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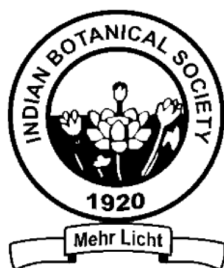

SALICYLIC ACID STIMULATES THE BIOSYNTHESIS OF LIGNANS IN THE CELL SUSPENSION CULTURE OF *GMELINA ARBOREA* ROXB.

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The present communication reports the induction of lignan biosynthesis in the cell suspension culture of *Gmelina arborea* Roxb. The calli were induced from the true leaf explant harvested from two-week old seedling over MS medium containing 13.5µM 2,4-D and 0.92µM kinetin. From these calli, a callus line was developed, which was friable and fast growing. Moreover, the selected callus line also synthesized lignan as indicated by the filter paper test and TLC analysis. The cell suspension culture was initiated from this callus line and its growth kinetics studied on the basis of cell count, packed cell volume and cell viability. Later the suspension culture was elicited with three concentrations each of yeast extract and salicylic acid during the log phase of growth. Elicitation with 250mg/l salicylic acid increased the biosynthesis of all the lignan derivatives detected in the culture. Thus, we recommend the use of 250mg/l salicylic acid to stimulate the lignan synthesis in *G. arborea* cell suspension culture.

Keywords: callus, elicitor, salicylic acid, lignan, Verbenaceae, yeast extract

Gmelina arborea Roxb. is a well-known timber plant. In addition, it is also used in traditional medicines. The juice of tender leaves mixed with cow's milk and sugar is used as demulcent. Moreover, the various plant parts of *G. arborea* are also useful in treating gonorrhea, catarrh of the bladder and cough (Tiwari 1995). The medicinal properties of plants, mostly, are due to the presence of a specific secondary metabolite in its tissues. Thus, the presence of an array of secondary metabolites in *G. arborea* is obvious. The stem bark and heartwood of *G. arborea* contains phytochemicals such as lignans, alkaloids, flavonoids, saponins, steroids etc. (Nair and Subramanian 1975, Anjaneyulu *et al.* 1977, Kaswala *et al.* 2012). Kaswala *et al.* (2012) has reported a number of lignans including arboreol, isoarboreol, methyl arboreol, gummadiol, gmelanone, arborone and 7-oxo-dihydrogmelinol from heartwood of the plant. Lignans are anti-cancerous compound effective against breast and prostate cancer (MacRae and Towers 1984). Moreover, they also reinforce durability, longevity and resistance against colon cancer. Lignans have anti-feedant, anti-bacterial and anti-fungal activity *in planta* (Hano *et al.* 2005).

The plant cell culture has been used by workers for various reasons like for bioassays, to study the metabolic pathways, in rDNA technology and to induce synthesis of commercially important phytochemicals. Production of lignans in cell culture has been reported earlier. In addition, the quantitative enhancement of the lignan production using *in vitro* techniques like elicitation, addition of biosynthetic precursors, and immobilization has also been reported (Verpoorte *et al.* 2002, Angelova *et al.* 2006, Capote 2012, Ramirez-Estrada *et al.* 2016).

Therefore, considering the importance of lignans an investigation was carried out with the aim to study the effect of biotic and abiotic elicitors on the accumulation of lignan in *G. arborea* cell culture.

MATERIALS AND METHODS

Raising the seedlings: The explants of *G. arborea* used for tissue culture were derived from the seedlings grown in the laboratory. The seeds were purchased from market and soaked in water for 24h before sowing in the autoclaved mixture of sand, coco-peat and vermicompost (1:1:1) in a tray. The trays were incubated in a seed germinator set at 90%

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relative humidity, 27 ± 2°C and photoperiod of 16h.

Surface sterilization of explant: The explants harvested from the seedlings of *G. arborea* were surface sterilized to remove the superficial microflora before inoculating them over the media. The explants were initially washed with sterile tap water for 2min. Then, they were washed sequentially with 0.1% $HgCl_2$ and 70% ethanol for 1min each. The explants were washed thrice with sterile distilled water for 1min each between the two sterilants. Similarly, the explants were also washed thrice for 1min. each after washing them with 70% ethanol. The surface sterilized explants were immediately inoculated over the media.

Callus induction: The explants such as hypocotyl, cotyledonary leaf, epicotyl, true leaf and shoot-tip were harvested from 1, 2 and 3-week old seedlings and inoculated over MS medium (Murashige and Skoog, 1962) fortified with various concentrations and combinations of cytokinin such as BAP (0.0, 0.44 and 0.88µM) and kinetin (0.0, 0.46 and 0.92µM) and auxin such as NAA (5, 10, 15 and 20µM) and 2,4-D (4.5, 9.0, 13.5 and 18.0µM). Twenty-five surface sterilized explants were inoculated over each medium in the culture tube and incubated at 25 ± 2°C and 16h photoperiod.

Maintenance and selection of calli to raise the suspension culture: The induced calli were sub-cultured after every 30 days over the same medium. Initially, the calli were slow-growing and compact. Later on, however, some of the calli picked-up the pace of growth and also became friable. Only such calli which were friable and fast-growing were maintained by sub-culturing over the same medium. In the next step the calli rich in the production of lignans were selected. Initially, the probable presence of lignans in the calli was tested using the filter paper test. A piece of callus was crushed over the filter paper to allow the contents of callus to be absorbed by the paper. Later, the spot was sprayed with the 10% ethanolic H_2SO_4 to detect the presence of lignans (Wagner and Bladt 1996). The filter paper was then heated in an oven at 60°C for 3-5min. Appearance of brownish colour indicated the presence of phenols (The class of phytochemicals to which lignans belong). All the samples showing intense colouration were then subjected to Silica gel G TLC analysis for confirmation of the presence of lignans (Wagner and Bladt 1996). The hand-cast TLC plates were activated by heating at 60° C overnight. 0.5g of callus were extracted with 10ml of 80% methanol and heated for 10min over the burner. The extract was cooled and 12µl of it was loaded onto the TLC plate. Podophyllum tincture was also loaded onto the TLC as standard. The samples loaded on TLC plate were resolved with Toluene : Ethyl acetate (7:3) solvent system. The TLC plate was sprayed with 10% ethanolic H_2SO_4 after the run was over and observed under visible and UV-254 light. The derivatives of lignans appear blue-grey under visible light and fluoresce yellow under UV-254 light. The R_f value for each band representing a lignan derivative was calculated.

Initiation of suspension culture: The callus line found positive for lignan production was multiplied to initiate the suspension culture. For this, a piece of callus was transferred to 50ml of sterile liquid medium and incubated in an orbital shaker at 120rpm, 25 ± 2° C and 16h photoperiod. The medium used to raise the suspension culture was the same over which the callus was raised. However, in this medium agar was omitted. After few days, a suspension was obtained, which was sub-cultured by transferring 3ml of it to the fresh medium. After about 5 such sub-cultures a uniform suspension was obtained.

Study of the growth kinetics of suspension culture: To study the growth kinetics of the suspension culture, 50ml of liquid medium was seeded with 300,000 cells. The growth of

suspension was studied using cell count, cell viability and packed cell volume. These parameters were determined after every 5 days of seeding. The cell count was determined using haemocytometer as per manufacturer's manual (Suprateck). The cell count was expressed in terms of cells per ml of culture. The cell viability was determined using Evan's blue staining method (Mustafa *et al.* 2011). To calculate the packed cell volume (PCV), 10ml of suspension culture was transferred to a graduated centrifuge tube. The tube was then centrifuged at 10,000rpm for 5min. The volume of cell pellet settle at the bottom of the centrifuge tube was directly read from graduated markings on the tube. The PCV was expressed as ml of volume occupied by the cells per ml of culture (Mustafa *et al.* 2011).

Preparation of elicitor solution: Two elicitors viz. yeast extract (biotic) and salicylic acid (abiotic) were used in the present study. 20mg of yeast extract powder was dissolved in requisite volume of distilled water to obtain 100ml of stock solution. The stock solution of salicylic acid was prepared by dissolving 250mg of powder in requisite volume of distilled water to obtain 20ml of solution. Both the elicitors were autoclaved before use.

Elicitation of the cell suspension culture : The suspension culture was elicited after 20 days of initiating the culture when it was in the log phase of growth. Prior to that the cell count and cell viability in the suspension culture was determined. The cultures were elicited with 50, 100 and 150µg/l of yeast extract and 150, 200 and 250mg/l of salicylic acid. The un-elicited cultures served as control.

Harvesting of the suspension culture: The cultures were harvested 5 days after elicitation. First, the cell count and cell viability of the culture was determined and later the cell suspension was filtered through a filter paper. The cell mass was collected and dried under cool conditions. The dried cell mass was used for quantification of the lignans.

Quantification of lignans: The lignan content in the samples was semi-quantitatively determined after resolving its derivatives by TLC. For this, the sample was extracted as described earlier. Subsequently, 7µl of each sample was loaded onto the pre-cast silica gel G plate (Merck, Germany) along with the *Podophyllum* tincture, which was used as a standard. The samples were resolved as mentioned earlier and lignan derivatives detected as described earlier and R_f value calculated. Later, the intensity of each band in the elicited sample was compared with the corresponding band in the control. For comparing the intensity of a particular band, the intensity of the band in control was designated as 1+. Now, if the intensity of corresponding band in the elicited sample was more than the control, then it was designated as 2+, 3+ and so on depending upon the proportional increase in the intensity. However, if it was less than the intensity of the band was designated as 1-.

Statistical analysis: The experiments carried out to study the growth kinetics of the suspension culture and to determine the effect of elicitation on growth and lignan synthesis were carried out in triplicate. All the data were analysed statistically by computing mean, standard deviation, standard error and Student's t-test using MS-Excel and XL-STAT.

RESULTS

Induction of callus: All the explants, except the hypocotyl, induced callus in the present investigation irrespective of the age of seedling. In general, the calli induced by cotyledonary leaf and epicotyl were greenish and compact, while those induced by true leaf and shoot-tip were yellowish and friable. The true leaf and shoot-tip explants responded better than other explants in terms of callus induction over the media tested. The true leaf induced calli with the frequency ranging between 16 and 72% over the medium containing either BAP or kinetin in

Table 1: Selection of calli induced from 2-week-old true leaf on the basis of friability and pace of growth.

Auxin (µM)	Cytokinin (µM)	Number of calli induced	Number of callus lines progressed after 5 sub-cultures	Number of friable and fast-growing callus lines retained
13.5 2,4-D	0.92 kinetin	240	150	25
5.0 NAA	0.46 kinetin	226	100	20

combination with 2,4-D. The frequency of callus induction was comparatively more with kinetin than BAP in combination with 2,4-D. However, the combination of BAP and kinetin with NAA was more supportive in inducing calli from true leaf. The frequency of callus induction with this combination was as high as 80%.

Like true leaf, shoot-tip explant also induced calli over all the media. However, the frequency was comparatively low with shoot-tip explants. The combination of 2,4-D with BAP induced calli with the frequency varying from 20 to 52%. The shoot-tip induced calli with higher frequency over media containing 2,4-D in combination with kinetin, which ranged between 44 and 60%. Similarly, the combination of NAA with either BAP or 2,4-D also induced the calli from shoot-tip explants with the frequency varying from 16 to 56%.

The explant derived from cotyledonary leaf induced callus only over the media containing 0.92µM kinetin and 2,4-D with the frequency ranging between 36 and 52% varying according to the age of explant and concentration of 2,4-D. The cotyledonary leaf explant did not respond over the MS medium containing PGR combination of 0.44 and 0.88µM BAP with 2,4-D, 0.46µM kinetin with 2,4-D and 2,4-D alone. Similarly, the cotyledonary leaf explant did not respond over the media containing NAA alone. However, in combination with either BAP or kinetin, NAA induced the callus from cotyledonary explant with varying frequency. The frequency of callus induction varied from 36 to 56% according to the cytokinin concerned, its concentration and the age of explant. The epicotyl explant was comparatively more responsive than hypocotyl or cotyledonary leaf over the media with respect to callus induction. The epicotyl explant induced callus over all the media except the 3-week-old epicotyl inoculated over MS medium containing NAA in combination with 0.00 to 0.88µM BAP. The frequency of callus induction from epicotyl varied from 24 to 72%.

Selection of callus line: The true leaf derived from two-week-old seedling induced calli with

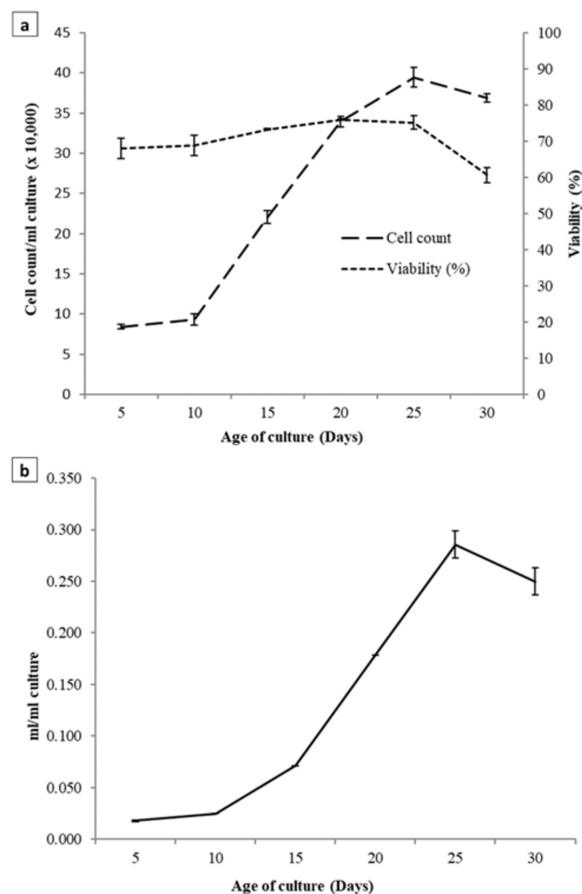

Figure 1: Growth kinetics of cell suspension culture. **a**. growth in terms of cell count and cell viability **b.** Growth in terms of packed cell volume

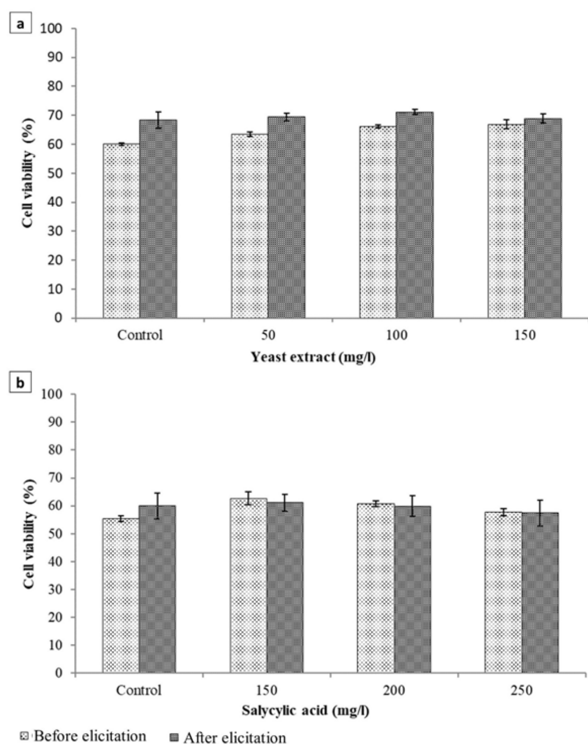

Figure 2: Effect of elicitation on cell viability in the suspension culture. **a.** Effect of yeast extract **b.** Effect of salicylic acid

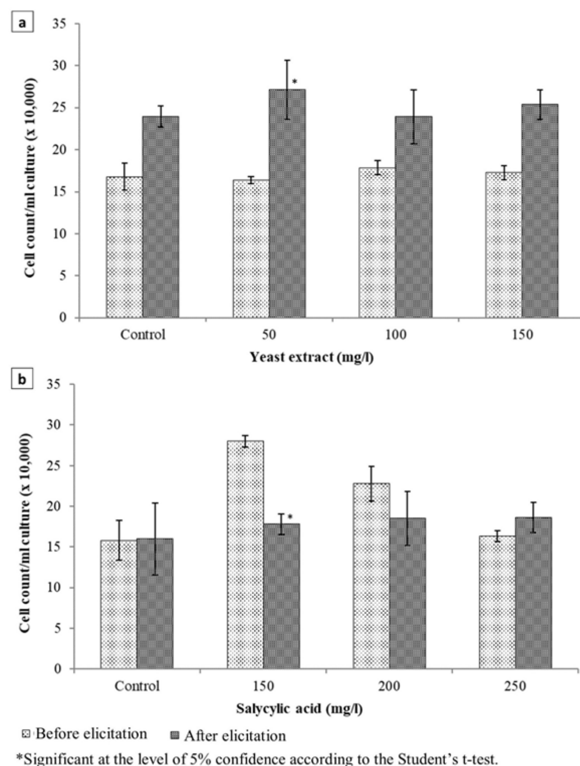

Figure 3: Effect of elicitation on cell count in the suspension culture. **a.** Effect of yeast extract, **b.** Effect of salicylic acid

desired friability and hence, only these calli were retained and sub-cultured over the same medium. The medium containing 13.5µM 2,4-D + 0.92µM kinetin and 5.00µM NAA + 0.46µM kinetin induced 240 and 226 such calli, respectively. With 5 sub-cultures the friability of a few calli increased and also, they picked-up the pace of growth. Later, the stricter selection criteria were implemented and only calli with high friability and growth rate were retained. This led to selection of 25 calli over the medium containing 13.5µM 2,4-D + 0.92µM kinetin and 20 calli over the medium containing 5.00µM NAA + 0.46µM kinetin (Table 1).

In the next step selection among these 45 calli was made on the basis of their lignan synthesizing ability. The filter paper test led us to the selection of 8 calli as they showed intense brownish spots over the filter paper indicating higher phenolic content and thus, probably, higher lignan content. Later, the extract of these 8 calli was subjected to TLC analysis, which identified 3 calli, which accumulated comparatively higher amount of lignans as evident from the greater intensity of the bands corresponding to lignans. These three calli were initiated over media containing 13.5µM 2,4-D + 0.92µM kinetin.

Growth kinetics of cell suspension culture: The selected calli were transferred to liquid MS medium containing 13.5µM 2,4-D + 0.92µM kinetin. The growth kinetics of the cell suspension culture was studied when the culture attained uniformity. The cell viability, cell count and PCV was determined till 30 days after seeding the culture at the interval of 5 days. The viability of cells remained around 70% throughout the growth phase studied except between 25 and 30 days after seeding when it dropped to about 60% (Fig. 1a). The cell suspension culture investigated in the present study had a lag phase till 10 days after seeding as evident from the marginal increase

Table 2: Relative intensity of the lignan derivatives in the cell culture elicited with yeast extract and salicylic acid as compared to control

Band number	R_f value	Control	Yeast extract (mg/l)			Salicylic acid (mg/l)		
			50	100	150	150	200	250
1	0.1	+1	-0.3	+0.3	+0.3	+1.0	+1.0	+1.3
2	0.4	+1	-1.0	-0.3	-0.3	+1.0	+1.0	+1.3
3	0.6	+1	-1.0	-0.3	-0.3	+1.7	+1.3	+1.7

in the cell count and PCV (Fig. 1a&b). However, later the growth of cells in the suspension picked up pace as evident from a massive increase in the cell count as well as the PCV (Fig. 1a&b). The growth reached the peak after 25 days of seeding and later declined (Fig. 1a&b).

Effect of elicitor treatment on the growth of cell suspension culture: The cell suspension culture of *G. arborea* was elicited with three different concentrations of yeast extract and salicylic acid to induce lignan synthesis. The cell viability was unaffected by the elicitor treatment (Fig. 2a&b). Similarly, the cell count was also mostly unaffected by the elicitor treatment. While the 50mg/l yeast extract enhanced the cell count, 150mg/l decreased it (Fig. 3a&b).

Effect of elicitor treatment on the synthesis of lignans in cell suspension culture: The effect of elicitation on accumulation of lignans in the cell suspension was evaluated in a semi-quantitative manner by comparing the intensity of the band corresponding to lignans in the elicited cells with that of control cells. Three derivatives of lignans with R_f values of 0.1, 0.4

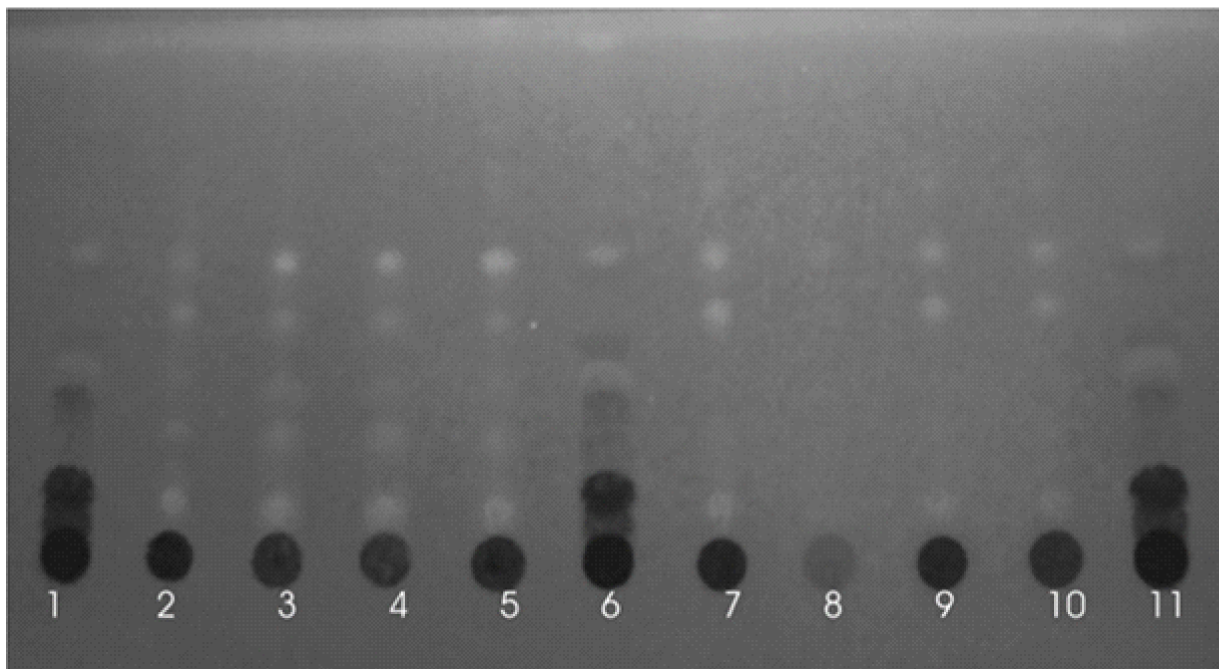

Figure 4: TLC analysis of the lignan derivatives induced in the cell suspension culture upon elicitor treatment. Lane: **1, 6 and 11–** Podophyllum tincture (Standard), **2 and 7**– Control, **3–** Elicited with 150mg/l of salicylic acid, **4–** Elicited with 200mg/l of salicylic acid, **5–** Elicited with 250mg/l of salicylic acid, **8–** Elicited with 50mg/l of yeast extract, **9–** Elicited with 100mg/l of yeast extract, **10–** Elicited with 150mg/l of yeast extract

and 0.6 were observed over TLC plate. Due to most of the treatments, particularly with yeast extract, the accumulation of lignans in the cell suspension was either unaffected or was reduced. It is noteworthy that 250mg/l salicylic acid increased the content of all the three derivatives of lignans. Similarly, the accumulation of lignan derivative with R_f value 0.6 was enhanced by salicylic acid at all the concentrations (Table 2, Fig. 4).

DISCUSSION

In the present investigation we have studied the effect of elicitors like yeast extract and salicylic acid on the lignan biosynthesis in the cell suspension culture of *G. arborea*. For this, we selected callus line suitable to initiate suspension culture. The callus induced by two-week-old true leaf over MS medium containing 13.5µM 2,4-D + 0.92µM kinetin was selected to initiate suspension culture. Generally, high concentration of auxin with low concentration of cytokinin in the medium promote abundant cell proliferation leading to callus formation (Shah et al. 2003). In the present investigation, high concentration of 2,4-D with low concentration of kinetin was found to be more effective in inducing calli in *G. arborea*. Gupta *et al.* (2014) have found 2,4-D with kinetin to be the best for callus induction in *Rauvolfia serpentina.* Similarly, Pandey et al. (2013) and Dalila *et al.* (2013) have reported 2,4-D with kinetin was effective against callus induction in *Psoralea corylifolia* and *Barringtonia racemosa.*

The suspension culture was initiated using the friable and fast-growing callus line induced by 13.5µM 2,4-D + 0.92µM kinetin. We first studied the growth kinetics of the culture to be treated with the elicitors. The growth of the culture was assessed in terms of cell viability, cell count and PCV. The growth of the cell culture studied using cell count and PCV followed an identical trend. Similar findings were reported by Warhade and Badere (2018) in the suspension culture of cockscomb. In addition, several investigators have found these parameters reliable to study the growth kinetics of cell suspension culture. For example, Singh and Chaturvedi (2012) used PCV in *Spilanthes acmella* and Sparapano and Bruno (2004) in *Cupressus arizonica* and *C. macrocarpa* used cell viability to study the growth of suspension culture. Inclusion of data on cell viability with either cell count or PCV enhances the reliability of the growth kinetics of the suspension culture as this gives a better estimate of the cells contributing to the growth and synthetic activities of the culture (Warhade and Badere 2018).

In the present investigation elicitation of the cell suspension culture with salicylic acid increased the accumulation of lignans at the concentration of 250mg/l. Similarly, the accumulation of lignan derivative with R_f value 0.6 was enhanced by all salicylic acid at all the concentrations. Elicitors could be used as enhancer of plant secondary metabolite synthesis in the cell cultures. Many investigators have attempted earlier to enhance the production of secondary metabolites through suspension culture using elicitors. For example, betalain content in *Celosia cristata* (Warhade and Badere 2018), monoterpene content in *Zataria multiflora* (Mohagheghzadeh *et al.* 2000) and essential oils in *Salvia macrosiphon* (Rowshan *et al.* 2010) and *Melissa officinalis* (Silva *et al.* 2014) have been enhanced in the cell cultures using elicitors.

The effectiveness of salicylic in triggering the biosynthesis of lignans in the cell culture might be because it is an endogenous growth regulator which participates in the regulation of physiological process (He *et al.* 2005). Moreover, salicylic acid plays an important role in the defence responses to pathogen attack and to several abiotic stress (Elizabeth and Munn-Bosch 2008). Exogeneous application of salicylic acid improves plant tolerance to heat (Dat *et al.* 1998), chilling (Janda *et al.* 1999) and salt stress (Borsoni *et al.* 2001). It is also effective in inducing secondary metabolite formation in plant cell culture

(Kiddle *et al.* 1991). Ghanati and Dehaghi (2011) have used salicylic acid to stimulate taxol synthesis in *Taxus baccata* cell culture. Dong *et al.* (2010) have also found it to be efficient in increasing the accumulation of phenolic compounds in cell cultures of *Salvia miltiorrhiza*.

Thus, based on the findings of the current investigation, it is concluded that salicylic acid stimulates the synthesis of lignans in the cell culture of *G. arborea*. The mechanistic details of the action of salicylic acid leading to enhancement of lignan content in the cell culture needs elucidation. This would not only increase our understanding of lignan biosynthesis in plants but also may pave a way to production of lignans in bioreactors.

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