BIOLOGIA PLANTARUM 38 (2): 185-190, 1996

Effect of *Calotropis* latex on laticifers differentiation in callus cultures of *Calotropis procera*

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Abstract

Laticifers differentiation in callus cultures of *Calotropis procera* (*Asclepiadaceae*) as affected by own latex and its fractions incorporated in Murashige and Skoog (MS) medium is described. Callus cultures have been maintained on MS medium with 2.3 μM 6-furfurylaminopurine (FAP) and 3.0 μM 1-naphthylacetic acid (NAA). Marked increase in laticifers differentiation (from 10.1 to 28.4 %) was observed on this medium supplemented with 1 % (v/v) of latex. Latex fractions containing proteins + complex polysaccharides or inorganic salts also increased laticifers differentiation (by 21.8 % and 24.1 %, respectively). Other fractions (free amino acid + saccharides, phenols and terpenes + sterols) had no marked effect on laticifers differentiation while alkaloid fraction inhibited it. Effect of latex on laticifers differentiation was much more profound than the reported optimal concentration of plant growth regulators (4.6 μM FAP + 1 μM IAA).

Additional key words: *Asclepiadaceae*, growth regulators.

Introduction

Cellular differentiation is a prerequisite where secondary metabolites are produced in specialized cells. Production of morphanian alkaloids was very low in cultures where cellular differentiation was lacking (for review see Roberts 1988). Alkaloid precursor supplement has been used successfully to enhance the production of ephedrine (Ramawat and Arya 1979) and several other secondary metabolites (Verpoorte *et al.* 1993, Ramawat 1995). Effect of cell constituents on the synthesis of own secondary metabolites is still lacking. However, this can be compared with the use of elicitors of fungal origin which generate a stress, under which host cells produce enhanced secondary metabolites (Eilert *et al.* 1985, Ramawat *et al.* 1995).

Cultures of *Calotropis procera* provide a unique system to study the cellular differentiation leading to laticifers formation (Dhir *et al.* 1984, Dutta and De 1986)

Received 6 February 1995, *accepted* 30 November 1995.
Acknowledgement: This research was supported by grant-in-aid for research from the University Grants Commission (No. F3-65/91SR II), New Delhi, to Dr. K.G. Ramawat.

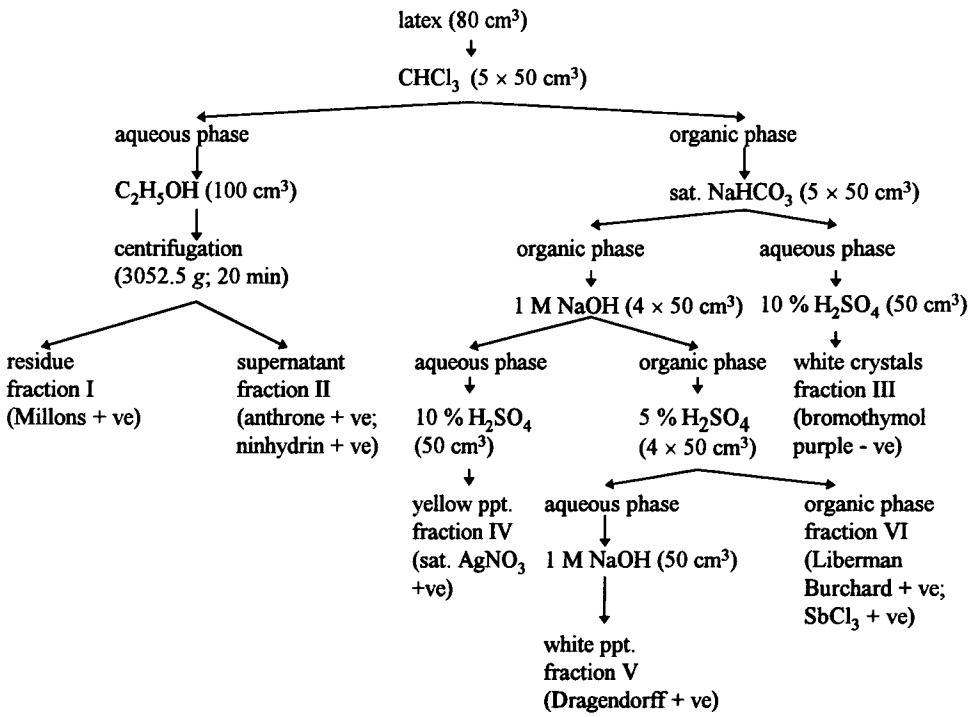
and its regulation by growth regulators (Suri and Ramawat 1995). Latex of *C. procera* is a complex mixture of proteins, carbohydrates, phenolics, terpenoids and alkaloids (Duke 1986).

In the present communication we report the effect of latex and its constituents on laticifers differentiation in callus cultures of *C. procera.*

Materials and methods

Seeds of *Calotropis procera* (Ait.) R.Br., *Asclepiadaceae,* were surface sterilized with 0.2 % aqueous $HgCl_2$ for three min followed by a quick dip in 70 % ethanol, rinsed several times with sterilized distilled water, and then were transferred on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) devoid of plant growth regulators. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 15 min. Callus cultures were initiated from cotyledon explants and maintained on MS medium supplemented with 2.3 μM 6-furfurylaminopurine (FAP) and 3 μM 1-naphthylacetic acid (NAA). Cultures were maintained in dark at 26 ± 1 °C and were subcultured every fourth week.

Process of fractionation of latex and tests performed:



Latex (80 cm^3) was collected in the month of October, when the latex yield was maximum (Dhir 1985), by making oblique incissions on the stem of field grown plants and was incorporated in various concentrations (0.1 to 2.5 %, v/v) in MS medium supplemented with 2.3 μM FAP and 3 μM NAA before autoclaving. Latex was fractioned into six different groups of compounds and each of these fractions were tested with specific reagents viz., Millon's reagent for fraction I, anthrone and ninhydrin reagent for fraction II, saturated $AgNO_3$ for fraction IV, Dragendorff's reagent for fraction V and Liberman Burchard's reagent and $SbCl_3$ reagent for fraction VI (Stahl 1969). Fraction III of *Calotropis* latex was analysed by titrimetric method for chloride ions (Piper 1950) and for Na, K, Ca ions by flame photometric method. All the fractions were then incorporated in various concentrations (0.2 to 10 mg dm^{-3}) in MS medium containing growth regulators as above. Cultures were grown in culture-tubes (25 × 150 mm, *Borosil*) each containing 20 cm^3 medium and single piece of callus (approximately 200 mg). Ten replicate cultures were used for each treatment. Four-week-old callus from each treatment was fixed in formalin:acetic acid:50 % alcohol (5:5:90) mixture (FAA) for 24 h (Johansen 1940), dehydrated in ethanol-xylol series and embedded in paraffin (mp 58 °C) containing few drops of oleic acid, which prevents the dissolution of coagulated latex in paraffin (Wimalaratna 1973). Three randomly selected blocks were cut at 8 - 10 μm on a rotary microtome and sections were fixed on slides using Haupt's adhesive. Sections were dewaxed, rehydrated and then stained with laticifer specific 1 % azure-I (aqueous). The sections were mounted in glycerine jelly, as canada balsam dissolves the coagulated latex (Wimalaratna 1973).

Laticifers differentiation [%] was calculated as $[L/(U + L)] \times 100$; where L and U are the number of laticifers and undifferentiated cells under a microscopic field, respectively. Data are the means of thirty separate observations.

Results and discussion

Some cells in a cultured cell population get differentiated into specialized cells called laticifer-initials, when exposed to appropriate exogenous growth regulators. Nuclei in these laticifer-initial cells become prominent, their cytoplasm turns dense and possesses vesiculating endoplasmic-reticulum. By the elongation of these specialized cells and the dissolution of end walls between such adjacent cells, they formed elongated tube-like structures which get branched at maturation and form a complete network. Laticifers of various shapes and sizes were observed in control cultures in the present investigation and in earlier studies (Dhir *et al.* 1984, Dutta and De 1986). It has been suggested that the appearance of tracheids in cell cultures may be of importance in identifying cellular differentiation that may lead to alkaloid accumulation (Roberts 1988). But we have observed that optimal conditions for tracheary-element differentiation were different than those for laticifers differentiation (Suri and Ramawat 1994).

Incorporation of latex in MS medium supplemented with 2.3 μM FAP and 3 μM NAA enhanced markedly the laticifers differentiation in callus cultures of *C. procera*. Laticifers differentiation increased significantly from 15.3 % to 29.8 % with the increase in concentration of latex from 0.1 % to 2.5 % (v/v) in the medium. The rate of enhancement was high up to 1 % (v/v) latex concentration (Table 1). At 1 % latex concentration laticifers differentiation was 2.8 folds over the control (10.1 %). Callus turned milky-white on the medium containing latex up to 1 % (v/v), and was light brown on higher concentrations of latex. The effect of latex on laticifers differentiation was much more profound than optimal concentration of plant growth regulators (4.6 μM FAP + 1 μM IAA) recorded in our previous experiments (Suri and Ramawat 1995).

Table 1. Effect of different latex concentrations on laticifers differentiation (mean ± S.D.) in cultures grown on MS medium containing 2.3 μM FAP and 3 μM NAA.

	Latex concentration [%]					
	0	0.10	0.25	0.50	1.0	2.5
Laticifers differentiation [%]	10.1± 2.1	15.3±2.9	20.0±1.3	24.4±1.5	28.4±0.9	29.8±1.5

Significant at 1 % value 3.34, S.E. = 0.41, C.D.= 0.81

Different fractions of latex separated gave positive group specific tests (Stahl 1969) and fraction III was found to contain chlorides of Na, K and Ca. Each of these fractions separated from 80 cm^3 of latex were categorized as follows: (I) proteins + complex polysaccharides (7.275 g), (II) free amino acids + saccharides (0.750 g), (III) inorganic salts (7.788 g), (IV) phenols (0.047 g), (V) alkaloids (0.026 g), and (VI) terpenes + sterols (7.835 g). The relative proportion of above fractions was 3:1/100:3:2/300:1/300:3, respectively. Each of these fractions showed different effects on laticifers differentiation when incorporated in MS medium (Table 2).

Table 2. Laticifers differentiation [%] on MS medium containing 2.3 μM FAP, 3 μM NAA and various concentrations of latex fractions. Mean ± S.D.

Fraction number	Concentrations [mg dm^{-3}] 0.2	0.5	2	4	6	10	C.D. (5 %)
I	-	10.6±2.5	13.5±1.3	15.4±2.7	21.8±3.6*	17.4±3.0*	2.49
II	9.6±1.5	11.6±1.9	12.8±2.6	10.3±2.5	8.0±1.6	7.7±1.0	1.74
III	-	10.9±2.4	11.5±2.3	16.8±2.1*	24.1±2.4*	19.4±1.4*	6.96
IV	-	10.3±1.3	10.5±1.6	11.6±1.1	14.6±1.2	12.8±2.6	0.74
V	10.9±2.4	10.1±1.2	6.5±1.4	4.1±0.7	1.9±1.5	-	2.44
VI	-	10.0±0.9	10.0±1.2	-	10.9±0.9	9.3±0.8	NS

* - large number of laticifer-initials in group were also observed; NS - non significant

Alkaloidal fraction (V) and neutral fraction (VI) were not effective at lower concentrations but higher concentrations (> 2 mg dm^{-3}) were inhibitory. Callus turned necrotic on the medium supplemented with 6 - 10 mg dm^{-3} of fraction V. Increase in laticifers differentiation was recorded on media containing 6 mg dm^{-3} of either of fractions I, III and IV. Laticifers differentiation was maximum (24.1 %) in the cultures grown in the presence of 6 mg dm^{-3} of fraction III, which was 2.5 times higher to that of the control. Laticifers differentiation on medium with 6 mg dm^{-3} of fraction I and IV was 21.8 % and 14.6 %, respectively. Fraction II had a marginal but statistically significant effect on laticifers differentiation.

The stimulatory effect of latex fractions may be due to stress generated by added inorganic salts (fraction III) or to an elicitor like effect of proteins and polysaccharides (fraction I). The high increase in laticifers differentiation by added whole latex could be a synergistic effect of these combined fractions. The inhibitory effect of alkaloids (Bell 1980) and terpenes (Komai and Ueki 1980) on cellular differentiation is well established.

Thus, it is clear from the present work that small amount of latex constituents can be used to induce high number of laticifers and consequently, laticifers-dependent product formation in cell cultures of *C. procera*. The system developed offers an opportunity to study the mechanism of laticifers differentiation.

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