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Hairy Root Cultures of *Taxus cuspidata* for Enhanced Production of Paclitaxel

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Hairy root cultures were established by transforming seedlings of *Taxus cuspidata* Sieb. Et Zucc (Korean yew) using *Agrobacterium rhizogenes*. Initially, 107 lines of hairy roots were induced by infection with 3 strains of *Agrobacterium* (R1000, A4, and 15384), however, only three lines generated from the R1000 strain were stably grown on hormone-free media over 10 months of successive cultures. These lines were designated as RC11104, RC11105 and RC11106. The hairy root line RC 11106 was selected for further experiments for taxol production based on its growth properties. Upon methyl jasmonate treatment, the RC11106 line accumulated 52.5 mg/L of taxol over 2 weeks of incubation at a 20-L culture scale.

Key words: Agrobacterium rhizogenes, *bioreactor, hairy root, methyl jasmonate, taxol, Taxus* cuspidata *Sieb. et Zucc*

Paclitaxel, commonly known as Taxol, a registered trademark of BMS (New York, NY), was originally isolated from the Pacific yew tree *Taxus brevifolia* [Wani *et al*., 1971]. It is a natural diterpenoid alkaloid with anti-cancer activity due to the promotion of microtubule assembly and the stabilization of microtubules against depolymerization [Schiff *et al*. 1979]. Since first approved by the US Food and Drug Administration for the treatment of refractory ovarian cancer in 1992, paclitaxel has been widely used as an effective chemo-therapeutic agent against various cancers, among others breast, non-small cell lung, and head and neck [Rowinsky and Donehower, 1995]. In addition, effectiveness of paclitaxel on the treatment of other diseases, e.g. kidney disease [Woo *et al*., 1994] and rheumatoid arthritis [Arsenaulta *et al*., 1998] has also been reported.

Paclitaxel was first isolated from the bark of the yew tree, which grows very slowly and contains only about 0.01% (dry weight of the bark) of paclitaxel [Vidensek *et al*., 1990], thus limiting its supply. Although a chemical method for total synthesis of paclitaxel was developed later, the method was not practical for industrialization [Holton *et al*., 1994a; Holton *et al*., 1994b; Nicolaou *et al*., 1994; Danishefsky *et al*., 1996] due to the complex steps of the synthesis reactions. As an alternative, paclitaxel was semi-synthesized from its natural precursor, 10-deacetylbaccatin III, which was extracted from the yew tree needles [Castor and Theodore, 1993]. In addition, microorganisms such as the taxol-producing fungi *Taxomyces andreanae* [Stierle *at al*., 1993] and *Taxodium distichum* [Li *et al*., 1996] were explored for the production of paclitaxel; however, the yield was too low to be commercially viable.

As a promising alternative for efficient production of paclitaxel and its related taxane compounds, cell cultures of various *Taxus* species have been explored [Christen *et al*., 1989; Kim *et al*., 1995; Srinivasan *et al*., 1995;

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Abbreviations: HPLC, high pressure liquid chromatography; IBA, indole butyric acid; mB5, modified Gamborg B5; MJ, methyl jasmonate; MPM, mycelia of the pine-mushroom; MS, Murashige Skoog; NAA, naphthalene acetic acid; PCR, polymerase chain reaction; SA, salicylic acid; SH, Schenk and Hildebrandt; WPM, woody plant medium; YEP, yeast extract peptone

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Cusido *et al*., 2002; Zhang *et al*., 2004]. However, the cell suspension culture was hampered by problems in maintaining the productivity of the cell lines over successive cultures.

Recently, attention has been rapidly moving toward the application of hairy root cultures for the production of many valuable secondary metabolites [Xu *et al*., 2008]. Hairy roots are adventitious roots induced at the infected sites following infection by *Agrobacterium rhizogenes* [David *et al*., 1984]. Their phenotypes are characterized by relatively fast growth independent of the exogenous hormone, lateral branching, loss of geotropism, and biochemical and genetic stabilities [Guillon *et al*., 2006]. Nonetheless, very few reports have been published on the *Taxus* hairy root cultures for the production of paclitaxel and the related compounds. In fact, we found only one report on the hairy root cultures of *Taxus* x *media* var. *Hicksii* Rehd. [Furmanowa and Syklowska-Baranek, 2000].

Here, we report on the establishment of the hairy root cultures derived from *T. cuspidata* Sieb. Et Zucc. and the optimal conditions for improved production of paclitaxel. Three strains of *A. rhizogenes* (R1000, 15384, and A4) were compared for the hairy root induction from *T. cuspidata* Sieb. et Zucc. in combination with different infection methods. Through successive sub-cultures over a 10-month period, a hairy root line showing stable and fast growth was selected for the production of paclitaxel in a bioreactor culture system. This hairy root produced a high level of paclitaxel in response to the methyl jasmonate (MJ) elicitation, suggesting that it could be used as an alternative source for the viable production of paclitaxel.

Materials and Methods

Bacterial strains and plant materials. *Agrobacterium rhizogenes* strains ATCC 15834, R1000, and A4 were grown on a yeast extract peptone (YEP) medium in the dark at 28°C. The mature seeds of *T. cuspidata* were collected from the trees at the So-Baek Mountain in Korea during the period from early September to late November, 2005. For the surface sterilization, the seeds were washed for 30 min under running tap water, immersed in 70% ethanol for 3 min, soaked in 2% NaOCl for 30 min, and finally washed three times with sterilized distilled water. For germination, the whole sterilized seeds or the embryos extracted from the sterilized seeds by dissection were mounted on a solid woody plant medium (WPM) medium [McCown and Lloyd, 1981] containing 3% sucrose and 0.15% gelrite at 25°C in a dark room for 3 weeks.

***Agrobacterium* infection for hairy root induction.** *A. rhizogenes* infection was carried out on 3-week-old *Taxus* seedlings, 1-2 cm in length, by the following methods: a direct infection method, in which the seedlings were wounded by a scalpel loaded with *A. rhizogenes* cultures; a liquid co-culture method, in which the seedlings were wounded and co-cultured with *A. rhizogenes* for 24 h in a liquid medium; a solid co-culture method, in which the seedlings were wounded and transferred onto the solid culture of *A. rhizogenes*, followed by 24 h incubation.

Hairy root formation. After the *Agrobacterium* infection, the seedlings were transferred onto a fresh WPM medium containing 500 mg/L cefotaxime and sub-cultured weekly onto the same medium until no signs of *Agrobacterium* growth were detected. Subsequently, the plantlets were sub-cultured for hairy root induction every 3 weeks on a fresh WPM medium lacking cefotaxime and plant growth regulators. The cultures were maintained in the dark for the first 3 weeks, and then the condition was changed to a 16 h/8 h light/dark cycle at 25°C. The sub-cultures were performed for 10 months to obtain three lines of the stable hairy roots (RC11104, RC11105, and RC11106).

Molecular analysis of hairy roots. The three hairy root lines (RC11104, RC11105, and RC11106) were verified for *Agrobacterium* transformation by polymerase chain reaction (PCR) analysis of the genomic DNA. Genomic DNA was isolated from the hairy roots using the standard method [Dellaporta *et al*., 1983]. The primers for PCR were designed to detect the *rol A* gene present on the pRi plasmid in *A. rhizogenes*: the forward primer (rol A-F), 5'-GGAATTAGCCGGAC TAAACG-3', and the reverse primer (rol A-R), 5'-CCGG CGTGGAAATGAATCG-3'. The PCR was performed in a volume of 25 mL containing 100 nM primers, 0.2 mg DNA, and 2 units of *Taq* polymerase (Elpis Biotech, Daejeon, Korea) for 40 cycles (94°C for 1 min, 52°C for 1min, and 72°C for 2 min), followed by a final elongation at 72°C for 10 min. The PCR products were visualized by 1% agarose gel electrophoresis.

Culture media and bioreactors. Four media with different compositions were tested to determine the optimum growth medium for the best-performing hairy root line RC11106: Schenk and Hildebrandt (SH) [Schenk and Hilderbrandt, 1972], WPM [McCown and Lloyd, 1981], Murashige Skoog (MS) [Murahige and Skoog, 1962], and modified Gamborg B5 (mB5) [Gamborg *et al*., 1968] media. Sucrose was added to each medium at 3%(w/v) as a carbon source, and gelrite was used at 1.5%(w/v) as a solidifying agent for the solid medium. For the large scale liquid culture, air-lift type bioreactors with 10 L total volume were used for both 3- and 5-L cultures.

Elicitations for paclitaxel production. The hairy roots were cultured in a WPM medium containing indole butyric acid (IBA) (1 mg/L) with 3% sucrose. Different elicitors were applied to the 6-week-old cultures. MJ was dissolved to 0.1 M in dimethylsulfoxide and added to the medium at a final concentration of 100 μM. Salicylic acid (SA) was dissolved to 0.1 M in 50% ethanol and added in the medium at a final concentration of 150 μM. Mycelia of mycelia of the pine-mushroom (MPM) obtained from *in vitro* cultures were air-dried and added to 0.1 % (w/v) in the media. A polystyrene resin (Diaion HP20) was also added at a final concentration of 0.2% (v/v) in the medium as an adsorbent of paclitaxel and taxanes excreted into the medium. The hairy roots were then harvested, dried, and extracted to quantify paclitaxel and its analogues.

Extraction and analysis of paclitaxel. Two weeks after elicitation, the hairy roots were harvested, oven-dried, and extracted for paclitaxel. Briefly, the dried roots were extracted with 100% MeOH using a Soxhlet extractor for 24 h at 65°C. The extract was filtered through Whatman No.1 filter paper, vacuum-dried, and re-dissolved in 20% MeOH. This solution was then subjected to the high pressure liquid chromatography (HPLC) analysis.

HPLC analyses were performed to quantify paclitaxel and its analogues using a reverse-phase μ-Bondapak C18 column (particle size 5 μm; 4.6×250 mm; Phenomenex, Torrance, CA) on the Agilent 1100 system (Agilent Technologies, Santa Clara, CA). The mobile phase (MeOH:MeCN:H_2O=20:40:40) was pumped isocratically at 1 mL/min, and the paclitaxel and its analogues were detected by a UV-VIS detector at 227 nm.

Results and Discussion

Transformation of *Taxus cuspidata* for hairy root induction. Because the *T. cuspidata* seeds were recalcitrant to germination, the embryo cultures were used to generate the seedlings (Fig. 1). For hairy root induction, 3-week-old seedlings of *T. cuspidata* were infected with different *A. rhizogenes* strains, including ATCC 15834, R1000, and A4, via three different methods (Table 1). *Agrobacterium* R1000 and 15834 strains successfully transformed the seedlings to induce the hairy roots by all three methods, whereas the A4 strain failed to generate the hairy roots regardless of the method used. These results suggested that there may be specificity to certain *A. rhizogenes* strains for the successful transformation of *T. cuspidata*. Previously, the *A. rhizogenes* strain, LBA 9402, was used to transform the plantlets of *Taxus* × *media* var. *Hicksii* for the hairy root induction

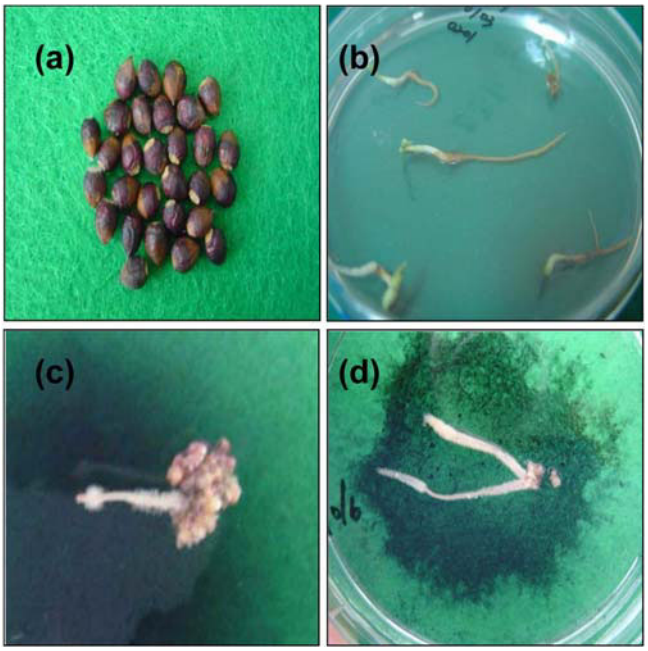

Fig. 1. Induction of hairy root from the seedlings of *T. cuspidata* Sieb. Et Zucc. (a), *T. cuspidata* seeds used in embryo culture; (b), the generation of the seedlings; (c), a newly induced hairy root from the *A. rhizogenes* infection; (d), 4-week-old hairy roots.

[Furmanowa and Syklowska-Baranek, 2000]. These hairy roots were different from the normal *T. cuspidata* in the number of adventitious roots and their shape (Fig. 2). They grew even in the absence of IBA, one of the auxin hormones, and contained lots of lateral branches.

The method of *Agrobacterium* infection also affected the transformation rate. Specifically, the direct infection method generated hairy roots at the highest rate, with approximately 26% of seedlings giving rise to hairy roots. Nonetheless, most new hairy roots obtained from these transformations did not grow into competent roots in the successive cultures, suggesting a possible gene silencing of the *rol* transgene as reported previously [Sinkar *et al*., 1988].

Characterization of the hairy root lines. After the induction of hairy roots, approximately 100 lines were successively sub-cultured on fresh WPM media every month for stabilization over a 10-month period. During the successive cultivation, however, the majority of the hairy root lines failed to grow stably and eventually lost their growth ability completely. Only three lines of hairy roots, designated as RC11104, RC11105, and RC11106, showing relatively fast and stable growth, were obtained (Fig. 3). Interestingly, they were all derived from the *Agrobacterium* R1000 transformations.

In a hormone-free solid culture growth on WPM medium, the three hairy root lines differed clearly in the

Table 1. Effects of different bacterial strains and infection methods on hairy root formation in *Taxus cuspidata*

Bacterial strains and transformation methods		Numbers of infected seedlings	Average days of hairy root induction	% of hairy root formation
R1000	Direct infection[a]	254	30.0± 1.5	26.4
	Liquid co-culture[b]	111	62.5±17.9	3.6
	Solid co-culture[c]	45	21.0± 0.0	2.2
15834	Direct infection	102	24.1± 2.5	25.5
	Liquid co-culture	67	32.4± 8.5	7.5
	Solid co-culture	36	19.8± 4.8	11.1
A4	Direct infection	100	Dried and dead	0
	Liquid co-culture	50	Dried and dead	0
	Solid co-culture	50	Dried and dead	0

[a]Direct infection was performed by wounding the 3-week-old seedlings with scalpels loaded with *A. rhizogenes* cultures and incubation for 24 h.
[b]For liquid co-culture, the 3-week-old seedlings were first wounded and then incubated in *A. rhizogenes*-growing liquid media for 24 h.
[c]Solid co-culture was performed by placing the wounded 3-week-old seedlings on the solid culture of *A. rhizogenes* before co-incubation for 24 h.
A. rhizogenes strains R1000, 15834, and A4 were used.

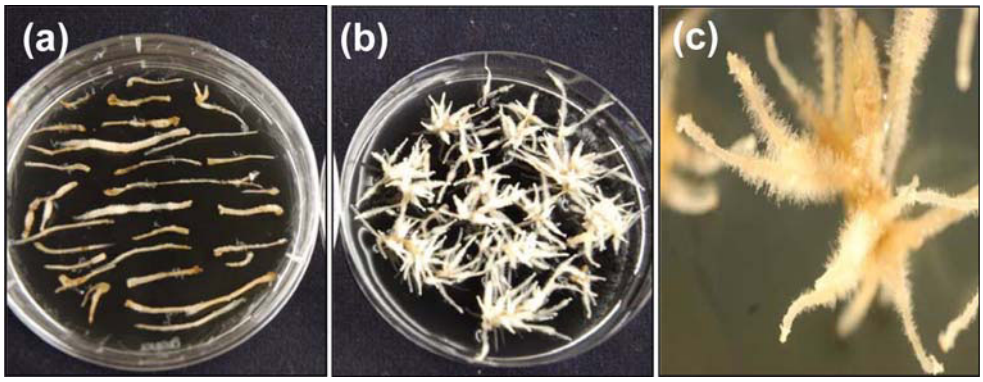

Fig. 2. Comparison of untransformed and transformed roots of *T. cuspidata* Sieb. Et Zucc grown on WPM media lacking exogenous hormones. Significant difference was observed in the growth between untransformed roots (a) and hairy roots (b). (c), magnified hairy roots showing massive adventitious roots.

growth pattern as well as in the growth rate (Table 2 and Fig. 3a). Over an incubation period of 1 month, the roots of the RC11104 line grew longest but thinnest with branching at a median rate (average of 13.4 branches per root), whereas the RC11106 line grew relatively thicker and shorter with branching at the highest rate (average of 35.4 branches per root). The RC11105 line branched at the lowest rate (average of 3.9 branches per root); however, it appeared to grow robustly for 60 days without showing any apparent aging phenotypes, in contrast to the other two lines that began to age after 45 days of incubation. PCR analysis revealed the presence of the *rolA* gene in the genomic DNAs of the three hairy root lines, but not in the untransformed root, verifying the genetic transformations of the three lines by *A. rhizogenes* R1000 (Fig. 3).

Growth conditions for the hairy roots. The RC11106 line was chosen for further characterization to optimize the growth conditions by analyzing the growth in all media and the hormonal effects. Compositions of the four media including MS, SH, mB5, and WPM lacking plant hormones were evaluated. In the solid culture, the root growth of RC11106 was the highest on the WPM medium based on the measurement of fresh weight increase of 19.3-fold during incubation for 2 months. In the mB5 medium, the hairy roots appeared to be slightly softened while growing most poorly (5-fold increase of fresh weight). The hairy roots on the SH medium grew very thin with red color, and in MS medium the colors and the appearances of the roots were good, but the overall growth rate (14.5-fold increase of fresh weight) was the second among the tested media.

Table 2. Growth evaluation of the three newly-established hairy root lines (RC11104, RC11105, and RC11106)

Hairy Root Lines	Average root lengths (cm)	Average numbers of root branches	% increase of the fresh weights
RC11104	3.95±0.38	13.4±1.9	1937
RC11105	2.93±0.28	3.9±0.7	1232
RC11106	2.50±0.14	35.4±4.8	1812

For comparison, the hairy roots were chopped into ~1 cm rootlets without branches, transferred onto the hormone-free solid WPM media, and incubated for 1 month in the dark at 25°C.

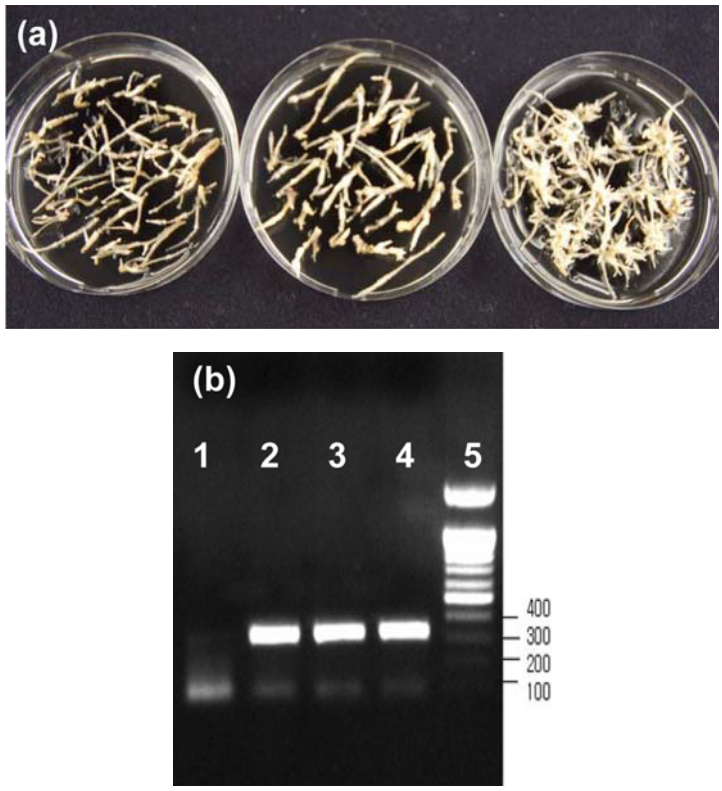

Fig. 3. Molecular verification of the hairy root lines and the growth of the three different hairy root cell lines on hormone-free media. (a) left, RC 11104; middle, RC11105; right, RC11106. (b), gel picture of PCR showing successful transformation of the roots of *T. cuspidata* Sieb. Et Zucc by *A. rhizogenes*. PCR was performed on genomic DNA from the taxus seedlings with the primers for *rolA* gene in *A. rhizogenes.* Lane 1, PCR with untransformed *T. cuspidata* seedlings; lane 2, PCR with the transformed hairy root lines RC 11104; lane 3, RC 11105; lane 4, RC 11106; lane 5, 1 kb plus ladder from Invitrogen. PCR products sized 329 bp are detectable only in lanes 2, 3, and 4.

To investigate the combinatorial effects of the plant hormones and the growth media on the growth of RC11106, different combinations of IBA and naphthalene acetic acid (NAA) were tested with various media. The addition of IBA at different concentrations ranging 1 to 5 mg/L had little effect on the growth of the hairy roots, whereas at NAA concentration over 1 mg/L, the root growth was significantly inhibited (Fig. 4).

Taxol production by the treatment of elicitors. Different elicitors including MJ, SA, and MPM were added to the 6-week-old cultures of RC11106 hairy roots to evaluate their effects on the production of taxol. Taxol production by the RC11106 line was greatly induced in the hairy roots treated with MJ. However, the treatments with SA and MPM did not affect the taxol production in the hairy roots as compared with the negative control (Fig. 5).

Upon MJ treatment for 14 days, the total content of taxol reached to 52.56 mg/L, a 23.7-fold increase from that of the day 1. Taxol was detected in the control, SA-treated, and MPM-treated RC11106; however, the amounts were almost negligible compared to the MJ-treated hairy roots. These results are in accordance with the previous experiments, in which the MJ treatment showed the most efficient elicitation effect for taxol production in the cell suspension cultures of *Taxus*

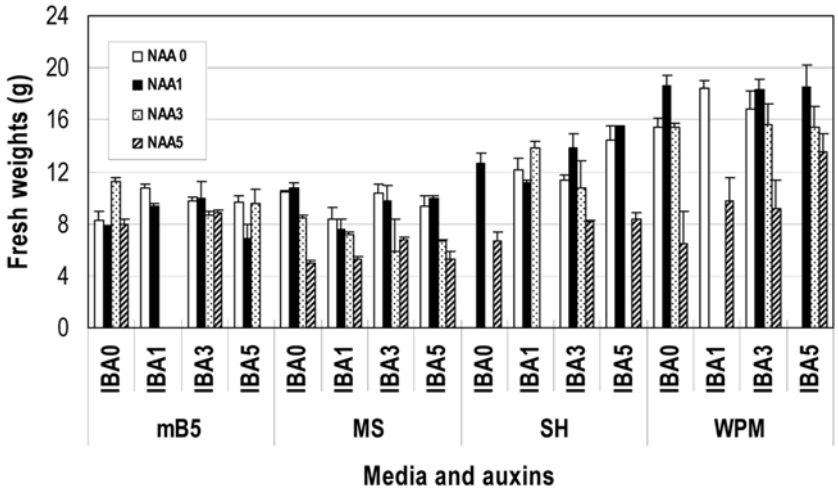

Fig. 4. Combinatorial effects of the auxin hormones IBA and NAA in various media on the growth of the hairy root culture RC11106. Before harvest, the hairy roots of 0.5 g (fresh weight) were incubated for 1 month on solid WPM, SH, MS, and mB5 media with different concentrations of IBA and NAA.

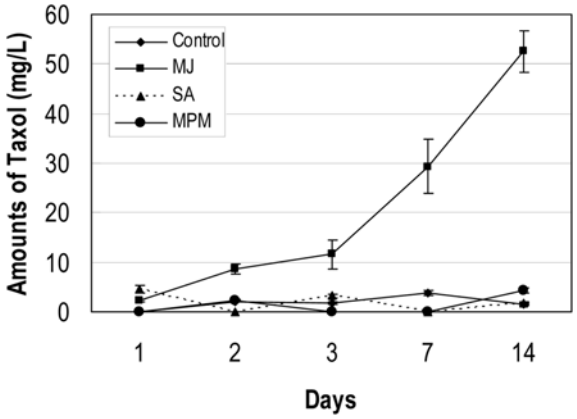

Fig. 5. Elicitation for taxol production. MJ (100 μM), SA (150 μM), and MPM (0.1% w/v) were added to the 6-week-old liquid cultures of the hairy root line RC11106, composed of WPM media supplemented with 1 mg/L of IBA. After 2 weeks, the hairy root cultures were harvested, dried, and extracted to determine the contents of taxol in the dried roots by HPLC analysis.

[Mirjalili and Linden, 1996; Yukimune *et al*., 1996; Ketchum *et al*., 1999].

In conclusion, the stable hairy root cell lines with high taxol content were successfully established from *T. cuspidata* by *A. rhizogenes* transformation. So far, to the best of our knowledge, there has been only one report on the generation of hairy roots from the *Taxus* genus, *Taxus* × *media* var. *Hicksii* [Furmanowa and Syklowska-Baranek, 2000]. Our newly established hairy root lines could be used to investigate the biosynthetic pathways of taxol and its derivatives in *T. cuspidata*.

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