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Influence of Hormones and Selection of Stable Cell Lines of *Plumbago rosea* for Accumulation of Plumbagin

P. Komaraiah, G. Jogeswar, R. Naga Amrutha, P. Sri Laxmi, B. Lavanya, S.V. Rama Krishna, P.B. Kavi Kishor*

Department of Genetics, Osmania University, Hyderabad 500 007, India

Abstract

Callus and suspension cultures derived from leaf explants of *Plumbago rosea* were established on Murashige and Skoog's medium containing 1 mg/L IAA, 0.5 mg/L NAA and 0.3 mg/L BAP. Callus cultures were tested for their growth and accumulation of plumbagin, a naphthoquinone and was identified by ^{1}H NMR and electron ionization mass spectroscopy. While auxins (not 2,4-D) influenced growth and plumbagin accumulation, cytokinins did not influence them much. Increasing concentrations of IAA in presence of NAA and BAP increased plumbagin in suspensions only up to 1 mg/L. Growth of callus was optimum (8.3 g DCW/l) at a hormonal combination of 1.5 mg/L IAA, 0.5 mg/L NAA and 0.3 mg/L BAP, but high plumbagin accumulation (4.9 mg/g DCW) was recorded at 1.0 mg/L IAA plus 0.3 mg/L BAP. Since instability in growth and secondary metabolite accumulation was noticed, several cell lines/clumps of callus were screened for plumbagin accumulation by visual and analytical methods. Biomass and accumulation of plumbagin showed a negative correlation in several cell lines. But one cell line showed stability both in terms of biomass and plumbagin accumulation over a period of 6 months.

Key words: *Plumbago rosea*, plumbagin, cell lines, secondary metabolism

Introduction

Plumbago rosea L. is a perennial shrub of the family Plumbaginaceae and is highly endemic in nature. The most exploited source of plumbagin is the roots of *Plumbago* species (*P. europea, P. zeylanica* and *P. rosea*). Plumbagin is an important naphthoquinone, which has anticancer (Parimala and Sachdanandam 1993), antibacterial, antifungal (Didry et al. 1994) antimutagenic and insecticidal activities (Kubo et al. 1983). *Plumbago* species grow slowly and the roots suitable for extraction may take years to grow (Kitanow and Pashankov 1994). Frequent harvesting of the natural population resulted in the near extinction of *P. rosea*. Therefore, the search for alternative sources of plumbagin is necessary. Plant cell cultures are a good source of secondary metabolites. Cellular variation is an important factor, which controls the synthesis of secondary products and has immense potential in improving biosynthetic capacity of cell strains. Although cell selection resulted in the isolation of high yielding cell strains (Matsumoto et al. 1982), the major constraint is the stability of the selected cell line. Many of the cultures are known to exhibit instability in regard to the synthesis of the secondary products (Zenk et al. 1977). We report here the influence of plant growth regulators on the production of plumbagin in callus cultures of *P. rosea*. Also, an attempt was made to isolate a cell line that is stable in biomass and plumbagin accumulation.

Materials and Methods

Establishment of callus and suspension cultures and growth measurements

Leaf derived callus cultures of *Plumbago rosea* (synonymous *P. indica*) were established and maintained in 150 mL Erlenmeyer flasks containing 40 mL of Murashige and Skoog' s (MS) basal medium (1962) supplemented with 1 mg/L IAA, 0.5 mg/L NAA, 0.3 mg/L BAP and 3% sucrose (Komaraiah et al. 2001). All the cultures were maintained under cool white, fluorescent light with 12 h photoperiod (30 μE m^{-2}S^{-1}) at 25$\pm$2°C. Suspension cultures were initiated using 2 g of callus as inocu-

* Corresponding author, E-mail: pbkavi@yahoo.com
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lum in a 250 mL conical flask containing 60 mL of above medium without agar and they were agitated on a rotary shaker at 100 rpm. pH of the media was adjusted to 5.7 with 1 M NaOH before autoclaving. Callus was removed from the flasks and fresh weight was measured. Dry weight (with-10% moisture) was determined after drying the cells at 60°C until a constant weight was obtained. Suspension cultures were initiated using 2 g callus as inoculum in a 250 mL conical flask containing 60 mL of the above medium without agar and they were agitated on a rotary shaker at 100 rpm. Suspensions were collected by filtration and blotted with tissue paper to remove excess water and fresh weight was measured. Dry weight was determined after drying the cells at 60°C until a constant weight was obtained.

Selection of cell lines

Callus cultures (500 mg) from the stationary phase were inoculated onto 40 mL of agar medium containing 1 mg/L IAA, 0.5 mg/L NAA, 0.3 mg/L BAP and 3% sucrose. Small callus masses differing in their colour (pale yellow, yellow or deep yellow or brown, green etc.) were isolated and analyzed for plumbagin accumulation. The biomass and plumbagin accumulation pattern of several cell lines from single leaf derived callus were studied for a period of six months.

Determination of plumbagin

At the end of each experiment, callus was extracted three times with equal volumes of ethyl acetate. The pooled fractions were reduced under vacuum and the residues re-dissolved in 1 mL of HPLC mobile phase. The cells were extracted thrice with methanol in a porcelain mortar using acid washed neutralized sand. Methanol was reduced under vacuum and re-dissolved in 1 mL of HPLC mobile phase and quantified. Data shown are mean of 6 replicates and ± S.E. values are presented as error bars. Plumbagin content in independent samples was determined by HPLC using two Shimadzu LC 10 AD pumps; SPD 10 A UV-VIS detector, and the column used was μ Bondapak C18 (3.9×300 mm^2) with a detection wavelength of 254 nm. The mobile phase was methanol, water (80:20) with 0.1% trifluoroacetic acid and the peak area was calculated by comparing with an authentic sample of plumbagin obtained from the Sigma Chemical Company, USA.

Results

Influence of auxin combinations on growth and plumbagin production in callus cultures of *P. rosea*

The influence of MS medium containing different concentrations (0.5 to 1 mg/L) of indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) keeping the BAP concentration constant (0.3 mg/L) on callus growth and plumbagin accumulation is shown in the Table 1. Plumbagin was purified 99% using TLC method before determining the spectra. Identification of plumbagin was carried out by 1H-NMR (Figure 1), electron-ionization mass spectra with a retention time of 0:24 (Figure 2) and GC/MS spectrum with a retention time of 33.958 (Figure 3). Maximum fresh weight of callus as well as plumbagin accumulation was observed in the medium containing 1 mg/L IAA + 0.5 mg/L NAA (4.21 g/culture and 0.89 mg/g DCW respectively), while the least in IAA (0.5 mg/L) and 2,4-D (0.5 mg/L) containing medium (Table 1). IAA plus NAA combinations were found better compared to IAA + 2,4-D and 2,4-D + NAA. The effect of IAA, NAA along with BAP on biomass production and accumulation of plumbagin is

Table 1. Influence of auxin combinations on growth and plumbagin production in callus cultures of *P. rosea*

Hormones (mg/L)			Fresh wt. (g/culture)	Plumbagin (mg/g DCW)
IAA	2,4-D	NAA		
-	0.50	0.50	1.44 (±0.11)	0.22 (±0.43)
0.50	-	0.50	2.13 (±0.25)	0.31 (±0.11)
0.50	0.50	-	1.28 (±0.25)	0.19 (±0.21)
-	0.75	0.50	2.00 (±0.33)	0.24 (±0.21)
0.50	-	0.75	3.34 (±0.27)	0.51 (±0.34)
0.75	-	0.50	3.69 (±0.21)	0.79 (±0.19)
-	1.00	0.50	2.69 (±0.15)	0.57 (±0.29)
1.00	0.50	-	2.00 (±0.21)	0.21 (±0.17)
0.75	1.00	-	1.59 (±0.15)	0.21 (±0.24)
1.00	-	0.50	4.21 (±0.34)	0.89 (±0.35)
0.50	-	1.00	3.60 (±0.45)	0.79 (±0.41)
1.00	-	0.75	4.16 (±0.28)	0.84 (±0.13)
1.00	-	1.00	2.01 (±0.18)	0.39 (±0.17)

MS basal medium + 3% sucrose; Inoculum: 500±30 mg fresh tissue in 40 mL medium. Data represent an average value of 6 replicates and data in parenthesis indicate ± SD. BA; 0.3 mg/L was used for all the treatments.

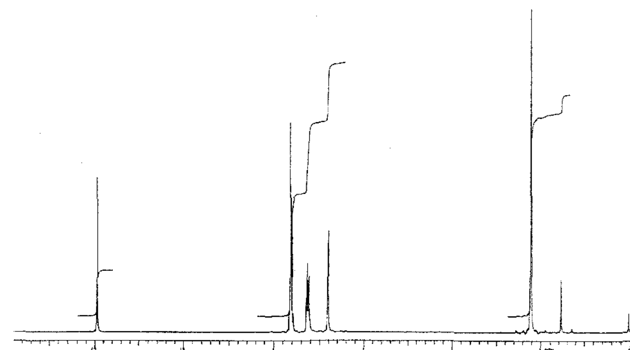

Figure 1. ^{1}H NMR spectrum of plumbagin

shown in the Table 2. While 1.5 mg/L IAA, 0.5 mg/L NAA and 0.3 mg/L BAP supported 8.35 g DCW/l callus growth, IAA (1.0 mg/L) and BAP (0.3 mg/L) combination resulted in the highest accumulation (4.92 mg/g DCW) of plumbagin. With drawl of BAP from the medium suppressed plumbagin (1.23 mg/g DCW) accumulation (Table 2).

Influence of cytokinins on growth and plumbagin production

Callus cultures initiated on MS medium containing 1 mg/L IAA, 0.5 mg/L NAA and 0.3 mg/L BAP were tested for their growth and plumbagin accumulation in presence of kinetin and BAP (Figure 4 and 5). All the media contained 1 mg/L IAA and 0.5 mg/L NAA in addition to the cytokinins. There was no significant difference in growth as measured in terms of fresh weight among calli grown in different concentrations of kinetin or BAP and also in plumbagin production.

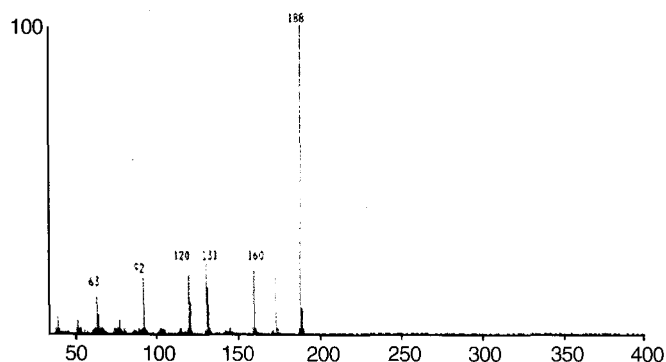

Figure 2. EI-Mass spectrum of plumbagin

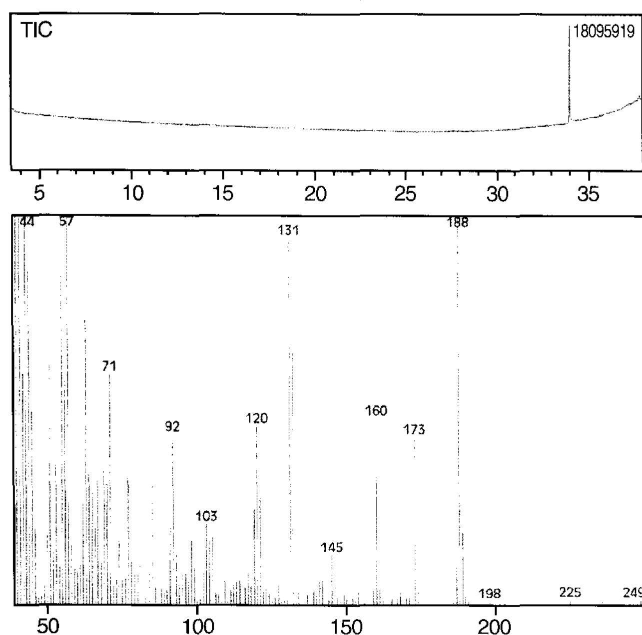

Figure 3. GC/MS spectrum of plumbagin

Stability of plumbagin accumulation through cell line selections

The pattern of biomass and plumbagin accumulation of cell lines from single leaf derived callus mass were studied for a

Table 2. Influence of phytohormones on growth and plumbagin production in cell cultures of *P. rosea*

Hormonal concentrations (mg/L)			Biomass (g DCW/l)	Plumbagin (mg/g DCW)
IAA	NAA	BAP		
0.0	0.5	0.3	4.25 (±0.51)	2.61 (±0.23)
0.5	0.5	0.3	5.81 (±0.41)	3.17 (±0.30)
1.0	0.5	0.3	7.79 (±0.31)	3.97 (±0.39)
1.5	0.5	0.3	8.35 (±0.24)	2.93 (±0.26)
2.0	0.5	0.3	6.43 (±0.64)	1.69 (±0.17)
1.0	0.0	0.3	5.19 (±0.36)	4.92 (±0.38)
1.0	1.0	0.3	7.13 (±0.59)	2.51 (±0.35)
1.0	1.5	0.3	6.45 (±0.46)	1.34 (±0.31)
1.0	2.0	0.3	5.84 (±0.35)	1.41 (±0.18)
1.0	0.5	0.0	5.16 (±0.27)	1.23 (±0.24)
1.0	0.5	0.1	6.86 (±0.36)	1.53 (±0.16)
1.0	0.5	0.2	7.41 (±0.42)	3.41 (±0.32)
1.0	0.5	0.4	6.65 (±0.61)	3.53 (±0.17)
1.0	0.5	0.5	6.14 (±0.57)	3.74 (±0.29)

Cultures were harvested after 16 days of incubation, biomass was measured in gram dry cell weight/L (g DCW/l) and plumbagin in mg/g dry cell weight (mg/g DCW). Figures in parenthesis represent standard error (n=6).

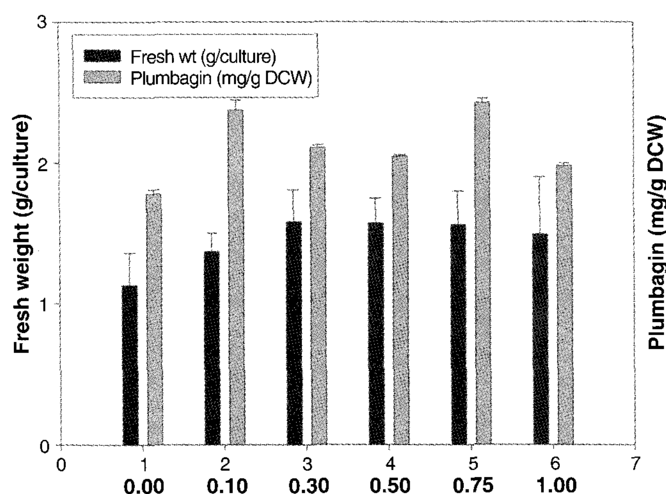

Figure 4. Inflence of kinetin (mg/L) on growth and plumbagin production in callus cultures of *P. rosea*

period of six months and data are presented in the Table 3. Growth of cells (not shown in the Table) and secondary metabolite synthesis showed a negative correlation. The cell lines PR8 and PR9 accumulated the highest biomass ranging from 12 to 15 g DCW/l but plumbagin synthesis in these lines was less (0.2 to 2.6 mg/g DCW). In case of cell lines PR3 and PR10, plumbagin accumulation was reasonably good but the biomass production was limited to 5 to 7 g DCW/l. Of all the cell lines, PR1 produced good amount of both biomass and plumbagin. Moreover, this cell line was stable and the stability in terms of both biomass and plumbagin accumulation was maintained for a period of 12 months (data shown only for 6 months) with regular subcultures. This cell line was therefore selected and used for further studies by testing its stability for growth and plumbagin accumulation now and then. In all other cultures, the degree of variation in biomass and plumbagin accumulation was very high.

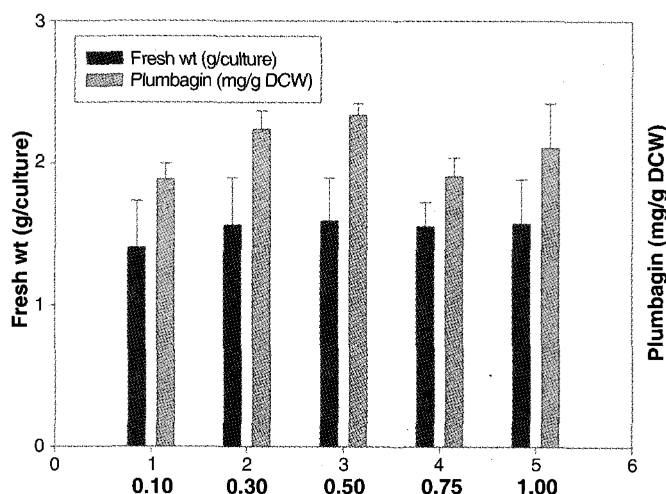

Figure 5. Influence of BAP (mg/L) on growth and plumbagin production in callus cultures of *P. rosea*

Table 3. Pattern of plumbagin accumulation in leaf derived cell lines of *P. rosea*

	Plumbagin mg/g DCW					
	Culture period (months)					
Cell line	1	2	3	4	5	6
PR1	4.3250	4.1320	3.9870	3.6540	4.4360	3.8720
PR2	2.5690	2.1680	1.3050	3.1450	0.3460	2.3150
PR3	4.4930	3.1570	2.1800	1.2360	0.3540	3.9840
PR4	2.5410	3.4160	0.2560	1.3200	3.1530	4.3210
PR5	2.4160	3.1860	4.3690	0.8750	3.4520	0.1560
PR6	1.3170	2.5310	1.0320	3.4120	0.2540	0.6540
PR7	1.3420	1.9870	0.5460	0.4160	2.6510	3.1450
PR8	0.9350	2.1540	0.2160	2.6350	1.6520	1.2560
PR9	1.7450	0.3460	1.6700	1.2360	2.3120	1.0690
PR10	4.5620	5.4960	4.1300	4.2680	3.4260	3.1570

Data represent an average of 6 replicates

Discussion

The concentration and balance between growth regulators in a culture medium would influence secondary metabolism. In *P. rosea*, it was found that optimum hormonal combination for cell growth (8.3 g DCW) was 1.5 mg IAA, 0.5 mg NAA and 0.3 mg BAP and for plumbagin accumulation (4.9 mg/g DCW) it was 1.0 mg IAA plus 0.3 mg BAP. On the other hand, Nahalka et al. (1996) reported enhanced plumbagin accumulation in cell suspensions of *Drosophyllum lusitanicum* with a combination of NAA, IBA and BAP. The reported plumbagin yields in cell cultures of *D. capensis* and *D. natalensis* were 0.0004% (Crouch et al. 1990) and 0.0001-0.003% in callus cultures of *Plumbago zeylanica* on fresh weight basis (Heble et al. 1974). In the present study, 0.02-0.035% plumbagin accumulation was recorded on fresh weight basis (0.43% on dry weight basis). Growth regulators that usually support high rates of growth do not induce the accumulation of secondary metabolites as was reported in *Beta vulgaris* (Jaafar 1992) and *Thalictrum minus* (Deus-Neumann and Zenk 1984).

In plant cell cultures genetic variation occurs spontaneously. In order to find the best possible variant cell line, large number of cultures needs to be screened for higher accumulation of secondary metabolite or pharmacologically important compounds. Subsequently, the variants must be isolated from the established lines, maintained and tested for their stability of production. In *P. rosea* cell cultures, initially a large degree of variation in growth and plumbagin production pattern was observed and the results were not comparable with one culture passage to the other. This variation is considered to be detrimental to the stability and homogeneity of the cultures (Yeoman and Yeoman 1996). To avoid this instability in growth and metabolite production, several cell lines/small clumps of calli were screened by visual and analytical methods. By this method, a high plumbagin yielding stable cell line No. PR1 was obtained. There are reports of such screening and selection of high and stable metabolite producing cell lines for longer periods (Yamamoto et al. 1987; Smith et al. 1987). Ogino et al. (1978) demonstrated that high yielding cell lines of tobacco maintained their ability to produce substantial amounts of nicotine over a 12-month period without reselection. In the present study, PR1 cell lines showed stability in plumbagin accumulation for more than 12-months. Cell lines selected from *Lithospermum erythrorhizon* were claimed to be stable for years for the accumulation of shikonin (Yamada and Hashimoto 1990). It is clear from the above results that cell line selection is very important not only

for enhancing but also for the stability of accumulation of secondary plant products.

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