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Production of agarwood fragrant constituents in *Aquilaria* calli and cell suspension cultures

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Abstract: Calli and suspension cell culture were established from *Aquilaria* species whose resinous portion was called agarwood and used as medicine and incense. Four different strains of calli were analyzed for fragrant compounds such as sesquiterpenoids and chromone derivatives which were the major components of agarwood. Main sesquiterpenoids detected from calli were α-guaiene, α-humulene and δ-guaiene, and those of chromone derivatives were phenylethylchromones (AH_3, AH_4, AH_5, AH_6). Amount of these compounds differed among the four strains, indicating that *Aquilaria* plants may have variation in capacity for fragrant compound production. Incubation temperature analysis was also done from 20°C to 40°C and resulted that cell growth was the best at 25°C, whereas the amount of fragrant compounds was largest at 20°C. Salicylic acid (SA) and methyl jasmonate (MJ) were added to calli and suspension cell culture respectively in order to induce production of fragrant compounds. Both SA and MJ apparently induced production of three sesquiterpenoids, α-guaiene, α-humulene, and δ-guaiene at early stage of treatment of SA or MJ, but did not induce that of chromone derivatives directly. Further studies of time course of chromone production and cell viability suggested that cell death may take part in chromone production, and that phenylethylchromones would be produced via oxydoagarochromones (OACs). These results indicate that sesquiterpenoids are synthesized in living cells, but chromone derivatives may be produced from debris of dying cells.

Key words: *Aquilaria*, calli, cell suspension cultures, sesquiterpenoids, chromone derivative.

Agarwood is the resinous portion of the trunk and branches of *Aquilaria*, *Gonystulus*, and *Gyrinops* species (Thymelaeaceae). It has been used as an incense due to its peculiar perfume, and the most fine pieces are highly valued in the Japanese incense ceremony. It is also used pharmaceutically as an anti-emetic, sedative, and digestive in oriental medical treatments. Worldwide trading of agarwood is facing a serious shortage of resources because of its chaotic collection in forests and the decrease in the tropical rain forest area. Genera *Aquilaria*, *Gonystulus*, and *Gyrinops* are endangered and listed in Appendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora (CITES) since 2005 (*The Official Gazette*, 2005), so its international import and export are now under strict control. Under these circumstances, the cultivation of agarwood trees and attempts to artificially produce agarwood using these trees are booming in Indochina. However, the mechanisms which produce these fragrant compounds produced in trees have not been unveiled. It is known that wounding and microbial infection are effective for resin production, though efforts to produce it artificially have not yet been successful.

All agarwood-producing plants are timber species which take a considerably long time to grow and the resinous portion is formed inside of the wood. These circumstances make studies using fresh plants difficult, so we used cultured cells of *Aquilaria* to study the mechanisms of production of these fragrant compounds. According to reports on the fragrant compounds of agarwood (Nakanishi et al. 1981; Shimada et al. 1982; Nakanishi et al. 1983; Ishihara et al. 1991; Ishihara 1993; Yagura et al. 2003), sesquiterpenoids and phenylethyl chromone derivatives are the principal compounds in the oleoresin of agarwood, and we found that these compounds are also produced in *Aquilaria* calli. In this report, the results of experiments concerning the patterns of fragrant compounds using cultured cells derived from four different origins are described. The effects of incubation temperature, the addition of salicylic acid

Abbreviations: MJ: methyl jasmonate; AH_1: (5S,6R,7R,8S)-2-(2-phenylethyl)-5e′,6e,7a,8a′-tetrahydroxy-5,6,7,8-tetrahydrochromone; AH_3: 6-hydroxy-2-(2-phenylethyl)chromone; AH_4: 6-methoxy-2-(2-phenylethyl)chromone; AH_5: 6-methoxy-2-[2-(3-methoxyphenyl)ethyl]chromone; AH_6: 6,7-dimethoxy-2-(2-phenylethyl)chromone; SA: salicylic acid
This article can be found at http://www.jspcmb.jp/

(SA), methyl jasmonate (MJ), and β-glucan to the media, and the relationship between the ratio of cell death and production of fragrant compounds were investigated.

Materials and methods

Plant materials

The *Aquilaria* leaves used for callus induction were *Aquilaria crassna* from Vietnam (n6) and from Thailand (ti and tr), and *Aquilaria sinensis* from Taiwan (sr), as described previously (Ito et al. 2005).

Establishment of Aquilaria calli and suspension culture

Fresh young leaves were disinfected by treatment with a sodium hypochlorite solution (effective chloride concentration 1.4%) for one minute and then with again sterile water. The treated leaves were cut into pieces and inoculated aseptically onto Murashige-Skoog (MS) medium (Murashige and Skoog 1962) (pH 7.0) containing 3% w/v of sucrose and 1.5 w/v % of gelangum (Instant Bio Brid, provided by the Research Institute for Industrial Bio-Science), which was prepared in tubes with hormonal combinations of 10^{-7}, 10^{-6}, and 10^{-5} M of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenin (BA). After incubation at 25°C for one month in the dark, callus induction and growth were assessed by visual inspection. Calli were inoculated onto fresh medium in tubes every 30 days. The medium contained 3% w/v of sucrose, 1.5% w/v of agar, and 10^{-6} M of 2,4-D and BA, which was the best hormonal combination. Suspension cell cultures were established from callus strain n6 and incubated with reciprocal shaking at 130 rpm at 25°C in the dark. The medium for the suspension cell culture was almost the same as that for the calli except that it did not contain agar, and was prepared in flasks. The suspension cell cultures were subcultured into fresh medium every two weeks.

Analysis of fragrant components of Aquilaria calli

The calli were incubated for 2 to 12 weeks after inoculation and were harvested every two weeks (n=6). Sesquiterpenoids were analyzed by GC-MS and chromone derivatives were by HPLC. Fresh cells for the GC-MS analysis were ground with a mortar and pestle and transferred into a flask with an airtight cap. Vapor from the flask was extracted using a solid phase micro-extraction (SPME) fiber (100 μm polydimethylsiloxane, Supelco) at 20°C for 30 min according to the manufacturer's protocol, and was injected into a GC-MS (M9000 GC3DQMS, HITACHI) equipped with a TC-WAX column (60 m×0.25 mm, film thickness 0.25 μm, GL Science) with a 5 minute desorption time at 250°C splitless. The conditions for the GC-MS analysis were as follows: the carrier gas was helium with a flow rate of 1 ml min^{-1}, the injector temperature was 250°C, the column oven program was started at 80°C and was increased by 5°C every minute until it reached 220°C, and was kept at this temperature for 10 min, and then increased by 10°C every minute until it reached 240°C, and was then kept at 240°C for 3 min. The ionization voltage for MS was 15 eV. Peaks were identified by comparing retention times and mass fragmentation patterns with those of samples and in database (WILEY), and were quantified by their TIC peak area. The standard samples were, authentic α-humulene (Nakarai Tesque Co.), and α-guaiene and δ-guaiene which were isolated from agarwood oil and identified by ^{13}C-NMR and GC-MS (Ito M et al. 2005). Fidelity of the quantitative analysis using SPME-GCMS was verified using authentic α-humulene and it turned out that peak area of TIC was proportioned to the amount of α-humulene exposed to the fiber (data not shown). For the HPLC analysis, ground cells were extracted with AcOEt (4 ml) overnight at room temperature. The extract was concentrated to dryness, dissolved in MeOH (ca. 0.4 ml), and then passed through a filter. The eluate was concentrated to dryness. The residue was dissolved in MeOH at 1 mg ml^{-1} and analyzed by HPLC (pump: L-7100, column oven: L-7300, UV detector: L-7420, HITACHI). The conditions for the HPLC were as follows: the column was a YMC-Pack ODS/R, 10 μm, 120Å, 250×4.6 mm I.D., the detection was carried out at 254 nm, the solvent was MeOH in water with a gradient from 37% (0 min) to 100% (30 min) MeOH, the flow rate was 1 ml min^{-1}, and the injection volume was 3 μl. Peaks were identified by comparing retention times with those of standard samples which were isolated from the extract of withered or artificially wounded wood of *Aquilaria* and identified by ^{1}H-NMR and ^{13}C-NMR (Yagura T et al. 2003, 2005), and were quantified by calculating according to the calibration curve of the samples. AH_3, AH_4, AH_5, and AH_6 in calli were also analyzed by LC-MS to confirm their structures.

Analysis of the incubation temperature

The tubes of calli cultured at 25°C for 6 weeks after inoculation were transferred into the temperature gradient incubator which was set at 20, 25, 30, 35, and 40°C (TG-200-AD, NIPPON MEDICAL & CHEMICAL INSTRUMENTS CO., LTD.). The calli were incubated in the dark at each temperature for 6 weeks, and then harvested for analysis by GC-MS and HPLC (n=5).

SA treatment of calli

SA (Nakarai Tesque Co.) was added to the MS agar medium containing 2,4-D and BA with a final concentration of 10 μM, 100 μM, 1 mM, or 10 mM. The calli cultured for 4 weeks after inoculation were transferred onto these medium with or without SA, and were incubated at 25°C in the dark for 2 to 16 weeks. They were harvested every 2 weeks for analysis by GC-MS and HPLC.

MJ and β-glucan addition to the cell suspension culture

MJ (Sigma) dissolved in DMSO (300 mM) and β-glucan (water soluble β-1,3-glucan, Wako) which is one of the cell components of fungi dissolved in water (0.1 mg ml^{-1}) were added to the cell suspension cultures at a final concentration of 22.4 μg ml^{-1} (0.1 mM) and 1 μg ml^{-1}, respectively, 5 days after inoculation. The cells were incubated at 25°C in the dark at 130 rpm, and were harvested by filtration 1 day, 3 days, 1 week, and 2 weeks after treatment to be analyzed in the same way as the calli (n=3). The MJ treated cells were also harvested at 2, 6, 12, and 24 hours after treatment.

Measurement of cell viability

The cells in the suspension culture were cultured for 2 to 6 weeks after inoculation and harvested once a week by filtration for cell viability measurement and for analysis of fragrant compounds by HPLC. In order to separate aggregated cells, the cells were transferred into 0.5 M mannitol solution with 0.3% SDS and 0.05% (w/v) pectolyase (Kikkoman) to enhance single cell release (Takebe et al. 1968). The treatment was carried out for 4 hours in standard culture conditions (25°C, 130 rpm in the dark). The treated cells were filtered through nylon mesh to collect single cells, and then stained with 0.025% (w/v) evans blue solution, which strains nonviable cells blue. The cells were observed under a light microscope to determine the proportion of viable cells.

Results

Fragrant components analysis of Aquilaria calli

Sesquiterpenoids and flindersia-type phenylethyl chromone derivatives were detected in all four types of *Aquilaria* calli (Figure 1). The amount of fragrant compounds and the starting time of their production varied among the types. The cell growth rates were also different among the four types of calli, which were tr, sr, n6, and ti in order of the highest growth rate (Figure 2).

The main sesquiterpenoids detected from the four types of calli were α-guaiene, α-humulene, and δ-guaiene (Figure 1). Only sr produced another sesquiterpenoid compound, and this seemed to be unique to the sr type, but has not been identified (data not shown). The amount of the three main sesquiterpenoids per weight followed the order sr, tr, ti, and n6 in order of the largest amount, and the time that the highest production rate was reached in following order: ti, n6, sr, and tr (Figure 2).

On the other hand, the principal chromone derivatives detected from these calli were AH_3, AH_4, AH_5, and AH_6 (Figure 1). The amount of the four chromones per calli weight was in the following order from highest to lowest: sr, tr, ti, and n6, which is the same as that of the sesquiterpenoids. Production of the chromone derivatives is believed to start later than that of the sesquiterpenoids (Figure 3).

The effect of incubation temperature on calli growth and production of fragrant compounds

The calli (tr) were incubated for 6 weeks at various temperatures and then analyzed for fragrant compounds.

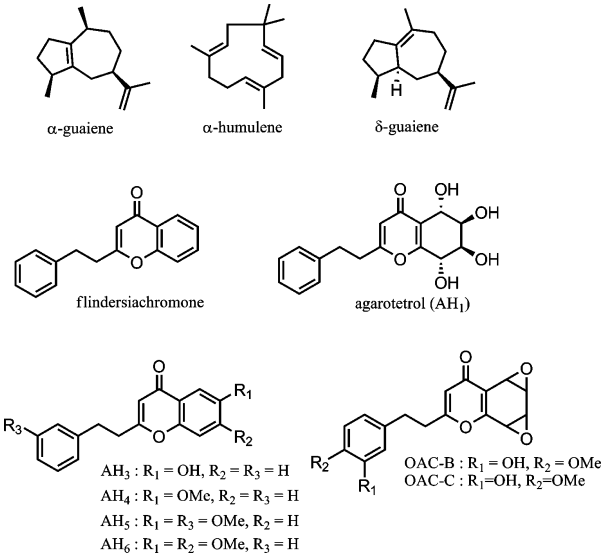

Figure 1. Chemical structures of sesquiterpenoids and chromone derivatives found in *Aquilaria* calli and suspension cultures.

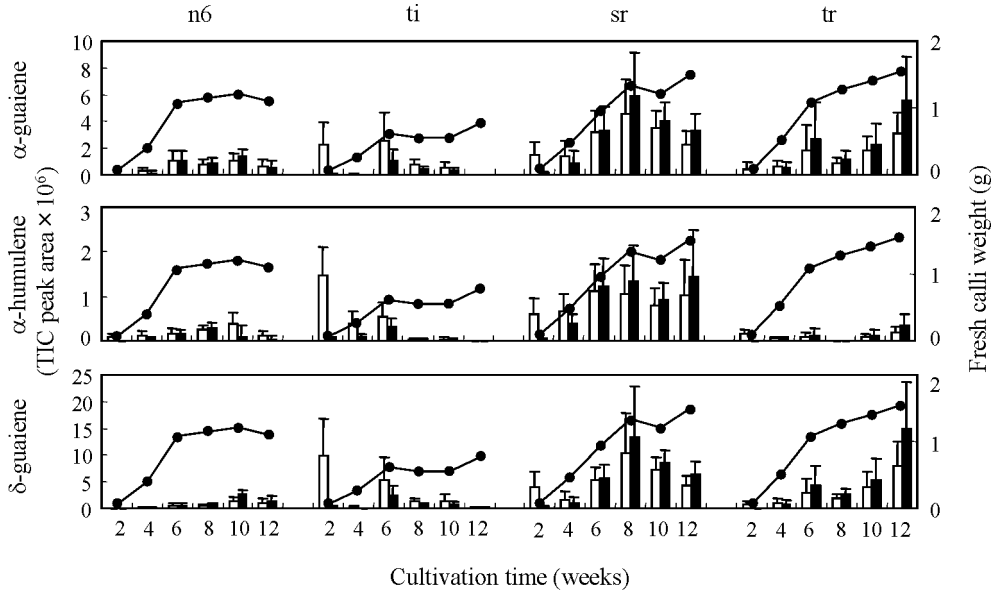

Figure 2. Time course of sesquiterpenoid content and calli weight of *Aquilaria* (n=6). Open columns show sesquiterpenoid content per calli weight, solid columns are for sesquiterpenoid content per tube, and line graphs show fresh calli weight per tube. Sesquiterpenoid values are the mean±SD. Calli weight are presented in mean value.

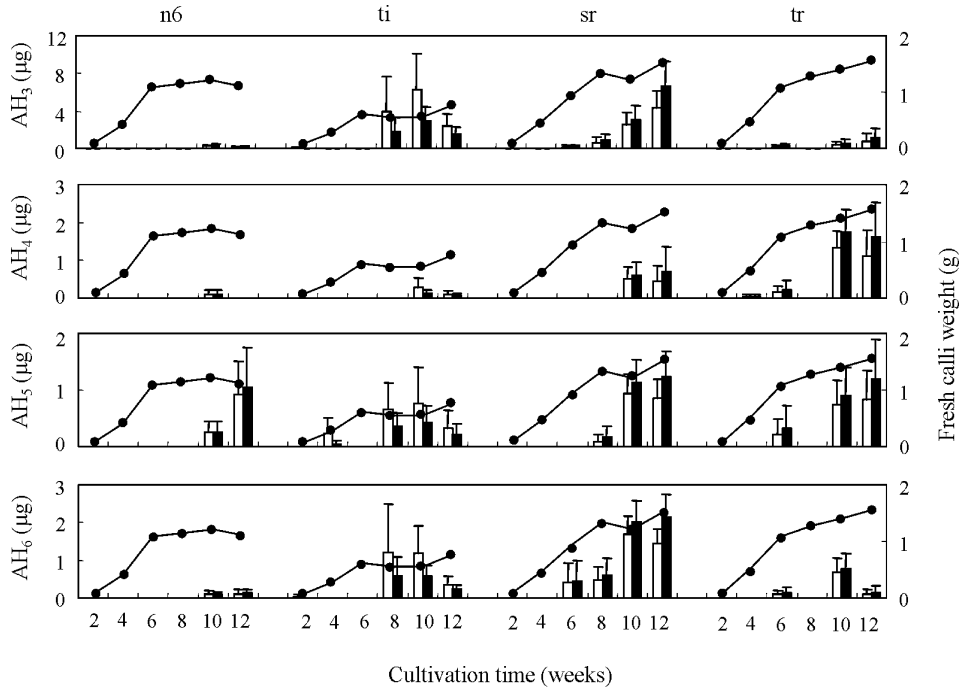

Figure 3. Time course of chromone content and calli weight of *Aquilaria* (n=6). Open columns show chromone content per calli weight, solid columns are for chromone content per tube, and line graphs show fresh calli weight per tube. Chromone values are the mean±SD. Calli weight are presented in mean.

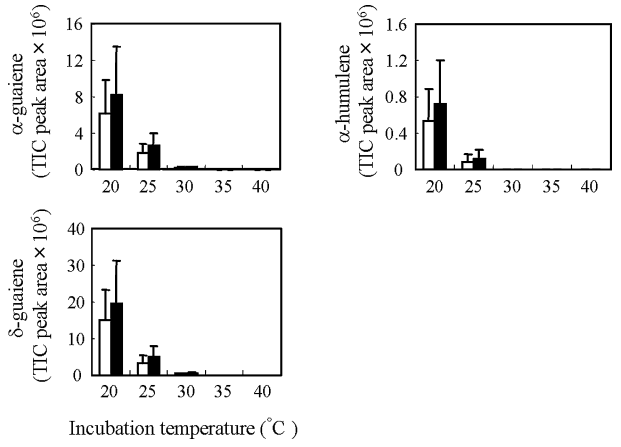

Figure 4. Sesquiterpenoid production of *Aquilaria* calli (tr) in various incubation temperature for 6 weeks (n=5). Open columns show sesquiterpenoid content per calli weight and solid columns are for sesquiterpenoid content per tube. Sesquiterpenoid values are the mean±SD.

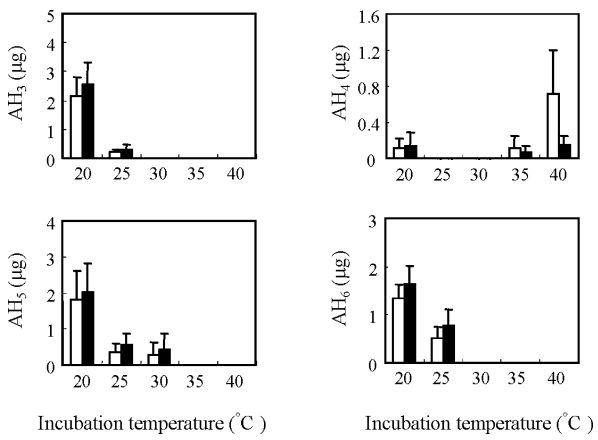

Figure 5. Chromone production of *Aquilaria* calli (tr) in various incubation temperature for 6 weeks (n=5). Open columns show chromone content per calli weight and solid columns are for chromone content per tube. Chromone values are the mean±SD.

Cell growth was highest at 25°C, and varied with temperature. When the fresh weights produced were shown in a ratio that compared to the fresh weight produced at 25°C, the ratio equaled 0.95, 0.88, 0.56, and 0.35 for 20, 30, 35, and 40°C, respectively).

Sesquiterpenoid content per calli weight was highest at 20°C, which was 2–5 times larger than one produced at 25°C. On the other hand, little sesquiterpenoid was detected when the incubation temperature was higher than 30°C (Figure 4). The content of the four chromone derivatives per calli weight was also highest at 20°C, which was 3.5 times that produced at 25°C. However, AH_4 was different from the others and was detected at 20, 35, and 40°C (Figure 5).

The effects of addition of SA to the media

Cell growth was slightly suppressed in calli on media containing 10 μM or 100 μM of SA (Figure 6, 7). The growth rate of calli as well as the production of fragrant compounds was very low on media containing 1 mM or 10 mM of SA (data not shown).

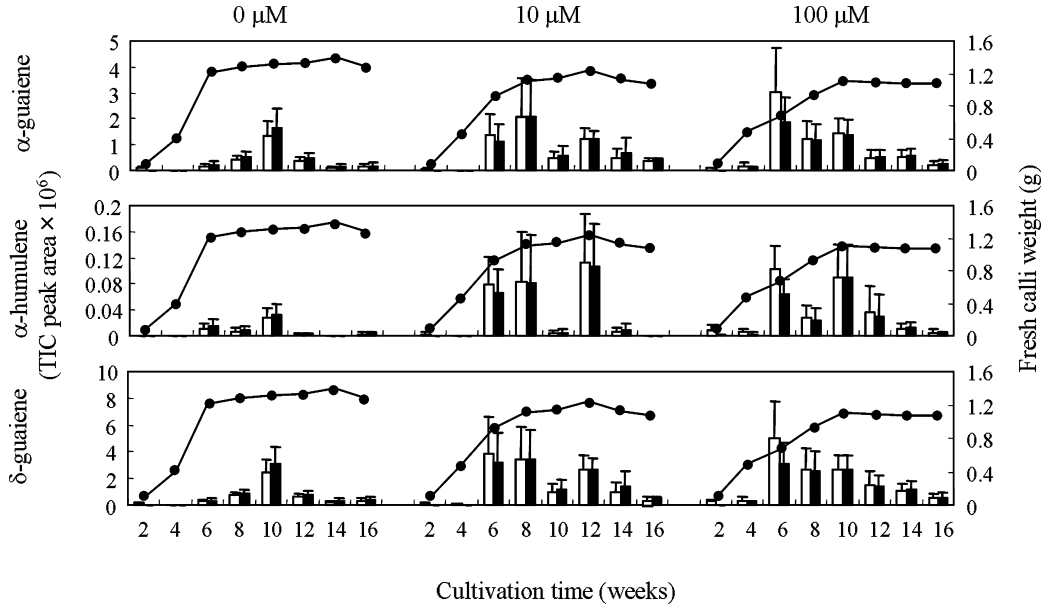

Figure 6. Sesquiterpenoid production and calli weight of *Aquilaria* on media containing SA (n=5). Open columns show sesquiterpenoid content per calli weight, solid columns are for sesquiterpenoid content per tube, and line graphs show fresh calli weight per tube. Sesquiterpenoid values are the mean±SD. Calli weight are presented in mean value.

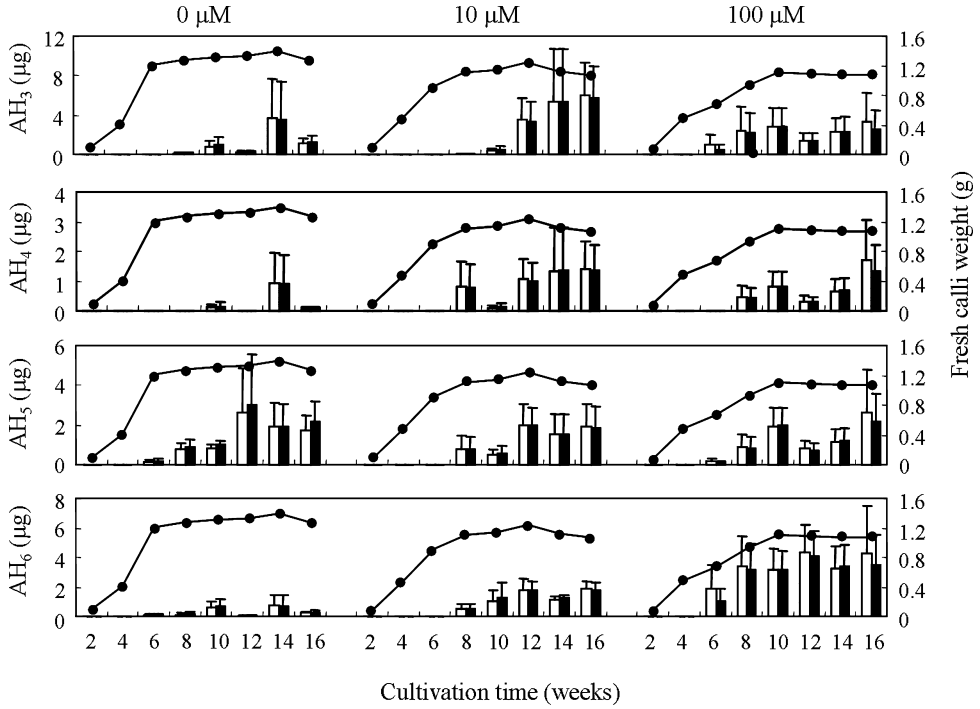

Figure 7. Chromone production and calli weight of *Aquilaria* calli on media containing SA (n=5). Open columns show chromone content per calli weight, solid columns are for chromone content per tube, and line graphs show fresh calli weight per tube. Chromone values are the mean±SD. Calli weight are presented in mean value.

The types of sesquiterpenoids detected from the calli growing on media containing SA were the same as that of the control calli, but the amount and peak time of production were different (Figure 6). The peak time for sesquiterpenoid production was 6 to 8 weeks after inoculation on the SA containing media, though it was 10 weeks in the control. The content ratios of α-guaiene, α-humulene, and δ-guaiene at the peak time of production were 1.4/1.8, 3.2/3.0, 1.8/1.6 (10 μM/100 μM) times as much as those of the control. This suggests that sesquiterpenoid production was increased on the SA-containing media in calli.

Chromone production in calli started slightly earlier on the SA containing-media than on the control, but not as early as that of the sesquiterpenoids. The amount of chromones accumulated in calli was increased on the SA containing media. The content of AH_3 increased, especially in calli on media containing 10 μM of SA, and that of AH_6 did so on media containing 100 μM of SA. The increases of AH_4 content in calli on media containing 10 μM or 100 μM of SA were almost the same. Among the four kinds of chromones, only AH_5 did not increase in the calli on the SA containing media (Figure 7).

The effects of the addition of MJ and β-glucan to the cell suspension culture

Our previous study showed that MJ induced production of three types of sesquiterpenoids in *Aquilaria sinensis* cell suspension culture, and that these sesquiterpenoids were not produced without MJ treatment (Ito et al. 2005). In this paper, a time course study of the fragrant compounds produced in cultured cells during the first day to 2 weeks after treatment with MJ or β-glucan is described. The same species of sesquiterpenoids as that found in the calli were detected in the MJ treated culture for 1 day, and then their amounts decreased. The cell growth rate was lower in the MJ treated cells than in the control. Sesquiterpenoids were hardly produced in the control and β-glucan treated cells (data not shown).

Since a significant amount of sesquiterpenoids was found in the MJ treated cells for 1 day treatment, analyses of samples harvested within a shorter period of time were performed. MJ treated cells of 2, 6, 12, and 24 hours of culture were analyzed. Sesquiterpenoids were not found in the control cells (Figure 8). The production of the three species of sesquiterpenoids in the MJ treated cells was at its peak 12 hours after the treatment.

As for the chromone derivatives, they were first detected in the cultured cells 3 weeks after inoculation. In contrast to the production of sesquiterpenoids, that of the chromone derivatives was very similar in the MJ and β-glucan treated cells both in its amount and its transition, which was also comparable to the controls (data not shown).

Measurement of cell viability and analysis of chromone in cell suspension culture

Chromone derivatives of the flindersia type were almost always found in calli which had been cultured for a comparatively long time after inoculation, namely, after 3 weeks of culture and the production of chromone compounds was homogeneous in the MJ treated and control cells of the cell suspention culture. These results indicate that the accumulation of chromones requires a long period so analyses of cells with a longer incubation time (4 to 6 weeks after inoculation, without MJ), was

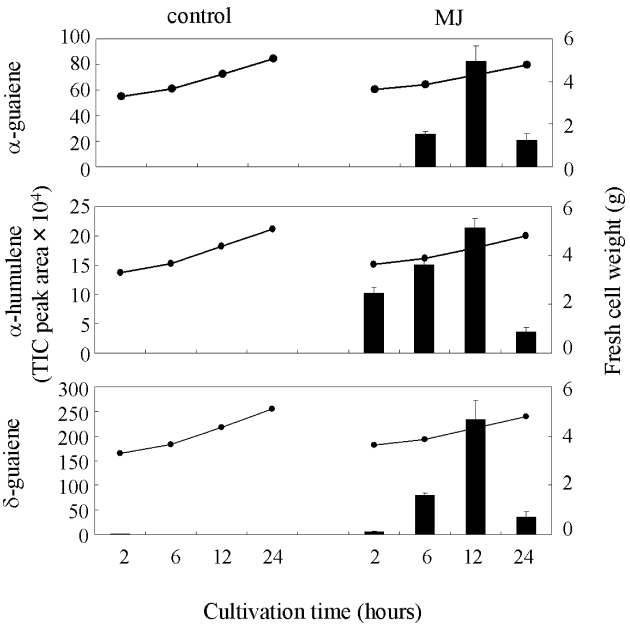

Figure 8. Time course of sesquiterpenoid content and cell weight of suspension culture after treatment with MJ (n=3–6). Solid columns show sesquiterpenoid content per flask, and line graphs are for fresh cell weight per flask. Sesquiterpenoid values are the mean±SD. Cell weight are presented in mean value.

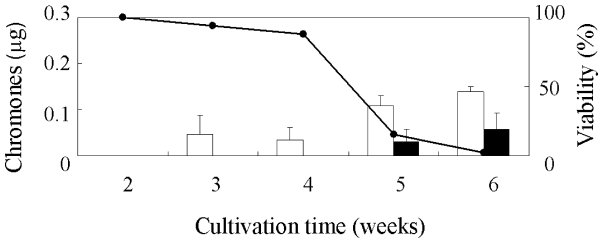

Figure 9. Time course of chromone content and cell viability of cell suspension culture (n=3–6). Open columns show agarotetrol (AH_1) content per cell weight, solid columns are for AH_4 content per cell weight, and a line graph shows cell viability. Chromone values are the mean±SD. Viabilities p are presented in mean value

performed, and at the same time measurement of cell viability was carried out.

The cell viability rate decreased rapidly from 4 to 5 weeks after inoculation (Figure 9, 10). Agarotetrol (AH_1) and AH_4 were detected after 3 weeks, and their amount increased as cell viability decreased (Figure 9). Among the chromone derivatives found in the cultured cells, some unusual types were detected. These chromones were oxidoagarochromones (OACs) (Figure 1), which are known to be produced in the wounded portion of *Aquilaria* plants soon after a deliberate injury (Yagura et al. 2005). OAC-B and OAC-C were the derivatives found in the cultured cells 3 weeks after inoculation, and the amount of OAC decreased as cell viability decreased (Figure 10).

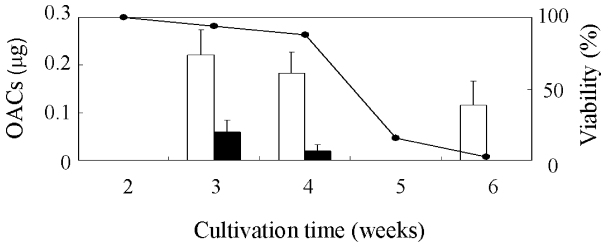

Figure 10. Time course of OAC content and cell viability of cell suspension culture (n=3–6). Open columns show OAC-B content per cell weight, solid columns are for OAC-C content per cell weight, and a line graph shows cell viability. OAC values are the mean±SD. Viabilities are presented in mean value.

Discussion

Agarwood can not be produced in healthy intact plants. Various attempts have been made to find out how agarwood is produced, though this question is currently unsolved. Many ideas about what is effective for the production of resin have been proposed such as physical wounding, microbial infection (Shu et al. 2005), and chemical administration (for example, solutions of $FeCl_3$ or acetic acid). In order to find an answer to its question, the mechanisms of induction of sesquiterpenoid and chromone production in agarwood were studied using calli and cell cultures of *Aquilaria*.

Four different types of *Aquilaria* calli (from Vietnam, Taiwan, and Thailand) were analyzed for their cell growth rate, amount of fragrant compounds, and the initial time of their accumulation. These turned out to be different among the four types, even between strains ti and tr, which were both from Thailand. Further studies are required to compare more types of calli in order to determine whether these differences are derived from source plant species or other conditions. However, these results suggest that *Aquilaria* plants may have variation in their capacity to produce fragrant compounds.

The cell growth of calli was highest at 25°C, which showed that the optimal temperature for culture was 25°C, although, the production of fragrant compounds was higher at 20°C than at 25°C. It was observed in some plants that the optimal temperatures for growth and for production of secondary metabolites were different (Rabino et al. 1986; Shichijo et al. 1993). However, higher temperature might cause greater loss of carbohydrates in calli through increased respiration (Amthor 1984), which might subsequently suppressed production of fragrant compounds. Some portion of sesquiterpenoids produced in calli might emitted into the air and media, and which could be another reason for the loss especially at higher temperatures.

As for production of sesquiterpenoids, it peaked and then rapidly decreased (Figure 2, 6, 8).Various reasons can be suggested for this decrease such as degradation by endogenous enzymes and secretion into the culture medium, but because the sesquiterpenoids detected in the calli were highly volatile, the most plausible reason was that they diffused into the air. In order to determine the reason, the volatile compounds in the head space of the tubes in which strain tr was incubated for 10 weeks were analyzed by SPME-GCMS, and α-guaiene and δ-guaiene were detected. This suggested that the sesquiterpenoids produced and accumulated in calli diffused into the air, and the same happened in the cell suspension culture. The sesquiterpenoid amount in the cells decreased when the amount of diffusion exceeded that of production during cell incubation.

The conditions for sesquiterpenoid production were different in calli on the gel-medium and in the suspension cells. Sesquiterpenoids could be found in the calli without treatment, but were not in the cells in the suspension culture. These two forms of cells are not fundamentally different though the ingredients of their culture media differed as to whether they contained agar or not. Agar mainly consists of polysaccharides derived from seaweed, and some of the constituents are known to act as elicitors (Etzler 1998). The production of sesquiterpenoids detected in the calli might have been induced by these polysaccharide elicitors; however, treatment with β-glucan or isomaltooligosaccharides (used as a food additive), both of which are categorized as polysaccharides, did not induce sesquiterpenoid production (data not shown), suggesting that the polysaccharides that are capable of acting as elicitors in *Aquilaria* cells might be limited, or that components of agar other than polysaccharides may act as elicitors. Apart from the agar constituents, oxidative stress on the calli caused by continuous exposure to air, might have been another elicitating factor.

Resin formation in trees seems to be triggered by mechanical wounding and infectious disease, and agarwood production is also believed to be an example of these phenomena, which is known as plant defense response. SA, MJ, and β-glucan which are experimentally used as elicitors of plant defense responses were added to the culture media, and this resulted in an enhancement of sesquiterpenoid content and a suppression of cell growth by SA and MJ treatments (Figure 6, 8). SA is well known as a signal transducer in pathogen responses (Shah 2003), and MJ is also associated with the response to wounding and pathogens. These two compounds and ethylene are reported to play important rolls in plant defense, in which a variety of defense genes are exposed through cross-talking of these signal transducers (Reymond and Farmer 1998; Felton and Korth 2000). The results of the experiments described here also suggest that SA and MJ are relevant to the synthesis of the sesquiterpenoids, which are often antibacterial and act as phytoalexins in plants. The time course of sesquiterpenoid production in

the *Aquilaria* suspension cells triggered by MJ treatment is shown here, so the next attempt at demonstrating the mechanisms of resin production should include cloning of the genes for sesquiterpenoid synthesis.

The administration of β-glucan did not induce sesquiterpenoid production in the cultured cells. The β-glucan used in this study was water soluble β-glucan extracted from *Letinus edodes* (Shiitake mushroom), but the result may have been different if β-glucans derived from another fungus had been used. A past report showed that recognition of the sugar chain structure of β-glucan elicitors varies among plant species (Yamaguchi et al. 2000).

Unlike the transition of the sesquiterpenoids, the content of the flindersia type chromone derivatives did not decrease after they were produced (Figure 3, 7, 9). Chromone derivatives are not very volatile and have a stable structure; therefore, they seemed not to diffuse into the air or culture medium or be degraded by endogenous metabolism.

Because chromones of the flindersia type were found in cells cultured for a long time after the inoculation of either calli or the suspension culture, and their amount increased as cell viability decreased (Figure 9), it was supposed that the production of chromones might have a relationship to cell death. In this context, the reason why chromone production in SA-treated calli started earlier than in the control and its amount increased is believed to be the alteration of the time of cell death in calli by SA treatment. SA is known to contribute to hypersensitive response (HR)-associated resistance via mechanisms that include the potentiation of reactive oxygen species (ROS) generation and cell death (Mur et al. 1997, 2000; Shirasu et al. 1997), whereas MJ is reported to suppress the cell death induced by SA treatment (Overmyer et al, 2003). Based on this, cell death would be induced in calli by SA treatment, but not in a cell suspension culture after MJ treatment, and consequently, the chromone production in the SA-treated cells would start earlier and its amount would increase.

The amount of chromones of the flindersia type increased as OACs decreased when cell viability was also decreased (Figure 9, 10). This suggests that flindersia type chromones are produced via OACs as precursors, which is supported by the fact that OACs are produced in the wounded portion of fresh *Aquilaria* plants soon after a deliberate injury (Yagura et al. 2005).

Constituents of the secondary cell wall start to be deposited when plant cells stop growing on the inner side of their primary cell wall, which consists mainly of cellulose. The main constituent of the secondary cell wall is in most cases lignin, which forms a net of aromatic compounds that has a phenylpropanoid skeleton by binding in various ways such as ester and ether bonds. It further gathers lignin, polysaccharides, and proteins to its structure. When a plant cell dies, it is often accompanied by vacuolar collapse, and the cellular content is degraded by enzymes from the vacuole such as hydrolases and oxidases. Chromone derivatives found in agarwood are produced concurrently with cell death and their structure includes phenylpropanoid skeletons. Chromone derivatives are not likely to be compounds whose biosynthesis is induced by signal transducers like sesquiterpenoids, but are produced by the degradation of cell wall constituents by endogenous enzymes during the process of cell death. Phytochemical analysis of the non-resinous portion of agarwood is currently being performed in order to clarify the source compound for chromone production. Our results described here suggest that the biosynthetic processes of sesquiterpenoid and chromone derivatives might be different.

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