Advances in Engineering Research, volume 194
5th International Conference on Food, Agriculture and Natural Resources (FANRes 2019)

In Vitro Bioassay of Allelopathy in Robusta Coffee Callus Using Sandwich Method

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ABSTRACT
The allelopathic potential of various plants, including coffee species, has been studied. Although the allelopathic potential of coffee leaves, stems, and roots has been reported, there is no detailed information about coffee callus. Since this callus produces bioactive metabolites, we focused on investigating the allelopathic potential of Robusta coffee (*Coffea canephora*) callus as follows. The sandwich (SW) method, a small-scale bioassay using dried leaves of test plants and recipient lettuce seedlings, was adapted to identify the allelopathic activity of target coffee calli. Test samples were prepared as 10 mg of dried calli under five drying conditions: oven drying at 40 °C, 60 °C, 80 °C, silica gel drying, and freeze-drying. The appearance of each dried callus and chemical features of its water extract varied depending on drying conditions. However, all Robusta coffee calli showed a strong suppressive effect on the growth of hypocotyls and roots of lettuce seedlings. Based on this finding, we analyzed the endogenous level of caffeine (1,3,7-trimethylxanthine), a known allelochemical of coffee and tea plants, using HPLC. In this report, we discuss 1) technical points such as moisture content, drying method, and managing sample quality in the SW method for calli, and 2) the relationship between allelopathic potential and endogenous caffeine level of Robusta coffee calli.
Keywords*: allelopathy, coffee callus, sandwich method*

I. INTRODUCTION

Allelopathy is a phenomenon by which plants biochemically exert influence overgrowth, survival, or reproduction of target organisms. It can be direct or indirect, have beneficial or adverse effects, and affect a plant's own or another plant's growth through the release of biochemical(s) into the environment. It affects plant distribution, community formation, intercrop evolution, and biodiversity conservation, and as such is a topic of international interest [1]. The biochemicals produced are known as allelochemicals. To ensure sustainable agricultural development, it is important to exploit cultivation systems that take advantage of the stimulatory/inhibitory influence of allelopathic plants to regulate plant growth and development and to avoid allelopathic autotoxicity. Allelochemicals can potentially be used as growth regulators, herbicides, insecticides, and antimicrobial crop protection products [2].

Coffee is one of the most important commodities cultivated worldwide and has a great economic impact in many countries [3]. The most cultivated variety is *Coffea arabica*, which has several important cultivars and is responsible for 60% of world production [4]. Another important variety is *C. canephora*, otherwise known as Robusta coffee. The allelopathic potential of various plants, including coffee commodities, has been studied. In previous research, aqueous extracts of leaves, stems, and roots of *Coffea arabica* significantly inhibited the seed germination and radicle growth of rye grass, lettuce, and fescue [5]. The allelopathic activity of coffee seed extract and powder was also reported by Paneva [6]. However, there is scant information about the allelopathic activity of coffee calli, especially that of Robusta coffee callus.

In this report, we focus on a small-scale bioassay named the sandwich (SW) method, originally established for investigating the allelopathic activitiy of dried leaves of test plants [7]. Allelopathic activity was determined by comparing the hypocotyl and radicle length of recipient lettuce seedlings grown in media with or without 10-50 mg of dried leaves for 3 days [8]. Although the scale of this bioassay is suitable to check allelopathic activity of target calli within a short time, there is no technical information for the SW method using coffee callus. The objectives of this study are 1) to discuss technical points, such as moisture content of samples, drying method, and management of sample quality for establishing the SW method for callus, and 2) to explore the relationship between allelopathic potential and endogenous caffeine level of Robusta coffee calli.

II. METHODS

a. Pre-measurement of moisture content

We used a moisture analyzer (Ohaus MB45) to monitor the relative moisture content of Robusta coffee callus (1 g fresh weight). The rate of water loss during linear drying at 60 °C was measured. Fresh samples (10 leaves and 10 calli of several plants, see **Table 1**) were dried using an oven drier (EYELA Natural Oven NDO-450) at 60 °C for 8 hours to identify the variation of moisture content in samples.

b. Comparison of drying conditions for sample preparation

We used 4-week-old Robusta coffee calli from a regular maintenance subculture period (every 4 weeks) on modified MS medium containing 10 □M 2,4-D. Fresh samples were collected and dried using an oven drier (EYELA Natural Oven NDO-450) at 40, 60, and 80 °C, or dried using silica gel (5 g silica gel in a glass jar) or a freeze dryer (TAITEC DC-120). After 8 hours of drying, dried samples were tightly packed in plastic zip-bags and stored at room temperature until use. Water extracts were prepared as follows: briefly, 10 mg dried sample was put into a 1.5 ml microcentrifuge tube and 300 □l distilled water was added. The sample was then processed for 20 minutes by ultra-sonication without any mechanical mashing. The supernatant obtained was examined by spectrum scanning (240–500 nm) using a microplate reader system (Varioskan Flash, Thermo Scientific).

c. The sandwich (SW) Method

For the SW method bioassay, we referenced the original method by Fujii, *et al* [7] and added a modification. Briefly, we prepared agar growth medium for the the two outer layers of the sandwich using purified agar (SeaKem GTG Agarose, Cambrex Bio Science Rockland, Inc., Rockland ME, USA). After weighing agar powder (0.5 % w/v), we added it and 100 ml distilled water to a 200 ml flask, then covered the flask with a plastic wrap. The agar solution was then heated using a microwave 1.5 min at 600W. After the solution boiled, we poured 5 ml molten agar solution into an individual well of a six-well plate to set the first layer. After putting 10 mg dried sample onto the first layer after setting, we then poured another 5 ml of agar solution on top of the sample to set the second layer. Then, 5 lettuce seeds (*Lactuca sativa* L., Great Lakes 366) were vertically placed into each well (**Figure 1**). For the control, we used 10 ml agar solution with 5 lettuce seeds on it. We covered the set six-well plate with a plastic lid and appropriately labeled it. We then incubated the plate at 20 °C in the dark.

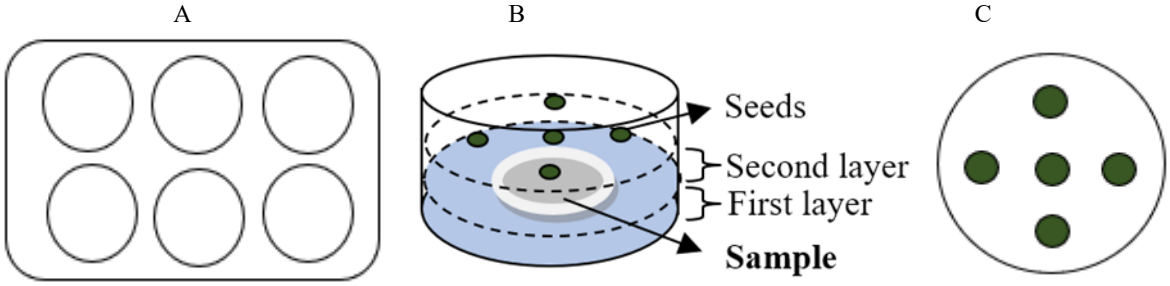

Figure 1. The SW method: A. Six-well plate; B. Sequence of sandwich layer: (1, first agar layer (5 ml); 2, dried sample (10 mg callus); 3, second agar layer (5 ml); 4, five lettuce seeds); C. Placement of lettuce seeds.

d. Growth Measurement

Length of hypocotyls and roots of germinated lettuce seeds were measured using Image J software after three days of incubation at 20 °C in the dark. The control consisted of seeds germinated in the absence of dried calli. Data were recorded as % growth of the control and averaged with standard error (SE).

e. HPLC Analysis

Robusta coffee callus (100 mg fresh weight) was extracted with 0.5 ml distilled water (0.2 w/v) in a microcentrifuge tube, and the supernatant was subjected to reverse-phase HPLC (column, Cosmosil Packed Column 5C18-MS-II, 4.6 ID x 150 mm from Nacalai Tesque (Kyoto, Japan); column oven, 40 °C; solvent, 20% methanol; flow rate, 0.6 ml min^{-1}; detection, 270 nm). Caffeine (MW 194.19, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the standard solution. Injection volume was set at 10 µl using a MICROLITER™ syringe (Hamilton, Reno NV, USA).

III. RESULTS AND DISCUSSION

a. Pre-measurement of moisture content

Measuring moisture content is one of the most important ways to identify the uniformity of plant materials. First, we measured the behavior of water in a mass of target callus during a linear drying process. As shown in **Figure 2**, coffee callus showed a high uniformity in 3 replicate measurements within a short time as measured by moisture analyzer. The average of the final moisture content was 88 % after 102 minutes of the drying process. Although variation (up to ~5-10 %) is expected in measurements made by moisture analyzer, the average value will be a useful reference to define suitable drying conditions for calli of interest in further experiments.

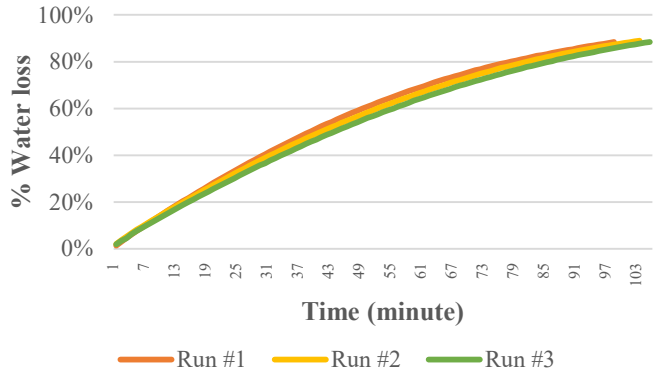

Figure 2. The behavior of water in a mass of coffee callus during a linear drying process at 60 °C.

Next, we investigated the variation of moisture content of leaves and calli from various plants. Fresh samples (10 leaves and 10 calli of several plants, see **Table 1**) were dried using an oven drier (EYELA Natural Oven NDO-450) at 60 °C for 8 hours according to the original SW method. When we used *in vitro*-grown leaves from 2 herbaceous plants (*Houttuynia cordata* and *Nicotiana tabacum*) and 2 woody plants (*Coffea arabica* and *Populus alba*), moisture contents varied in the range of 78.1 % to 93.5 %. When we used field-grown leaves from 2 bamboo plants (*Bambusa oldhamii* and *Dendrocalams latiflorus*) and 4 woody plants (*Akebia quinata*, *A. trifoliate*, *Gardenia jasminoides*, and *Populus* sp.), the variation of moisture contents had a wider range, from 56.2 % to 76.2 %. Based on these results, we suggest that the value of the moisture content of target leaves should be carefully discussed before their use as experimental materials for evaluating bio-activities. On the other hand, all the 10 calli tested showed a high degree of similarity, with most having more than 95 % moisture content. These values were the same as the moisture content preliminarily measured with the moisture analyzer (see **Figure 2**). Although the moisture content of each callus did not vary in this experiment, we must note a few key points.

Table 1. Comparison of moisture content of 10 different plant leaves (A) and 10 different callus strains (B).

A : Plant leaves

Category	Scientific name	Common name	Moisture content (%)
In vitro	*Nicotiana tabacum*	Tobacco	93.5
	Houttuynia cordata	Dokudami	89.7
	Populus alba	Poplar	85.6
	Coffea arabica	Arabica coffee	78.1
Field	*Akebia trifoliata*	3Leaves Akebi	76.2
	Populus sp.	Poplar (Field)	74.7
	Gardenia jasminoides	Cape jasmine	66.7
	Bambusa oldhamii	Green-bamboo	59.6
	Dendrocalamus latiflorus	Ma-bamboo (Giant bamboo)	59.1
	Akebia quinata	5Leaves Akebi	56.2

B : Callus strains

Category	Scientific name	Common name	Moisture content (%)
Tree	*Chamaecyparis obtusa*	Hinoki	97
	Sonneratia ovata	Mangrove	95.5
	Derris indica	Pongame oil tree	95.1
	Coffea canephora	Robusta coffee	94.3
Fruit	*Prunus persica*	Peach/momo	96.9
	Punica granatum	Zakuro	95.3
	Ficus carica L.	Ichijiku	91.3
Bamboo	*Phyllostachys nigra Munro var. Henonis*	Hachiku	96.9
	Bambusa multiplex Raeush	Houraichiku	96.4
	Dendrocalamus gigenteus Munro	Kyochiku	96

When we perform a maintenance culture of a target callus, a series of growth stages can be seen. These stages are: 1) lag phase, in which metabolite mobilization starts and synthesis of proteins and specific metabolites occurs without cell multiplication; 2) exponential phase, in which cell division reaches the maximum; 3) linear phase, in which cell division is reduce; 4) deceleration phase, in which cell division decreases and cell expansion occurs (at this point, we have to transfer the callus to a new culture medium due to the reduction of nutrients, agar dryness and accumulation of toxic substances); 5) stationary phase, in which no cell division or weight increase occurs, but the secondary metabolite accumulation sometimes increases; and 6) decline phase, in which weight is lost due to cellular death [9][10][11]. It is important to carefully define the culture period and growth stages of calli before using them experimentally.

b. Comparison of drying condition for sample preparation

In order to optimize the drying process for callus samples, we tested 3 oven drying conditions: 40, 60, and 80 °C. Non-heating conditions, both silica gel drying and freeze drying, were also examined to minimize damage to the sample and its components. **Figure 3** shows the difference in percent water loss from each sample after 8 hours. With the exception of the silica gel drying condition, samples were highly or fully dried. Oven drying at 80 °C and freeze drying show the highest percent of water reduction, reaching 96 % of fresh weight. Oven drying at 60 °C was slightly less effective, with samples achieving 94 % water reduction, and oven drying at 40 °C produced 89 % water reduction. Silica gel drying shows the weakest ability to reduce water in the sample during the drying process (46 % water reduction). It was expected that the relative concentration of endogeneous chemicals per unit weight of the callus would be ~2-10 times higher than that of the original sample. As shown in **Figure 4**, it was recognized that color and texture of the samples varied depending on the drying process.

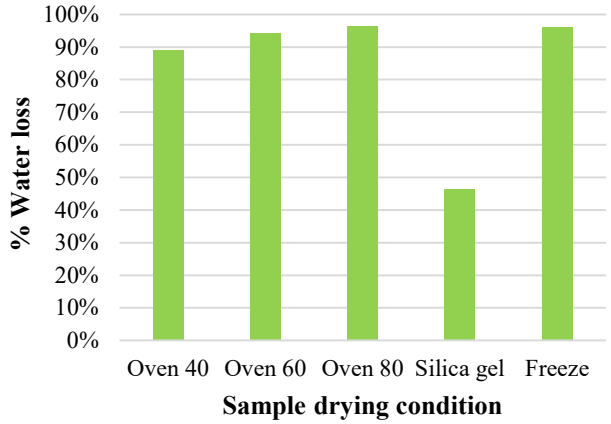

Figure 3. Water loss percentage in different drying processes after 8 hours. One g fresh weight of coffee callus was measured in each condition. Note: freeze drying takes 24 hours.

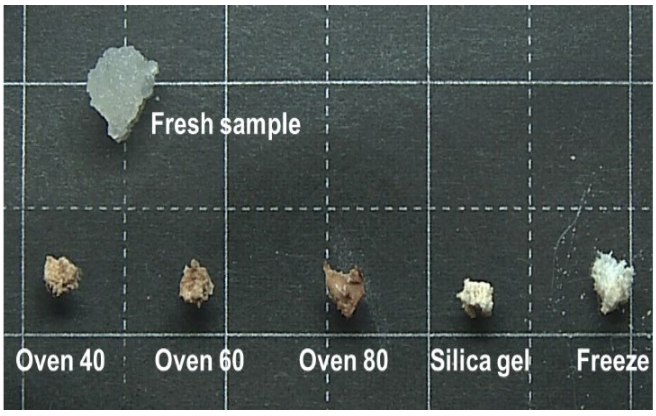

Figure 4. Characteristics of dried callus tissues in the different drying processes. Grid indicates 1 cm.

When oven-dried (40-80 °C), callus tissue gradually turned brownish in color, especially at higher temperatures. The texture also varied from crunchy to hard, tracking the color changes observed. In non-heating conditions, the color of callus tissues remained whitish under both conditons. However, a texture difference could be seen between the silica gel-dried sample and the freeze-dried sample. The former was crunchy on the outer surface but retained moisture in the middle, while the latter became crumbly in texture. We then confirmed the leaching efficiency of metabolites from dried calli using the water extraction method described in the Methods section.

Since this extraction method is a non-destructive process, it is expected to be a simulative environment in the SW method bioassay. The supernatants were examined by spectrum scanning (240–500 nm) using a microplate reader system (See **Figure 5**). **Figure 5A** shows profiles of the UV-vis absorption spectrum of oven-dried calli. The spectra of oven 40 and 60 °C conditions overlapped considerably, but the spectrum of the oven 80 °C condition showed a weak absorbance pattern. **Figure 5B** shows a clear difference between the silica-drying and freeze-drying processes. The spectrum of silica drying had a similar pattern to that of oven 40 and 60 °C conditions. As the overall estimetion,

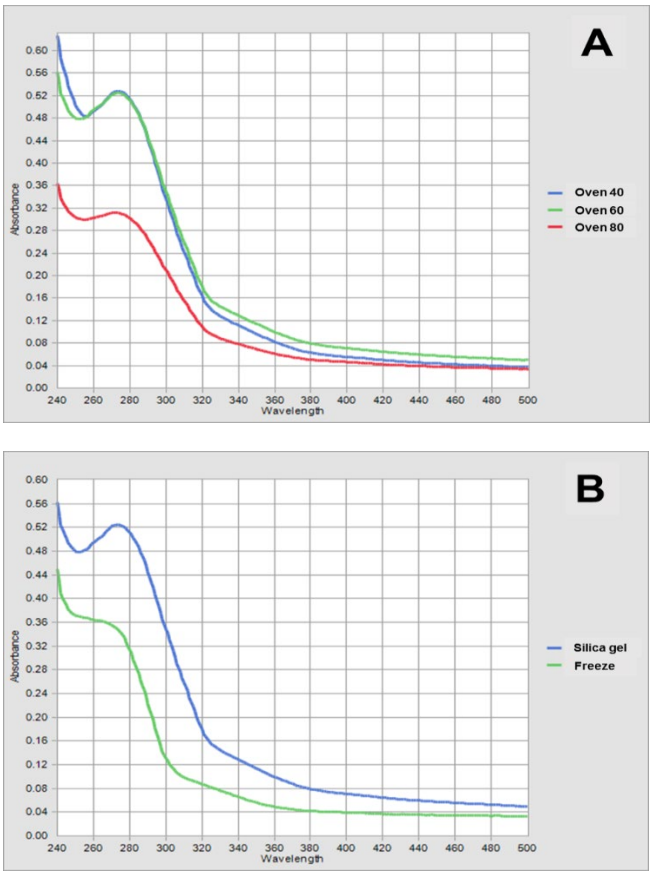

Figure 5. The profiles of UV-vis absorption spectrum of A. oven-dried samples and B. non-heating dried samples.

c. The SW Method

All dried calli were evaluated using the SW method to determine allelopathic activity. Strong inhibitory effects on the growth of lettuce seeds could be seen. As shown in **Figure 6**, we captured the germination pattern using a digital camera and measured the length of hypocotyls and roots in each sprout using Image J software.

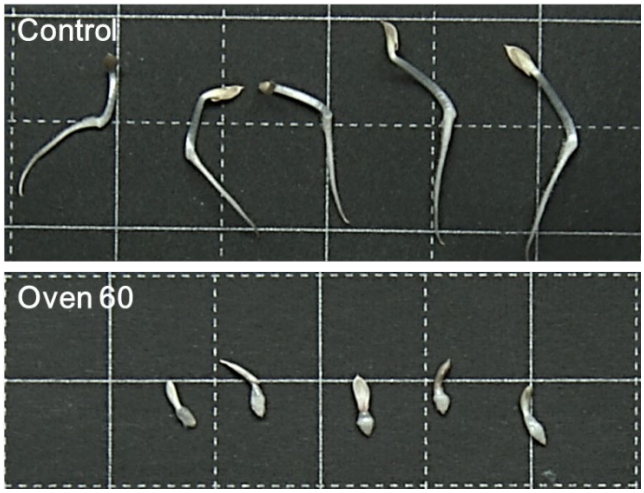

Figure 6. Images of germinated lettuce seeds after three days of incubation at 20 °C in the dark. Grid indicates 1 cm.

d. Growth Measurement

The percent lettuce seed growth is summarized in **Figure 7**. Leachates from dried Robusta coffee calli were considered strong inhibitory factor(s) since all the oven-dried samples and freeze-dried samples showed more than 85 % inhibition of hypocotyl and root elongation. On the other hand, the sample from silica gel-drying showed weaker inhibition (only 40 % inhibition to root growth). This might be due to a lower water-loss percentage (see **Figure 3**).

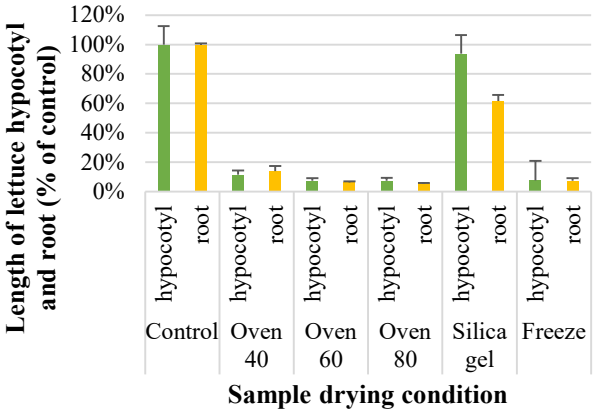

Figure 7. Length of lettuce hypocotyl and root (% of control) after three days of incubation at 20 °C in the dark.
Averages with SE (N = 5).

Chou and Waller [5] have suggested that the phytotoxins present in coffee tissue are caffeine, theobromine, theophylline, paraxanthine, scopoletin, and caffeic, chlorogenic, vanillic, ferulic, p-coumaric, and p-hydroxybenzoic acids. Caffeine (1,3,7-trimethylxanthine) and its derivatives are thought to be strong allelochemicals [12][13][14]. Sasamoto, et al. reported that the effect of caffeine and theophylline at 1000 µM is to completely inhibit the proliferation of lettuce cells derived from protoplast culture [15]. We carried out HPLC analysis to measure the endogenous caffeine level of Robusta coffee calli, described in the next section.

e. HPLC Analysis

Robusta coffee callus showed more than 85 % inhibition to elongation of hypocotyls and roots. To clarify the endogenous caffeine level, we analyzed the water extract of 4-week-old Robusta coffee callus via HPLC. The endogenous caffeine level was averaged with standard error, (e.g. 22.3±4.6 nmol/100 mg fresh weight) from 5 independent measurements. Several unknown metabolites were also detectable at 270 nm (data not shown).

IV. CONCLUSION

1) The SW method is applicable to investigate allelochemicals in target calli. 2) Several technical points for optimizing the SW method are suggested. 3) Further detailed allelochemical analysis should be carried out using HPLC.

ACKNOWLEDGMENT

The author (MIA) acknowledges the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) Scholarship for funding while studying in Japan. The author also expresses gratitude to the Prefectural University of Hiroshima for providing me with financial support to the international conference (IC-FANRes 2019, Ternate, Indonesia).

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