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RESEARCH ARTICLE

Plumbagin production by root cultures of *Plumbago rosea*

Pharkphoom Panichayupakaranant *
Department of Pharmacognosy and Pharmaceutical Botany
Faculty of Pharmaceutical Sciences
Prince of Songkla University
Hat-Yai, Songkhla, 90112, Thailand
Tel: 66 74 428220
Fax: 66 74 428220
E-mail: ppharkph@ratree.psu.ac.th

Supinya Tewtrakul
Department of Pharmacognosy and Pharmaceutical Botany
Faculty of Pharmaceutical Sciences
Prince of Songkla University
Hat-Yai, Songkhla, 90112, Thailand
Tel: 66 74 428220
Fax: 66 74 428220
E-mail: supinyat@yahoo.com

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Root cultures of *Plumbago rosea* Linn. were established from young leaf explants on solid Gamborg's B5 (B5) medium supplemented with the combination of α-naphthalene acetic acid (NAA) and kinetin in the concentration ranges of 0.5-2.0 mg/l and 0.1-0.5 mg/l, respectively. The production of plumbagin, determined by TLC-densitometry was higher [0.016 ± 0.0030% dry weight (DW)] in cultured roots obtained from B5 medium supplemented with 1.0 mg/l NAA and 0.1 mg/l kinetin. Plant selection increased the plumbagin production to 0.129 ± 0.0139% DW, while variation of sucrose and nitrogen (as $(NH_4)_2SO_4$) concentration in B5 media slightly increase the plumbagin synthesis to 0.023 ± 0.0017 and 0.020 ± 0.0015% DW, respectively.

Plumbagin (2-methoxy-5-hydroxy-1,4-naphthoquinone) is a natural naphthoquinone possessing various pharmacological activities *i.e.* antimalarial (Likhitwitayawuid et al. 1998), antimicrobial (Didry et al. 1994), anticancer (Parimala and Sachdanandam, 1993), cardiotonic (Itoigawa et al. 1991) and antifertilityaction (Bhargava, 1984). At present, the most exploited source of plumbagin are the roots of *Plumbago* spp. (*Plumbago europea, P. rosea, P. zeylanica*). However, these plants grows quite slowly and it takes long time until the roots are suitable for use (Kitanov and Pashankov, 1994). Therefore, it is advisable to search for alternative sources of plumbagin. Previous experiments to obtain this quinone *in vitro* showed that plumbagin was best synthetized and accumulated in *Plumbago zeylanica* (Heble et al. 1974) *Drosophyllum lusitanicum* (Nahalka et al. 1996), *Drosera natalensis*, *D. capensis* (Crouch et al. 1990) and *Drosera gigantea* (Budzianowski, 2000). The aim of this work was to establish root cultures of *Plumbago rosea*. Medium manipulation and high yielding plant selection were also studied to increase plumbagin production.

Materials and Methods

Plant material and chemicals

Plumbago rosea plants were grown in the botanical garden of the School of Pharmaceutical Sciences, Prince of Songkla University, Thailand. All chemicals used were analytical grade and were purchased from Sigma (USA) or Merck (Darmstadt, Germany).

Plumbago rosea root cultures

Young leaves explants were used to obtain *Plumbago rosea* root cultures using the initiated cultures on solid B5 medium supplemented with a combination of NAA and kinetin in the concentration ranges of 0.5-2.0 mg/l and 0.1-0.5 mg/l, respectively. The root cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of liquid B5 medium supplemented with the same hormonal factors as the solid media. The flasks were incubated on a rotatory shaker at 25ºC, 120 rpm with a 16 h light (light intensity 1,000 lux) photoperiod. The root cultures were maintained

* Corresponding author

under these conditions and subcultured every 4 weeks.

Medium manipulation

The concentration of sucrose in liquid B5 media were 1, 2, 3 and 4%, while those of $(NH_4)_2SO_4$ were 0-, 0.5-, 1- and 2-fold of the initial concentration in the basal B5 medium.

Selection of high-plumbagin yielding plants

Extraction: fifty-seven rootsamples were separately collected from 2-years old *Plumbago rosea*plants.The roots were dried at 50ºC and 2.0 g of the dried powdered roots were extracted with 20 ml methanol under reflux for an hour. The extracts were filtered and adjusted to the volume of 1.0 ml with methanol. The concentration of plumbagin was determined spectophotometrically by densitometry as described below.

Determination of plumbagin production

The 30-day-old root cultures were harvested and extracted under reflux with methanol. Plumbagin content was determined using a TLC-densitometric method (Panichayupakaranant, 2001). Silica gel 60 F_{254} was used as a stationary phase and toluene:glacial acetic acid (99:1) was used as the mobile phase. The solvent system allowed a clear separation of plumbagin from other extract constituents. The identityof thecompound was confirmed by the obtained Rf value, co-chromatogrphy with an authentic sample in at least three different solvent systems and UV absorption spectrum. Plumbagin content in each crude methanol extract was determined by TLC-densitometry. Some 5.0 µl of the extracts was applied as the bands on silica gel 60 F_{254} TLC plates using an automatic spotter. After development in the above mentioned solvent system, absorbance was measured using a TLC densitometer. The conditions of the instrument were: UV detection, 254 nm; slit dimention 10.0 x 0.2 mm; monochromator band width 20 nm. The measurement was set to the mode of adsorption/reflection with scanning speed of 20 mm/sec. The area under the peaks of plumbagin was integrated and converted to concentration using its calibration curve. The calibration curve of plumbagin was established from an authentic sample at the concentration range of 0.062 - 1.0 mg/ml. and displayedas a linear equation of $Y = 3507.66X + 448.28$ ($r^2 = 0.9932$).

Time-course of growth and plumbagin production

The 30-day-old root cultures (2 g fresh weight) were transferred into 250 ml Erlenmeyer flask containing 50 ml of liquid B5 medium as described above. The cells were harvested every 6 - 7 days for 48 days. The dry weight was recorded after drying at 50ºC for 24 hours. The concentration of plumbagin was determined by TLC densitometry and is presented as percentage of plumbagin on the basis of dry plant material. To obtain growth and plumbagin production curves, the dry weight and plumbagin content of the root cultures were plotted against time. All the process was done in duplicate.

Results and Discussion

Plumbago rosea root cultures

Even plumbagin is mainly accumulated in the roots of *Plumbago rosea* (Evans, 1996), the root cultures were not established by the root explants, because the survival rate was very low due to high contamination, slow growth and development of the root explants. Thus, the young leaf explants were alternatively used to establish the root culture of *P. rosea*. The root organogenesis took place after 2-week period of the initiation. The white puffy roots were formed on the solid medium supplemented with the combination of NAA and kinetin in the concentration range of 0.5 - 2.0 and 0.1 - 0.5 mg/l, respectively. After that the color of the culture root turned to dark brown. The combination of 1.0 mg/lNAA and 0.1 mg/l kinetin was found to be bestsuited for growth promotion. Thus, the root cultures were maintained in liquid B5 medium supplemented with 1.0 mg/lNAA and 0.1 mg/l kinetin. The culture roots in liquid medium appeared as a small rosette aggregation. The root biomass increased about 18 times of the inoculated root within a month. The growth cycle of the root cultures during the period of 48 days showed that there was a period of 7 days for the lag phase followed by a rapid growth (exponential) and linear phase (Figure 1). There was a continuous increase in the biomass during 34 days. Thereafter, the dry weight of the biomass remained relatively constant indicating that the culture reached the stationary phase at day 41. The highest dry biomass was 0.83 g/250 ml-flask at day 41, about 21 times the biomass of the inoculated roots.

Plumbagin production by the root cultures

After a few subcultures, the culture roots were examined for their ability to produce plumbagin. TLC-densitometric method was used because this method is simple, rapid and without prior purification steps (Tewtrakul et al. 1992). It was found that the 4-week-old root cultures accumulated plumbagin at 0.016 ± 0.0030% DW. The root cultures of *Plumbago rosea* produced higher plumbagin concentration than those of *Drosera capensis* and *D. natalensis in vitro* cultivations [0.0004% Fresh weight (FW)] (Crouch et al. 1990) and the calluses of *Plumbago zeylanica* (0.0001 – 0.003% FW) (Heble et al. 1974). The concentration of plumbagin was, however, less than that of *Drosophyllum lusitanicum* suspension cultures (3.5% FW) (Nahalka et al. 1996). In addition, the present study found that the intact roots of the 3-year-old *Plumbago rosea* contain 0.32 -

1.16% DW of plumbagin. An attempt toincrease the plumbagin production was undertaken using medium manipulation and high yielding plant selection. It was found that B5 medium supplemented with 1% sucrose was able to increase plumbagin production in the root cultures. The growth of the culture root, however, decreased (Table 1). Taking into account the production per litre, the root cultures treated with 3% sucrose yielded higher plumbagin production. In contrast, the influence of $(NH_4)_2SO_4$ exhibited that 2-fold of $(NH_4)_2SO_4$ slightly increased plumbagin production but not affected the growth of cultured roots (Table 1). The effect of sucrose on increasing plumbagin production might be related to the retardation of the growth, while increasing $(NH_4)_2SO_4$ concentration or NH_4^+/NO_3^- ratio should be affected on the improvement of plumbagin production by the root cultures.

To select high plumbagin producing *Plumbago rosea* plants, the variation of plumbagin content was assessed in fifty-seven plant samples. The average yield of plumbagin in the root samples was 0.86 ± 0.186% DW. The highest and lowest yields were 1.16 ± 0.020 and 0.32 ± 0.026% DW, respectively. The high-yielding plant was selected and its leaves were used as an initial explant for the establishment of *P. rosea* root cultures in the conditions described above. It was found that the root culture initiated from high plumbagin producing plant produced 0.129 ± 0.0139% plumbagin DW. Although the roots under these conditions produced less plumbagin than the intactroots of *Plumbago rosea*, the growing time is shorter when compared to field growing plants. This study also indicated that the use of high producing plants for the initiation of tissue culture is the way to succeed in an increasing of secondary metabolite production by plant tissue cultures.

During the 48-day period of the culture growth, it was found that plumbagin was initially accumulated in the exponential phase (after day 7) and actively biosynthesized until reaching the late linear phase. The highest content of plumbagin was observed at day 34; after that the production rate began to slow down (Figure 1). This fact suggests that the biosynthesis of plumbagin took place at the same time that other primary metabolites used for growth promotion. This phenomenon is different from most secondary metabolite production, which usually takes place when the growth rate is declined.

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APPENDIX

Table

Table 1. Influence of sucrose and nitrogen (as (NH4)2SO4) concentration on the growth and plumbagin production of Plumbago rosea roots culture.

Experimental conditions	Biomass/litre (g fresh weight ± SEM)	Plumbagin content (% DW ± SEM)
Sucrose concentration (g/l)		
10.0	4.92 ± 0.5673	0.023 ± 0.0017
20.0	5.809 ± 0.5673	0.020 ± 0.0044
30.0 *	7.662 ± 0.5673	0.016 ± 0.0030
40.0	7.605 ± 0.5673	0.015 ± 0.0023
$(NH_4)_2SO_4$ concentration (mM)		
0.000	6.369 ± 0.5673	0.017 ± 0.0017
0.507	6.011 ± 0.8610	0.015 ± 0.0012
1.015*	6.001 ± 0.5243	0.016 ± 0.0030
2.030	6.567 ± 0.9557	0.020 ± 0.0015

*concentration used in basic B5 medium

Figure

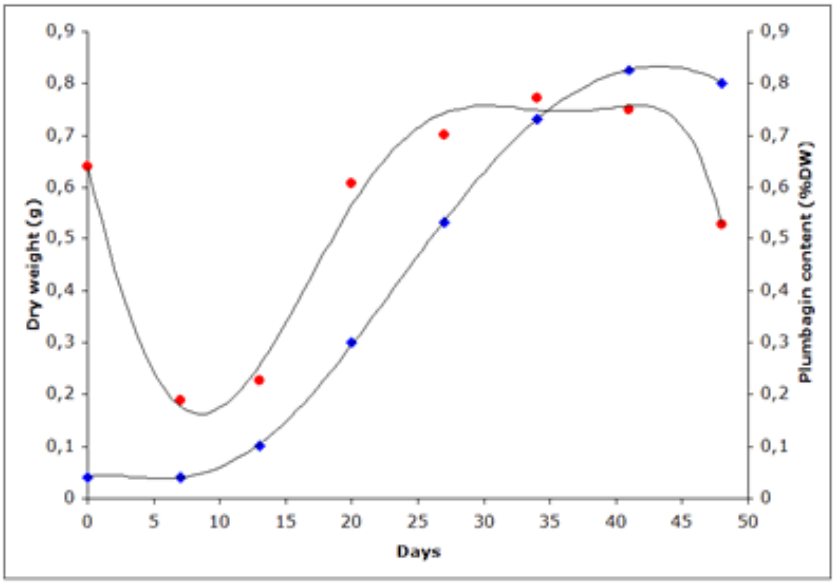

Figure 1. Time courses of growth (•) and plumbagin production (•) in P. rosea root cultures.