lettuce seed cv. Great Lakes 366, with sandwich testing[11] containing the leaves from the transgenic *Eucalyptus camaldulensis* conferring *codA* and non-transgenic counterpart trees as a biological assay. A dried leaf was placed in each well of a 6-well multi-dish plastic plate (φ35 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 hours at 25 ºC under dark conditions. The plates were then frozen at –20 ºC for 1 day to stop growth. After defrosting the agar, the germination rate and the lengths of roots and hypocotyls were recorded. Original measurements were made on the length of the hypocotyls and roots in mm, respectively. Ten seeds per replication were used in a sandwich medium with four replications.

Soil germination

Soil used in growing transgenic and non-transgenic eucalypts was used in seeding lettuce cv. Great Lakes 366. One hundred ml soil was placed in a plastic pot with a diameter of 8 cm as one replication. The potts were put in a growth chamber at 25 ℃ at dark for 72 hours. Irrigation was made daily. Thirty seeds / replication with four replications were tested.

Gas Chromatography

Gas chromatography was conducted on the volatile substances sampled from the plants according to National Institute of Agro-Environmental Sciences.[12,13] A plastic container of 6. 5 litter volume was used to cover and seal off a branch of each tree in a growth room to sample precisely the volatile substances. The container was placed for one hour, and the inside air sample was made by a suction pump in to a glass samaple vial via a teflon tube. The glass vial was heated to make internal temperature to 200 ℃, and He gas was flown with the rate of 30 ml / min for one min, then sample was introduced for the gas chromatography. Thermon-1500 glass column ( 3.2 mm φ * 2.1 mm). He was the carry-gas for the analysis. Column temperature raised from 50 to 210 ℃ at the rate of 4 ℃ / min, and detection was made with a flame ionization detector (FID).

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was conducted on the phenolic compounds from the leaves accordingly to the methods described by Shiomi et al. for the substantial

equivalence testing purposes.[13] Twenty gram of fresh leaves were crashed in eight-time volume of 80% ethanol, and filtrated after overnight incubation at room temperature. The extract was dried with a drier, and an amount of 100 ml of distilled water was added and, pH was adjusted to 2.8 with 6N HCl. This solution was placed in a separating funnel and mixed with an equal amount of ethyl acetate, and shaken for 30 min then to overnight incubation. Then ordinary ethyl acetate extraction was conducted and the extract was dried and dissolved in an amount of 5 ml of methanol solution for HPLC. A Shimpak ODS column (6.0 mm φ * 150 mm, Shimazu LTD. Co.) was used with a sample amount of 10 $\mu$ l. Sample flow rate was 1.8 ml / min by 2 % acetic acid solution with concentration of 15 to 40 % methanol. Detextion was made at 254 nm UV absorption.

## Results

### Sandwich method

The replications did not show significant difference and it seems that the reproducibility of the testing is sufficiently support the results. (Table 1). There was no substantial variation between non-transgenic and transgenic lines with respect to the hypocotyl growth and root enlargement, and consequently there is no substantial allelopathic effect from the transgenic materials compared with the non-transgenic ones. The results corresponded well with the previous report.[10]

### Soil germination

Germination rate was around 70% for all soil samples obtained from the individual eucalyptus genotypes. Table 2 revealed no alteration on the germination rate of the lettuce seeds.

## Gas Chromatography

Gas chromatographic evaluation did not provide qualitative variation in the presence of the peaks on both according to reported by Kikuchi et al.[10] However, there may be possibility of the individual gas chromatography peak pattern and there may be quantitative difference of the each peak. So that quantitative assessment was made on the major peak which may have difference indicated in the previous study[10]: Peak was scored by three different samples / day at the same time and its average was used as one repetition. Five repetitions sampled at alternative days were used for the analysis of variance (ANOVA) assessment. The results indicated that there is no substantial difference between transgenic and non-transgenic ones (Table 3). While the sampling difference was observed, the genotype difference was not significant.

## High Performance Liquid Chromatography (HPLC)

No qualitative difference was detected in the presence of the peaks of HPLC as to reported by Kikuchi et al.[10] However, as the same as gas chromatography, there may be possibility of the alteration of the individual gas peak pattern in quantitative manners. So that the major peak which may have difference obtained in the previous study was assessed quantitatively.[10] Three measurements from the same sample were regarded as a repetition, and five sampling was made each alternative day. The results indicated that there is no substantial difference between transgenic and non-transgenic ones, but the difference in repetition was observed (Table 4).

## Discussion

In the present report, four different tests were conducted for the assessments of allelepathy in

the transgenic eucalypts conferring a salt tolerance gene: 1) sandwich bioassay; 2) soil mixing test, 3) gas chromatography; and 4) HPLC;. There was no significant difference between transgenic and non-transgenic trees over the four experiments related to the allelopathic examination. With respect to the risk assessment, it is clear that no environmental risk is expected on allelopathy aspect.

Comparing each assessment method, there may be primary choice for further prompt evaluation of allelopathy. On both gas chromatography and HPLC, there was no qualitative difference in the presence of the peaks (Fig. 1). However, as to the reproducibility and stability of the assessment methods and the consequence for the judgment of the allelopathic effects, both results indicated the significant variation in sampling that was revealed by repetition difference in corresponding ANOVAs in Tables 3 and 4. Chromatographic analyses have more fluctuation of data variation due to non-genetic factors.[14] On the other hand, the biological assays by sandwich method for growth inhibition and soil mixing testing for the germination, indicated constant results in the repeated trials. For the environment biosafety assessments, it gives priority to a biological evaluation rather than chemical one to have a wide scope of evaluation on the integral effect of the subject, GM plants.

Having thought on the logistics required to conduct the environmental risk assessment, it is cardinal to have simple system and requirements to make a primary evaluation. The chromatography requires specific sampling, extraction and/or facility set-up. Considering also the cost performance of the evaluation, the biological assays shall be the primary test, in contrast chromatography is optional one. Regarding the non-technological factors such as public perception, [3,15] which may influence the interpretation and decision-making for the scientific risk assessment results toward deliberate release to the environment of the transgenic plants, the reproducibility and stability of the scientific data shall be well accepted by diverse stakeholders, in order to avoid the misinterpretation and/or miss-uses of the scientific

results.

Having overall aspects, the biological assays with relevant repetitions are at ease, useful and at easy-comprehension for the primary risk assessments on the allelopathic effects. Options for the detailed testing on the chromatographies are not deniable in case any possibility of the higher allelopathic effects recognized by the bioassays, however, there shall be careful setting of the sampling to make uniformity and reproducibility of the results in order to avoid the miss-interpretation of the data.

In the present risk assessment of our Eucalyptus, all of the four methods were employed for the documentation for the proof of substantial equivalence to the environmental effects associated with allelopathy in the transgenic *Eucalyptus camaldulensis* for the governmental inspection and decision to the deliberate release of the transgenic trees. However, for the future assessments of the allelopathy of similar property of transgenic materials, a biological assay shall be tested at first then other options upon the result of the first choice, shall be considered to make systematic flow of the uses of such assessment methods.

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Table 1. Analyses of variance on growth measurements of hypocotyls and roots were made on germinated lettuce seed cv. Great Lakes 366, with sandwich testing[11] containing the leaves from the transgenic *Eucalyptus camaldulensis* conferring *codA* and non-transgenic counterpart trees as a biological assay. Original measurements were made on the length of the hypocotyls and roots in mm, respectively. Ten seeds per replication were used in a sandwich medium with four replications.

Hypocotyl

| Degree of freedom | Sum of square | Mean Square | F value | Probability | Significance |
|---|---|---|---|---|---|
| Line | 3 | 28.7213 | 9.5738 | 1.5513 | ns |
| Replication | 3 | 18.3412 | 6.1137 | 0.9907 | ns |
| Line x Rep | 9 | 6.5438 | 0.7271 | 0.1178 | ns |
| Error | 144 | 888.6754 | 6.1714 | | |
| Total | 159 | 942.2817 | | | |

Root

| Degree of freedom | Sum of square | Mean Square | F value | Probability | Significance |
|---|---|---|---|---|---|
| Line | 3 | 5.872 | 1.9573 | 0.9243 | ns |
| Replication | 3 | 18.477 | 6.159 | 2.9086 | ns |
| Line x Rep | 9 | 2.247 | 0.2497 | 0.1179 | ns |
| Error | 144 | 304.914 | 2.1175 | | |
| Total | 159 | 331.51 | | | |

ns: non-significant

Table 2. Analysis of variance on seed germination testing with the soil used for growing the transgenic and *Eucalyptus camaldulensis* conferring *codA* and their non-transgenic counterpart trees.

| Degree of freedom | Sum of square | Mean Square | F value | Probability | Significance |
|---|---|---|---|---|---|
| Line | 3 | 0. 0075 | 0. 0025 | 0. 2308 | ns |
| Error | 12 | 0. 13 | 0. 0108 | | |
| Total | 15 | 0. 1375 | | | |

ns: non-significant

Table 3. Gas chromatograpy peak variation assessment obtained from Kikuchi et al. (2006) between transgenic and non-transgenic *Eucalyptus camaldulensis* trees.

| Degree of freedom | Sum of square | Mean Square | F value | Probability | Significance |
|---|---|---|---|---|---|
| Line | 3 | 3.3687 | 1.1229 | 0.5467 | ns |
| Repetition | 4 | 212.2623 | 53.066 | 25.838 | ** |
| Error | 52 | 106.7963 | 2.0538 | | |
| Total | 59 | 322.4273 | | | |

ns: non-significant, ** significant at the 1% level.

Table 4. Testing of the high performance liquid chromatography peak indicated in Kikuchi et al. (2006) among the transgenic and non-transgenic *Eucalyptus camaldulensis* trees.

| Degree of freedom | Sum of square | Mean Square | F value | Probability | Significance |
|---|---|---|---|---|---|
| Line | 3 | 2.41E-05 | 8.02E-06 | 0.2471 | ns |
| Rep. | 4 | 0.002392 | 0.000059 | 18.4285 | ** |
| Error | 52 | 0.001687 | 3.24E-05 | | |
| Total | 59 | 0.004103 | | | |

ns: non-significant, ** significant at the 1% level.