Research paper

Production of Acteoside in Hairy-Root Culture of *Paulownia fortunei* Hemsl.

Kuo-Lung Ku,[1] Ching-Fen Hsu,[2] Yue-Ken Liao[2,3]

【Summary】

In vitro grown paulownia (*Paulownia fortunei* Hemsl.) seedlings were inoculated with *Agrobacterium rhizogenes* (BCRC 15011; (ATCC 43057)) to produce hairy roots for acteoside (AS) production. Hairy-root lines 7-6, 23-1, and 55-1 were first cultured in MS liquid medium (50 mL flask^{-1}) to monitor their AS production, and line 55-1 was determined to have the maximal productivity (13.12±2.36 mg flask^{-1}). Subsequent examinations of this culture line revealed that MS medium and its dilutions (×1/2 and ×1/4 strength) all provided suitable nutrient components for AS production and were better than the other concentrations tested ($p < 0.05$). It was observed that when the medium was supplemented with 60 g L^{-1} sucrose, better AS production was also achieved ($p < 0.05$) due to enhancement of root growth. Ethanol (EtOH; 9.5% (v/v)), a commonly used solvent for elicitor preparation, was determined to be inhibitory and caused a correspondingly low AS content when applied to the medium. A 24-h pulse treatment of methyl jasmonate (MeJA), dissolved in an appropriate concentration of EtOH and adjusted to a final concentration of 0~50 μM, was tested on a 28-d-old root culture and was noted to have had a non-significant effect on AS accumulation. Further investigation is necessary to define a better combination of pulse duration and elicitor concentration to improve AS production with this culture system.

Key words: acteoside (AS), hairy root, *Paulownia fortunei*, secondary metabolite, verbascoside.

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研究報告

培養泡桐毛狀根生產洋丁香酚苷

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摘 要

試管內培養的泡桐(*Paulownia fortunei* Hemsl.)實生苗接種農桿根群菌(*Agrobacterium rhizogenes*, BCRC 15011 (ATCC 43057))之後可誘導毛狀根(hairy root)發生並生產洋丁香酚苷(acteoside, AS)。先從泡桐毛狀根群7-6、23-1及55-1號進行高產量篩選，其中以55-1號培養在MS液體培養基(50 mL flask^{-1})，有最高產量(13.12±2.36 mg flask^{-1})。後續以該根群進行之研究顯示，MS培養基或將其濃度稀釋1/2或1/4，皆能有效提供培養基中之營養成分供毛狀根生長並產製AS，且均優於受測之其它濃度($p < 0.05$)。在不同蔗糖濃度影響AS生產的試驗中，含有60 g L^{-1}蔗糖的培養基促進了根群的生長，因此可獲得最佳的AS產量。乙醇(ethanol; EtOH)為配製多種誘引因子常用之溶劑，試驗顯示乙醇劑量達9.5% (v/v)時有抑制AS產量的結果。以適量的乙醇為溶劑配製甲基茉莉酸(methyl jasmonate; MeJA) (0~50 μM)成為誘引因子時，在根群培養至第28天時給予24 h的短暫刺激，對AS的生產並未形成顯著的影響。未來可將此誘引刺激的時間及濃度做進一步的調整，以改善從毛狀根生產AS的情形。

關鍵詞：洋丁香酚苷(AS)、毛狀根、泡桐、二次代謝物、毛蕊花苷。

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INTRODUCTION

When acteoside (AS) was first isolated from *Verbascum sinuatum* L., it was given the name “verbascoside”. The chemical structure of this compound was elucidated by Birkofer et al. (1968), who extracted it from the violet flowers of *Syringa vulgaris* L. and introduced the new name of “acteoside”. This compound was identified as a phenylpropanoid glycoside and was later extracted from many other plant species, e.g., *Plantago* spp. (Jensen et al. 1996, Franzyk et al. 1998, Li et al. 2005), *Verbena officinalis* L. (Calvo et al. 1997), *Picconia excelsa* (Ait.) DC. (Damtoft et al. 1997), *Faradaya* spp., *Oxera* spp. (Grayer and de Kok 1998), *Fraxinus ornus* L. (Iossifova et al. 1999), *Olea europaea* L. (Ryan et al. 1999, Owen et al. 2003), *Clerodendrum trichotomum* Thunb. (Hsien 2001), *Teucrium chamaedrys* L. (Avula et al. 2003), *Cistanche tubulosa* (Schenk) Wight (Chen et al. 2005), *Eremostachys glabra* Boiss. (Delazar et al. 2005), and *Dolichandrone serrulata* (DC.) Seem. (Sinaphet et al. 2006). However, in most of the aforementioned documented reports, only detection of this compound in the respective examined plants was described and no further application was pursued.

AS exhibits antioxidant and free radical-scavenging properties (Chiou et al. 2003) as well as having antihypertensive (Ahmad et al. 1995), hepatoprotective (Lee et al. 2004, Morikawa et al. 2010) and neuroprotective attributes (Sheng et al. 2002, Tsai 2006, Wang et al. 2009). It was also determined that AS presents antiviral (Kernan et al. 1998), antiproliferative (against lymphocytic mouse leukemia L-1210 cell growth) (Herbert et al. 1991), and immunosuppressive (Kimura et

al. 1987) properties. All of this means that the possibility of commercial production of AS is worth intensive study.

Advanced callus or cell-suspension culture methods have been used to maintain target plant cells in the rapid proliferative phase. The increased cell mass can be used as a source for AS extraction (Ellis 1983, Dell et al. 1989, Inagaki et al. 1991, Nezbedova et al. 1999, Skrzypek and Wysokinska 1999, Lu and Mei 2003, Chen et al. 2007, Estrada-Zuniga et al. 2009). In addition to studies where plant materials were collected from proliferating cells or calli, AS production is also possible from hairy-root culture (Wysokinska and Rozga 1998, Fons et al. 1999, Nishikawa et al. 1999, Kovacs et al. 2004, Dhakulkar et al. 2005, Grabkowska et al. 2010).

The "hairy root" is a genetically modified organ derived from *Agrobacterum rhizogenes*-mediated gene transformation. It has a high growth rate under *in vitro* conditions with no need for incubation with plant growth regulators (PGRs). This criterion is important, even crucial, when one is considering a culture system to produce valuable secondary metabolites for pharmaceutical or nutraceutical purposes. Another advantage derived from hairy roots is that the root cultures are usually able to produce the same metabolites found in the wild-type counterparts of the same plant species, without the loss of concentration frequently seen in callus or cell-suspension cultures (Kim et al. 2002).

Based on the above-mentioned circumstances, the present report deals with *in vitro* studies establishing hairy-root culture of *Paulownia fortunei* Hemsl. for mass AS production. Culture conditions (parameters) that influenced AS production were briefly examined, providing some basic information for possible application of this system in a scaled-up experiment. Tolerance to solvent concentrations applied for elicitor preparation and elicitation by methyl jasmonate (MeJA) were also investigated for their feasibility of manipulating AS accumulation in stressed cultures.

MATERIALS AND METHODS

Hairy-root culture preparation

Hairy-root cultures (lines 7-6, 23-1, and 55-1) were obtained from our prior study (Liao and Jhuang 2007). They had been subcultured every 4~6 wk for 4 yr on agar-solidified, PGR-free MS medium (Murashige and Skoog 1962) (Fig. 1A). In the current study, the experimental materials were prepared by excising sections of a hairy-root tip (ca. 2.0 cm) and then transferring them into MS liquid medium (Fig. 1B). The procedures applied for this liquid culture are further described below and for simplicity defined as "standard culture procedures" (SCPs). A 250-mL Hinton's flask (horizontally baffled across the bottom) was used in the procedures as the culture vessel. It contained 50 mL of liquid MS medium (pH 5.8; 0.3% (w/v) sucrose) for root growth. The flasks were shaken at 80 rpm on a rotary shaker and incubated at 21±1℃ with a 16-h photoperiod under cold white fluorescent light (TFC HSU-GUANG, FL40D/38, Taipei, Taiwan) mixed with incandescent light (PT. Philips, GLS A55; 100W/E27, Sidoarjo, Indonesia) at 33.3 μmol m^{-2} s^{-1}. Hairy roots were subcultured on the same medium every 21 d to increase the biomass which was used as the stock material for subsequent experiments. The same SCPs were followed throughout the entire study unless otherwise stated.

Confirmation of the genetic transformation

An examination was first carried out to confirm that the hairy-root cultures which

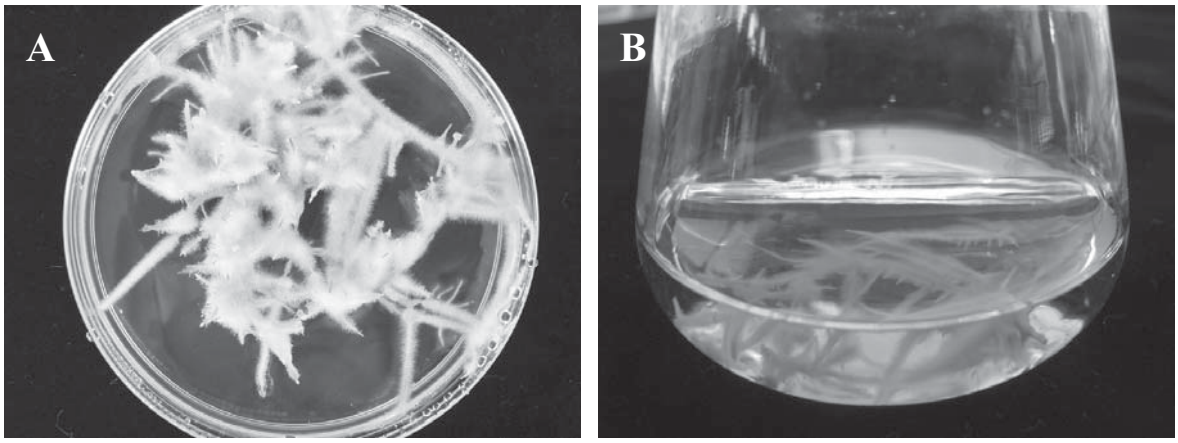

Fig. 1. Hairy-root culture (line 55-1) of *Paulownia fortunei* maintained *in vitro* for 4 yr (A) and root tip sections inoculated in MS liquid medium for preparation of stock material (B).

had been genetically transformed by *A. rhizogenes* (BCRC 15011; (ATCC 43057) purchased from the Food Industry Research and Development Institute, Hsinchu, Taiwan) still included the inserted tDNA after such a long period of time. Bacterial plasmid DNA was extracted utilizing a commercial extraction kit (High-speed Plasmid Mini Kit, Geneaid, Sijhih, Taiwan) as a positive control. Another extraction kit (Plant Genomic DNA Mini Kit, Geneaid) was used for extracting genomic DNA from 3 hairy-root lines and an *in vitro* grown (non-infected) root sample of a paulownia seedling (used as a negative control). All these DNA samples were subjected to polymerase chain reaction (PCR) processing to amplify the tDNA *rol*C gene fragment with primers of 5'-CTGTACCTCTACGTCGACT-3' and 5'-TCAGTCGAGTGGGCTCCTTG-3' (Hosokawa et al. 1997). The DNA extraction process was carried out according to instructions provided by the kit manufacturer, and the PCR conditions and gel electrophoresis methods followed procedures described by Liao and Jhuang (2007). The PCR-amplified products were further isolated from the gel for DNA sequencing. Sequence data were compared with the NCBI database to provide evidence of genetic transformation events.

Measurement of hairy-root growth and AS accumulation among culture lines

Root-tip sections were excised form SCP-incubated stocks of culture lines 7-6, 23-1, and 55-1 (0.5 g per inoculum) before being transferred into separate 250-mL flasks. The subsequent incubation processes were as described in the SCPs. However, the growth (increase in biomass) of the root cultures and their internal AS accumulation were determined after 35 d of incubation (without subculturing). The basic measurements of hairy roots included the fresh weight (FW) increase (mg flask^{-1}), dry weight (DW) increase (mg flask^{-1}), AS content (mg (g DW)$^{-1}$) and overall AS production (mg flask^{-1}). The best culture line (55-1), was determined by measuring the overall AS production, and was used for subsequent experiments.

Selection of medium strength

The same inoculation method as described in the preceding section was followed for incubation of hairy-root line 55-1, which also proceeded according to SCPs.

The strength of the MS medium was adjusted to 5 levels (×2, ×1(control), ×1/2, ×1/4, and ×1/8) to determine the most suitable concentration for supporting root growth and AS accumulation. Root cultures were grown for 35 d (without subculturing), and basic measurements were carried out as described above. The best medium strength (×1/2) thus determined was then applied in subsequent experiments.

Time-course changes in root growth and AS accumulation

Kinetic studies on root growth and AS accumulation in the hairy-root line 55-1 were carried out by incubating the cultures in 1/2 MS liquid medium for a 35-d culture period, with monitoring at 7-d intervals. SCPs were followed, and basic measurements were taken each sampling day. Information provided by this experiment showed that AS production entered a stationary phase after day 28; therefore, the culture period was extended to no longer than this stage in subsequent experiments.

Selection of sucrose concentration

Hairy-root line 55-1 was incubated according to SCPs with different amounts of sucrose (60, 30, 15, 7.5, and 3.75 g L^{-1}) supplemented as the carbohydrate source in 1/2 MS liquid medium for 28 d. These cultures were then collected for basic measurements. The experimental results provided evidence of which sucrose concentration was superior, and a level of 60 g L^{-1} was used thereafter.

Determination of the ethanol (EtOH) level for elicitor preparation

Combining all optimized variables determined in the above experiments, hairy-root line 55-1 was incubated in 1/2 MS liquid medium (with 60 g L^{-1} sucrose) for 28 d under SCPs. The entire root mass was collected from the flask and blotted dried to remove any residual medium. The root mass was then aseptically transferred to a Petri dish atop 5 Whatman No. 1 filter pieces (sterilized). Five milliliters of 1/2 MS liquid medium (with 60 g L^{-1} sucrose) with the addition of 95% (v/v) EtOH was filtered and aseptically poured into the dish for a 24-h pulse treatment. The final EtOH concentrations were adjusted to 0, 0.0475, 0.190, 0.475, 4.75, and 9.5%. Basic measurements were taken after this pulse treatment. The inhibitory concentration (9.5%) of EtOH was determined and eliminated from future applications.

MeJA elicitation

The same culture preparation method as described for the EtOH determination test was again applied in this experiment. However, the addition of 5.0 mL of the EtOH-containing medium to the Petri dish was replaced by the same volume of 1/2 MS liquid medium supplemented with a MeJA solution for culture elicitation. The final concentrations of MeJA were 0, 2.5, 5.0, 10, 20, and 50 μM prepared using 95% EtOH as the solvent, and the final EtOH concentration was adjusted to 1.9%. The MeJA-containing medium (5 mL) was also (0.2-μm) filter-sterilized and added to the dish for a 24-h pulse treatment after which basic measurements were taken.

AS extraction and high-performance liquid chromatographic (HPLC) analysis

At the end of the each experiment, hairy roots grown in the flask or treated in the Petri dish were collected for an HPLC analysis. They were first weighed (FW measurement) and then oven-dried at 70℃ to reach a constant weight (DW measurement). Samples were then ground up into a fine powder before being extracted 3 times (15 min for each

extraction) in methanol (MeOH; 3×15 mL). The extracts were combined and adjusted to 50 mL with MeOH, before being filtered through a 0.2-μm filter and injected into the HPLC system.

The HPLC system was equipped with a pump (L-7100, Hitachi, Tokyo, Japan), a reverse-phase C-18 column (250×4.6 mm i.d., 5 μm particle size; Hypersil Gold, ref. no. 25005-254630, Thermo, Waltham, USA), and a UV detector (330 nm; Waters 484, Milford, USA). Gradient elution of the moving phase for fraction separation was programmed to maintain a constant flow rate of 1.0 mL min^{-1} with MeOH (solvent A) and H_2O containing 1.0% acetic acid (solvent B). The gradient profile with the following proportions of solvent A was applied (T_{min}, %A): (0, 24), (8, 27), and (25, 80).

To determine the quantity of AS in the MeOH extracts, a calibration curve (y = 11730x – 12247; R^2 = 0.9999) of AS responses (area readings of the AS-specific peak), correlated to the reference AS concentrations of 1, 5, 10, 25, 50, 100, 250, and 500 ppm, was first established. The AS peak of the MeOH extracts was separated and identified by comparing the retention time with the reference compound (Fig. 2), which was kindly provided by Prof. Tung-Hu Tsai (National Yang-Ming Univ., Taipei, Taiwan) and prepared as a 50% MeOH solution.

Statistical analysis

In this present study, a completely randomized design was applied for all experiments. The experiments were repeated twice with each treatment covering 5 bottles of hairy-root cultures (replicates). The initial hairy-root sample (0.5 g FW) was placed into the culture vessels for different incubation periods, depending on the treatment designs. Basic measurements were then taken on specified sampling days or at the end of the experiments (28 or 35 d). Data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's new multiple-range test. The HPLC analysis data of methanolic extracts from every single vessel, with each measurement done in triplicate, were calculated and averaged to obtain the AS content (mg (g DW)$^{-1}$) and overall production (mg flask^{-1}).

RESULTS

Confirmation of the genetic transformation

The PCR-amplified products presented clear evidence verifying genetic transformation of all hairy-root lines by *A. rhizogenes*. The amplified *rolC* gene fragment (ca. 1100 bp) was obtained from the bacteria plasmid and lines 7-6, 23-1, and 55-1, but not detected in the non-infected control (Fig. 3). Further comparison of the nucleotide sequence of amplified fragments with the *rolC* gene sequence obtained from the NCBI database demonstrated a 99% similarity (accession no. K03313.1). The results revealed that the hairy-root lines had truly been genetically transformed because the inserted tDNA was still present after 4 yr.

Hairy-root growth and AS accumulation among culture lines

Hairy-root cultures incubated in MS liquid medium for 35 d exhibited different growth rates. The DW and FW increased more in line 55-1 ($p < 0.05$), recorded at 260 ±2 mg and 5.12±0.6 g flask^{-1}, and the lowest increase in biomass was for culture line 7-6 (Fig. 4). However, there was no corresponding increase in the amount of AS accumulation with growth-rate changes. The greatest AS content was obtained from line

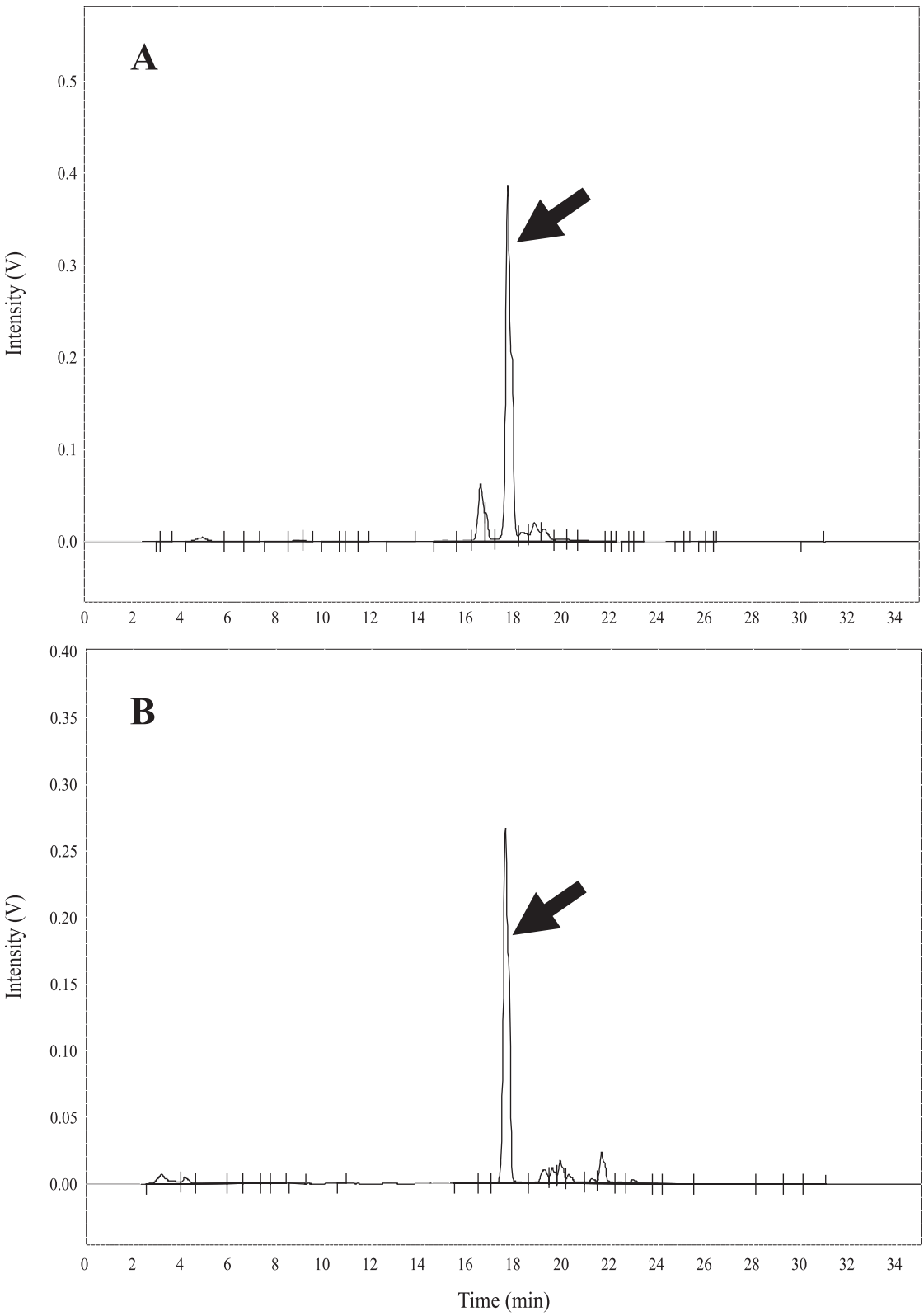

Fig. 2. HPLC chromatograms of acteoside (AS) standard (A) and purified methanolic extracts of hairy-root culture 55-1 (B); detector responses vs. retention times (min) are shown.

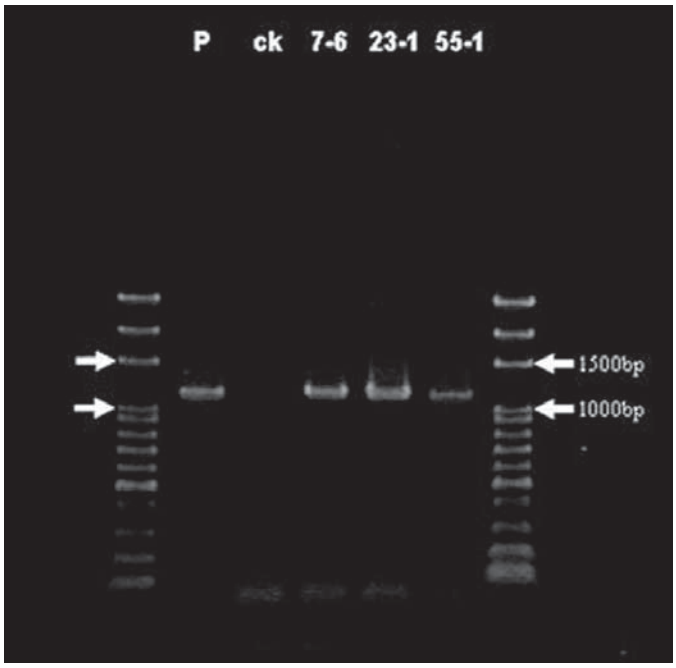

Fig. 3. Polymerase chain reaction-amplified DNA fragment of *rol C* genes from plasmid DNA of *Agrobacterium rhizogenes* (lane P); hairy-root cultures derived from *A. rhizogenes* transformation of *Paulownia fortunei* (lane 7-6, 23-1, and 55-1). Lane ck is the negative control where the DNA template was extracted from non-infected plant roots.

23-1 (103.72±10.53 mg (g DW)$^{-1}$), not from line 55-1. It should be noted that when the AS content and changes in the biomass were both considered, a better overall AS production was obtained from line 55-1 (13.12±2.36 mg flask^{-1}) (Fig. 4). This culture line was therefore selected for subsequent experiments.

Selection of medium strength

In the experiment conducted to select the medium strength, significant inhibition of hairy-root growth was observed when the MS medium was prepared at double (×2) strength (Fig. 5). Similarly, when the medium strength was diluted from ×1 (the original formula) to ×1/2, ×1/4, and ×1/8, the increase in biomass of hairy-root line 55-1 slowed down in a concentration-dependent manner. Interestingly, the AS content increased in a manner inversely proportion to the gradual depression of root growth, showing an opposite trend of variation due to medium dilution (Fig. 5). Following the above-mentioned AS measurement with equal consideration of both variables (root growth and AS content), better AS productions were obtained from ×1, ×1/2, and ×1/4 MS medium-cultured roots ($p < 0.05$), with recorded yields of 24.55±2.02, 29.34±1.97, and 26.27±2.05 mg flask^{-1}. The results of this experiment showed that 1/2 MS medium was the most suitable strength for routine use in the following studies.

Time-course changes in growth and AS accumulation

Hairy-root line 55-1 was cultured in 1/2 MS liquid medium for 35 d and showed a rapid increase in the root biomass during the first 7~28 d, followed by a stationary stage lasting for the remainder of the culture period (Fig. 6B). This was apparently an indicator of the occurrence of limiting conditions in the

culture system. However, such a variation was not seen during monitoring of the AS content (as indicated by the solid bars in Fig. 6B). The AS content remained relatively constant during the entire culture period, while overall AS production actually increased (Fig. 6A)

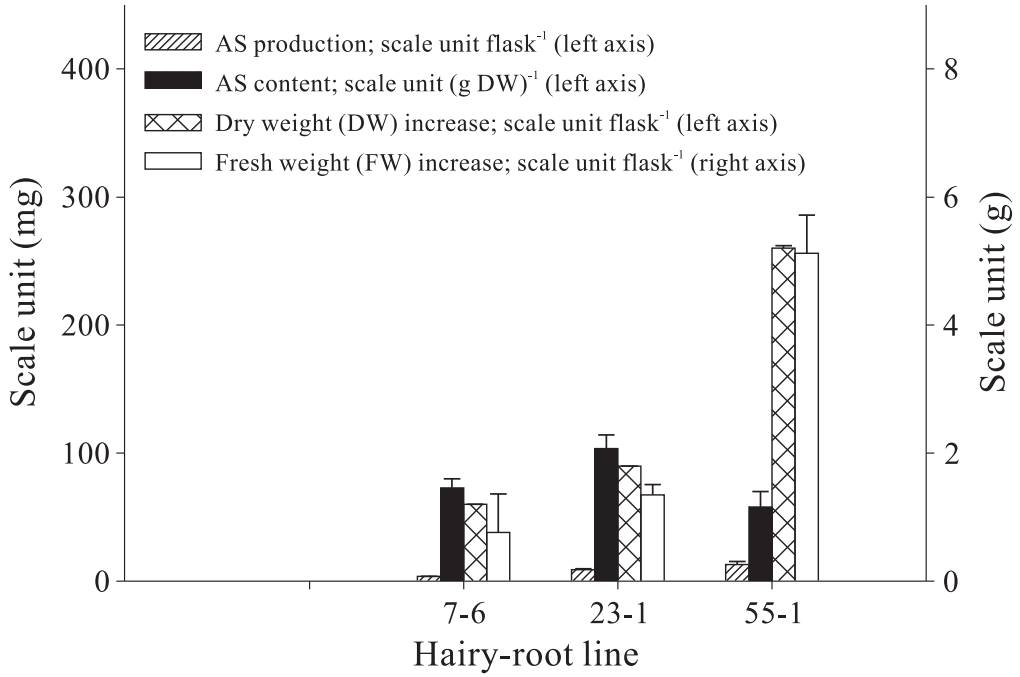

Fig. 4. Root growth and acteoside (AS) accumulation recorded from the three hairy-root lines of *Paulownia fortunei*.

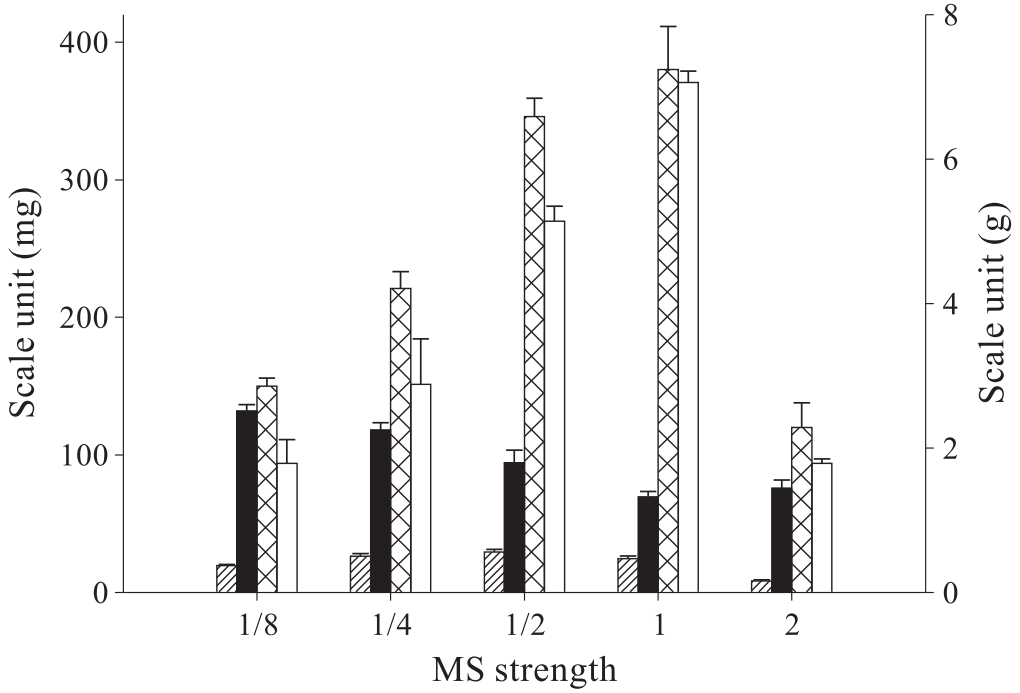

Fig. 5. Root growth and acteoside (AS) accumulation recorded from hairy-root line 55-1 incubated in different concentrations of MS medium. See Fig. 4 for histogram symbols.

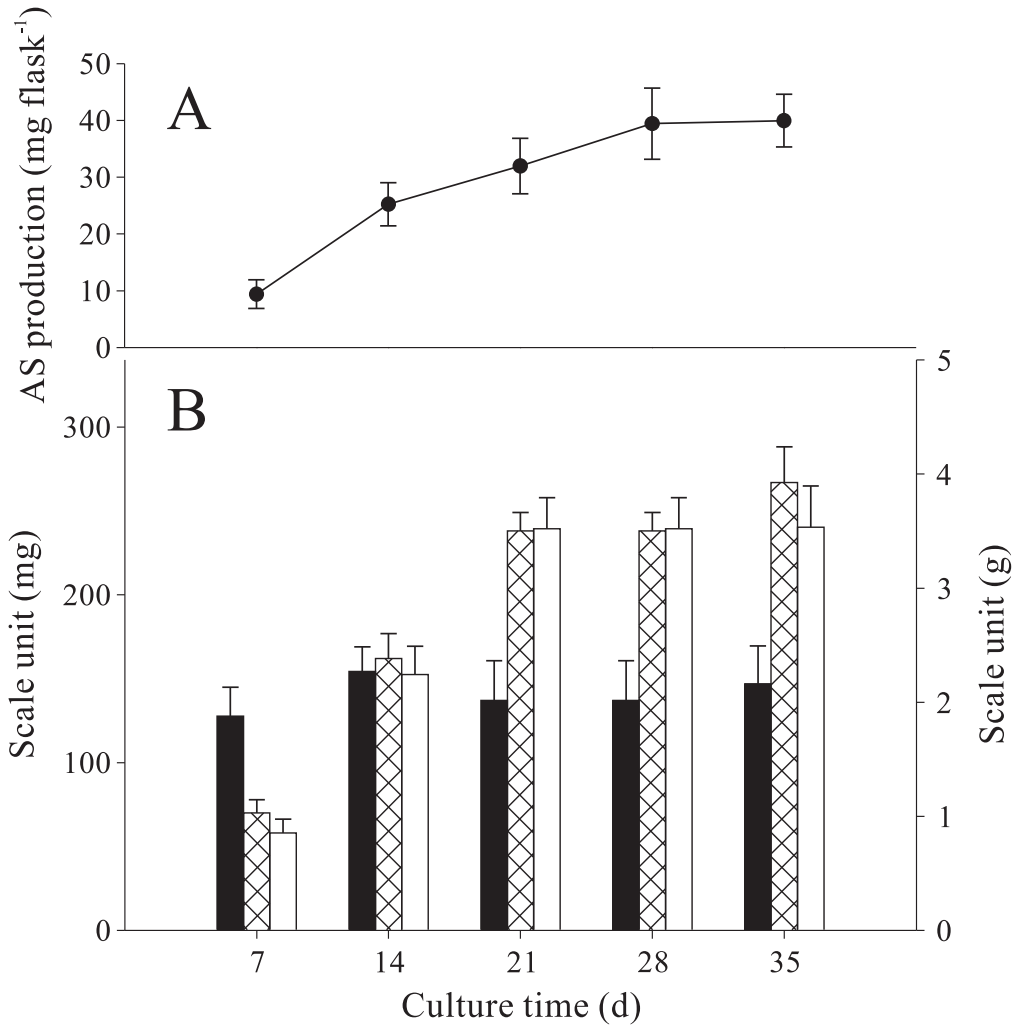

Fig. 6. Kinetic changes in root growth and acteoside (AS) accumulation in hairy-root line 55-1 incubated in 1/2 MS medium for a 35-d period. Stationary phase of AS production (A) and root biomass increase (B) observed on day 28. A relatively constant recording of AS content (solid bars in B) was obtained during the culture period. See Fig. 4 for histogram symbols.

showing a growth-associated pattern. Since we saw no further increase in AS production after day 28 (Fig. 6A), it was determined appropriate to terminate the culture period at that point.

Effects of sucrose addition on the biomass increase and AS production

The effect of the sucrose level on manipulating root growth was examined next. It was shown that the root biomass decreased as the sucrose concentration was gradually diluted. Obviously, a high sucrose concentration (30 or 60 g L^{-1}) was essential to provide a sufficiently large carbohydrate source to support the rapid growth of hairy-root culture. However, in contrast to changes in the root biomass, the AS concentration remained relatively constant, regardless of the sucrose level being tested (Fig. 7). It was determined that the overall AS production was better when the sucrose level was 60 g L^{-1}, while less was

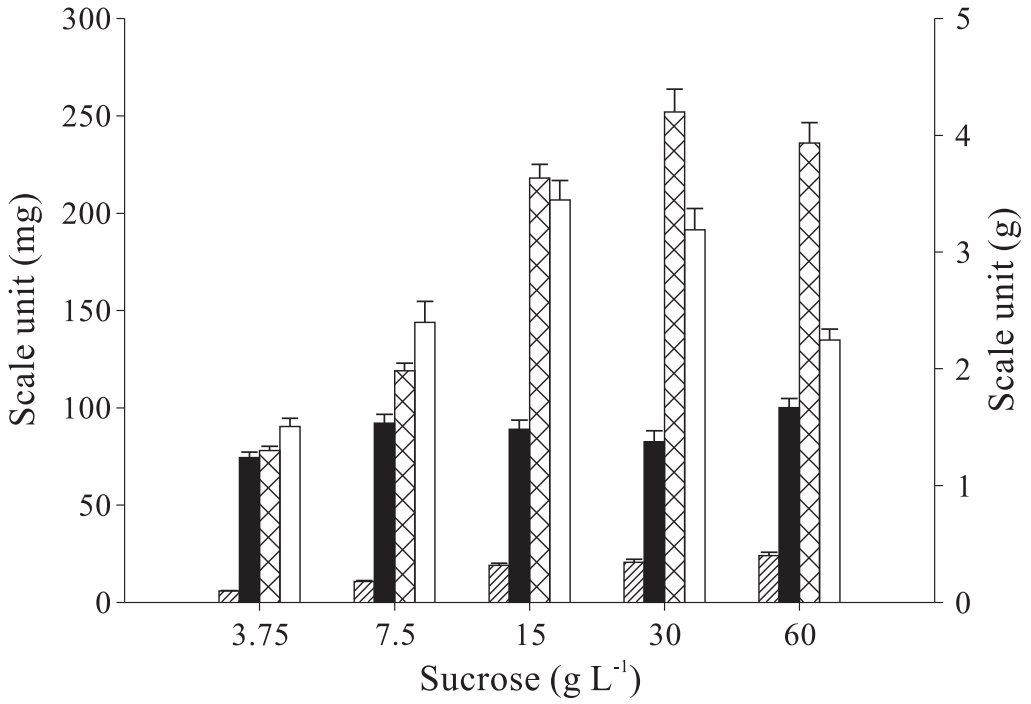

Fig. 7. Effects of sucrose concentration on root growth and acteoside (AS) accumulation in hairy-root line 55-1. See Fig. 4 for histogram symbols.

produced when the sucrose concentration was diluted.

Determination of the proper EtOH level

The application of different doses of EtOH to the hairy-root cultures had an inhibitory effect which was subsequently observed in the AS content measurement ($p < 0.05$). However, increases in the DW and FW were not significantly influenced ($p > 0.05$) (Fig. 8). Given this background, the addition of EtOH at 0.19~4.75% was determined to be harmless, and eventually better AS production was achieved, compared to the EtOH dose of 9.5% which resulted in the lowest AS content.

AS production under MeJA elicitation

There was no distinct elicitation effect of MeJA on promoting of AS production in our experiments. Although there was a slight decrease in the AS content when the MeJA concentration was gradually increased, the overall AS production per flask was not significantly affected (Fig. 9). For all MeJA concentrations we tested, there was no sign of repression of root growth either, which indicated that the MeJA dosage and the EtOH (solvent; 1.9%) level added were both below the threshold that would damage cultured roots. This was also a clue indicating that the impact of elicitation we provided was insufficient to alter physiological conditions of the cultures.

DISCUSSION

In the present study, hairy-root cultures were demonstrated to be a very stable plant material as far as the genetic transformation event was concerned. These root cultures had been transformed and were routinely subcultured on PGR-free MS medium for 4 yr. There was no doubt that the inserted wild-type *rol* genes remained and were expressed. These cultures maintained their transformed

phenotype without loss of the easy-growth characteristic. Their genetically modified nature meant that the hairy roots were suitable for plant tissue culture operations.

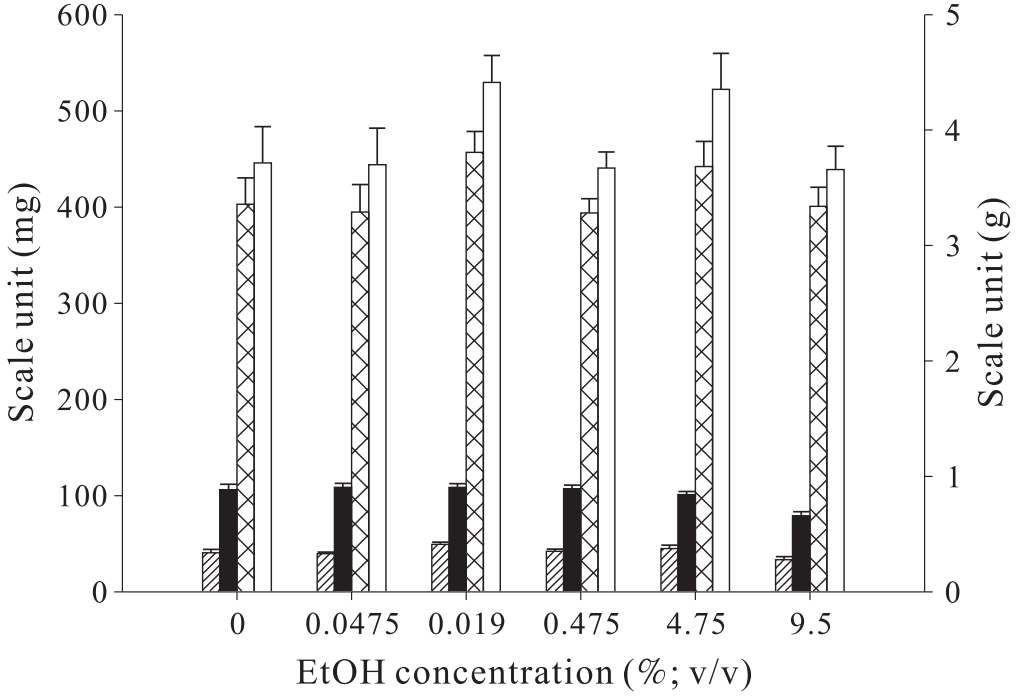

Fig. 8. Root growth and acteoside (AS) accumulation recorded from hairy-root line 55-1 incubated in EtOH-containing medium to test the tolerance of the root culture to the solvent additive. See Fig. 4 for histogram symbols.

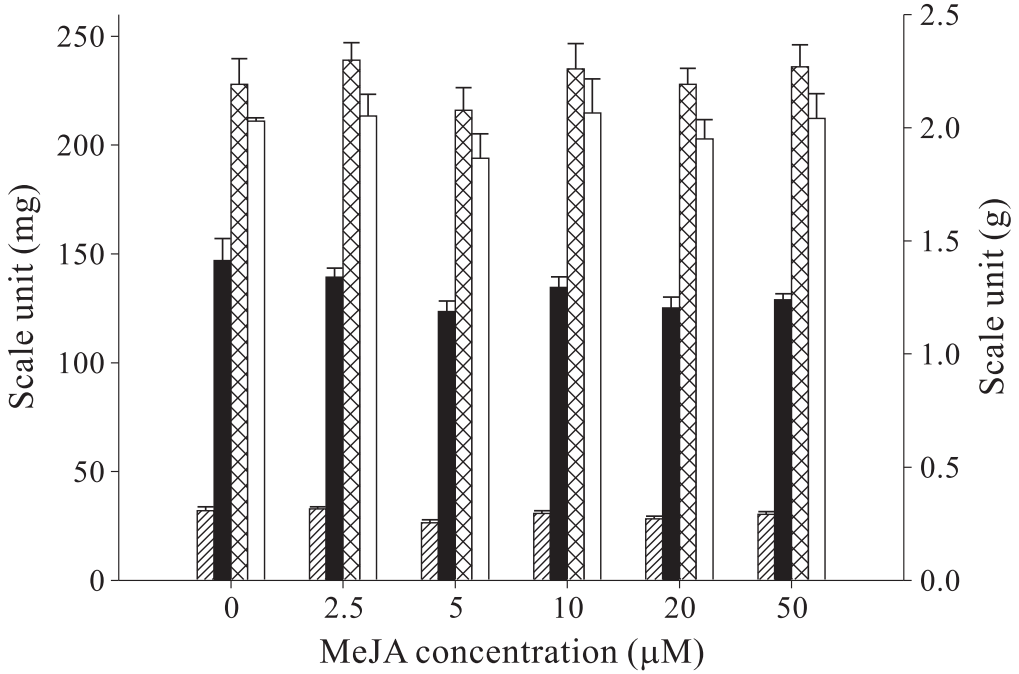

Fig. 9. Root growth and acteoside (AS) accumulation in hairy-root line 55-1 after 24-h pulse elicitation of methyl jasmonate (MeJA). See Fig. 4 for histogram symbols.

During the selection process, as observed in our study, the measurement units (mg (g DW)$^{-1}$) commonly used for calculating secondary metabolite accumulation was less indicative of the total production of plant material. In the first experiment conducted to select the hairy-root line which would give the greatest AS production, the lowest AS content recorded on a mg (g DW)$^{-1}$ basis was from line 55-1. However, the low AS content was obviously compensated for by the rapid biomass increase as the AS production reached a maximal value in the same culture line (Fig. 4). Therefore, it is not recommended that the AS production from plant sources be monitored in mg (g DW)$^{-1}$ units as the only basis for selection, especially for a fast-growing culture line (55-1), even though the internal concentration of the target compound was low. Both the plant biomass and AS content per unit biomass should be taken into account (e.g., measurements should be made per culture vessel basis). This would present more-accurate information to evaluate AS production. This conclusion is in agreement with results of Chang (2005), in which that author also selected a fast-growing culture line (GP-01, *Gynostemma pentaphyllum* (Thunb.) Makino) for use as experimental material.

It was demonstrated in the 2nd experiment that the medium strength (richness of the nutrients) played an important role in manipulating hairy-root growth. However, it seems that increasing the medium strength only promoted root growth rather than overall AS production. The low AS production was due to an associated reduction in the AS content extracted from roots grown in the higher-strength medium (Fig. 5). This unsynchronized and seemingly contradictory phenomenon, observed only in this experiment, can be explained as being due to the root-culture's fast-growing characteristics, where the constant production of AS would be equally distributed in tissues with a greater biomass. This allocation should inevitably lower the AS content per (g DW).

Interestingly, such a phenomenon was not seen in either the time-course change experiment (Fig. 6) or the sucrose determination test (Fig. 7). In both these experiments, as the culture period was prolonged or sucrose concentration increased, the AS content remained relatively stable when the root biomass gradually increased. To maintain such a stable concentration in the culture system, there was an apparent increase in overall AS production in the growing roots. Similar results were reported elsewhere, although measured on a mg (g DW)$^{-1}$ basis, where the production of a secondary metabolite might be considerably growth-associated, showing a linear or synchrotic relationship between the yield and biomass production (Ellis 1983, Inagaki et al. 1991, Nezbedova et al. 1999). However, opposite conclusions were also reached when addressing a non-growth-associated relationship, where the highest yield was obtained during the stationary phase of growth (Bhadra and Shanks 1997). Obviously such observations are species- or culture environment-dependent and require detailed examinations of each newly established and selected hairy-root culture before any final conclusions are made.

Since culture elicitation is now a common and effective approach to stimulate plant material to produce greater amounts of secondary metabolites, certain organic elicitors are routinely used and co-cultured with plant cells. These compounds (elicitors) might not all be water-soluble and hence need specific organic solvents for preparation. In plant tissue culture procedures, the proper application of an organic solvent to the culture system is a considerably important researchers' concern to prevent the cultured plant materials from

being damaged. Therefore, we first tried to determine the tolerance of our hairy-root line to the EtOH dose for a follow-up elicitation test in which an EtOH-soluble elicitor was investigated. The result showed that a dramatic decrease ($p < 0.05$) in AS production was observed as the EtOH was added at a final concentration of 9.5% (v/v). This reduction was mainly due to a decrease in the AS content (Fig. 8). For all other concentrations tested, there was no great change in the remaining measurements, especially in root-growth data. This result provides us with a wide range of EtOH concentrations which can be used, since different kinds of elicitors (which may have different solubilities in EtOH) may be needed for future experiments.

MeJA, an EtOH-soluble elicitor, is a commonly used compound to enhance secondary metabolite production under experimental conditions. We initially tried a higher dose of this elicitor (50~300 μM) for a long duration (7 d), but observed a significant decrease in AS production in our root cultures (data not shown). However, the data of growth and AS production showed no obvious influence when the dosage during elicitation treatments was relatively low (< 50 μM, see Fig. 9). Based on these experimental results (Fig. 9) with similar elicitation responses ($p > 0.05$), we therefore concluded that the cultures would have a high probability of being able to sustain stronger MeJA elicitation. A more-effective MeJA elicitation is expected to have a stimulation impact of > 50 μM (24 h) but less than the intensity previously applied in our preliminary tests (50~300 μM, 7 d).

Taking the units (mg (g DW)$^{-1}$) as a standardized estimation for comparison, although not recommended in this study, our maximum AS concentration was at 146.88±10.12 mg (g DW)$^{-1}$, as seen in Fig. 9. This is greater than the concentrations extracted from hairy roots of *Paulownia tomentosa* Steud. (9.5% of their dry weight; equivalent to 95 mg (g DW)$^{-1}$) (Wysokinska and Rozga 1998), *Plantago lanceolata* L. (15 mg (g DW)$^{-1}$) (Fons et al. 1999), *Scutellaria baicalensis* Georgi (30 mg (g DW)$^{-1}$) (Nishikawa et al. 1999), *Gmelina arborea* Roxb. (0.13 mg (g DW)$^{-1}$) (Dhakulkar et al. 2005), and *Harpagophytum procumbens* (Burch.) DC. ex Meisn. (8.12 mg (g DW)$^{-1}$) (Grabkowska et al. 2010). However, the amount was still less abundant than that obtained in some other studies dealing with different species of plant materials (Nezbedova et al. 1999, Skrzypek and Wysokinska 1999). Our results suggest that maintaining or enhancing the AS content in root cultures is a basic requirement to contribute to increasing overall AS production. A proper culture procedure which supports rapid root growth is another crucial factor in improving the metabolite yield. The proper adjustment of the elicitation impact (elicitor type and intensity) is a possible approach to meet the first requirement. The use of enriched sucrose as a carbohydrate source may be a solution for supporting rapid growth in hairy roots. The applicability of both strategies for better AS production, from our paulownia hairy roots, is worth further investigation.

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