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Somoclonal variation studies on *Passiflora mollussima* (H.B.K.) bailey using phytochemical methods

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ABSTRACT

The present study was aimed to develop a somoclonal variant for *Passiflora mollussima* (H.B.K.) Bailey using the inter-nodal segments and leaves as explants. Maximum percentage of callus formation (inter-nodal segments 75.2 ± 0.90 and leaves segments 78.7 ± 0.51) was obtained on Murashige and Skoog's basal medium supplemented with 3% sucrose and 1.0mg/l of 2, 4-Dichlorophenoxy acetic acid. The calli mediated plantlets showed the morphological variation in the leaves texture and size. These morphological variations were confirmed as somoclonal variant through the phytochemical, anti-bacterial and isozyme (peroxidase) analysis. Preliminary phytochemical analysis and extraction was performed on 4 weeks old leaves segments derived calli. The phytochemical study confirmed the presence of more alkaloids, saponins, tannins, flavanoids, phenolics etc., from leaves segments derived calli. Antimicrobial activity of different extracts (benzene, methanol, ethanol, isopropanol, and chloroform and petroleum ether) of mother plants and somoclonal variant were investigated by well-diffusion method against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella aerogenes, Aeromonas sps, and Serratia* and *Escherichia coli*. Of these, ethanol extract of leaves segment derived calli showed the maximum solubility and antimicrobial activity with the MIC ranged from 100 to 250µl. Extracts of petroleum ether and isopropanol were ineffective in inhibiting the selected bacteria. The isoperoxidase banding profile showed the different banding profile in inter-nodal and leaves segments derived calli and mother plants. The isozyme banding patterns of the somoclonal variant is used as a molecular marker for the future plant breeding or genetic improvement programme.

KEYWORDS

In vitro;
In vivo;
Calli;
Bio-efficacy;
Antibacterial;
Phytochemistry;
Somoclonal variant.

ABBREVIATIONS

MS: Murashige and Skoog, SA: *Staphylococcus aureus*, PA: *Pseudomonas aeruginosa*, KA: *Klebsiella aerogenes*, A: *Aeromonas sps*, S: *Serratia* EC: *Escherichia coli*

INTRODUCTION

Passiflora mollissima (H.B.K) Bailey climber belongs to the family Passifloraceae, are distributed in many tropical countries. The plant is cultivated for ornamental purposes and its fruits having medicinal value. Its

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force is exerted chiefly upon the nervous system. It proves especially useful in the spasmodic disorders, insomnia of infants and old people. It is praised for its control over the spasms of childhood, whether from dentition, worms or undigested ailment. It is also been successfully employed in woughing cough with convulsions. *Passiflora* has given relief and in hysteria with spasmodic movement it is reputed equally successful. A special feature of angiospermic plants is their capacity to produce a large number of organic chemicals of high structural diversity. The so-called secondary metabolites have contributed more than 7000 different compounds in use today as cardiac drugs, anticancer agents, hormones, antibiotics, laxatives, diuretics, analgesics, anaesthetics, drugs for ulcer treatments and antiparasitic compounds. In USA, 74% of drugs are based on plants[1]. The accumulation of phytochemicals in the plant cell cultures has been studied for more than thirty years, and the generated knowledge has helped in the realization of using cell cultures for production of desired phytochemicals[2]. Already the direct regeneration and organogenesis was achieved in our laboratory[3]. Currently the various applications of genetic engineering works are implemented in medicinal plants to increase the secondary metabolites production[4]. Since 1930s, electrophoresis joined with the zymogram technique has been used tool for the studies of heritable variation. Isozymes are widely used for its relative efficiency and cost effectiveness, particularly in studies of intra and inter specific variation[5-8]. A lot of reports are available on phytochemical, antimicrobial, antifungal, antiviral and antibacterial activities on plants[3, 9-13], but a few reports were available for phytochemical and antimicrobial analysis in *in vitro* derived cultures[6,7,14]. Due to its medicinal and horticultural value there is great demand in the market. In the present study, the production of somoclonal variant using inter-nodal and leaves segments as explants was emphasized. In addition to this, an attempt was made to produce biochemical (isozyme) and phytochemical markers for the medicinally and horticulturally important plant. The present study will strengthen the phytochemical and antibacterial study on cell lines and isozyme usage in the intra and inter specific and somoclonal variation studies.

MATERIALS AND METHODS

Callus induction

Inter-nodal and leaves segments of *Passiflora mollissima* (H.B.K) Bailey were collected from the young top shoot cuttings of mature plants. The explants were washed thoroughly under running tap water for 5 min. and then washed with a commercial detergent tween-20 for 3 min. followed by thorough washing with sterile distilled water. Surface sterilization was done with mercuric chloride solution (0.5% w/v) for 2 min. then washed thrice with sterile distilled water, the explants were cut into 0.7 cms in length and cultured on Murashige and Skoog[15] solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration of 2, 4-D either alone or in combination. The pH of medium was adjusted to 5.8 before autoclaving at a pressure of 1.06Kg/cm^2 (121°C for 15 min.). After few weeks, the *in vitro* proliferated callus cultures were subcultured onto MS medium supplemented with BAP, Kin, and NAA either alone or in combinations for organogenesis (Data's are not included). The cultures were incubated at 25± 2°C with 12/8 h photoperiod under white fluorescent tubes (1500lux). Each and every experiment was performed with ten replicates and repeated thrice. The callus cultures were maintained for a period of over 10 months by periodic sub-culturing with 2 to 4 weeks intervals on to fresh multiplication medium.

Phytochemical analysis

Dried whole plants as also the *in vitro* leaves segments derived callus were powdered using the electric homogenizer exhaustively extracted with 150ml of solvent (benzene, methanol, ethanol, isopropanol, chloroform and petroleum ether) for 8-12 h by using the soxhlet apparatus[14]. The preliminary phytochemical screening was performed by Harborne[16] method.

Antibacterial activity

The extracts were concentrated and these extracts were subjected for their antibacterial activity against the selected bacteria. Stock cultures were made fresh every seven days on agar slants during this scheme of work. Pure bacterial cultures, namely *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella aerogenes, Aeromonas sps, Serratia* and *E. coli* were maintained on nutrient broth at 37°C for 24 hrs. Different concentrations of extracts ranging from 0 to 250µl were used for bacterial sensitivity test. Antibacterial efficacy was performed by well diffusion

method and incubated for 24 hrs at 37°C[17]. The inhibition zone and antibacterial activity against the bacteria were recorded. The experiments were repeated in triplicate and the results were documented.

Isoperoxidase analysis

For the electrophoresis studies, mother plant and inter-nodal and leaves segments derived calli and calli mediated plants young leaves were harvested and ground on ice cold mortar and pestle with 0.1M phosphate buffer (pH 7 .0). The slurry was centrifuged at 10,000rpm at 4°C for 10 min. and the supernatant were collected and separated by native poly acrylamide gel electrophoresis. The native (PAGE) gel electrophoresis was performed by Anbalagan[18] method. The gel was stained with O-dianisidine (100mg) acetate buffer (90ml, pH 4.2), ethanol (5ml), 30% H_2O_2 (1ml) and distilled water (4ml)[19]. The banding patterns were documented and Rf values were calculated using Biogene Software. Variation in banding pattern was determined by the migration from the origin towards the anode. Isozymes region were designated to define the general area on the zymogram with in which the bands migrated.

RESULTS AND DISCUSSION

Callus induction was observed on the inter-nodal and leaves segments on MS medium supplemented with 2, 4-D. Based on the concentration of plant growth hormone the callus formation frequency was varied (TABLE 1). Maximum percentage of callus formation (inter-nodal segments 75.2 ± 0.90; leaves segments 78.7 ± 0.51) was obtained on Murashige and Skoog's basal medium supplemented with 3% sucrose and 1.0 mg/l of 2, 4-Dichlorophenoxy acetic acid (Figure 1 A, B and C). Different types of calli were obtained of which, the friable, semi friable and creamy white coloured showed high proliferation rate. In high concentration of auxins the callus was hard and dark yellowish brown in colour. The semi friable callus was showed highest rate of shoot proliferation. Friable calli were showed highest percentage of cell division and cell multiplication. The MS medium augmented with BAP 1.0mg/l in combination with 0.5mg/l of NAA produced maximum percentage and number of shootlets on the *in vitro* derived calli (TABLE 1).

The present study emphasized on the phytochemical comparative study between the mother plant and leaves segment derived calli mediated one to confirm the phytochemical constituent's presence. The calli mediated tissues showed the higher percentage of metabolite constituents compared to the *in vivo* and nodal derived plants (Results are not included). The phytochemical study revealed the high quantity presence of saponins, alkaloids, phenolic compounds etc in calli mediated tissues and calli. Different kinds of solvents were used for extraction, of which ethanol extracted solvents showed maximum values (9/9) compared to others (TABLE 2).

The result of the present study revealed that, antibacterial efficacies of ethanol, chloroform, methanol, benzene, isopropanol and petroleum ether extracts were varied in effectiveness which may be attributed to the presence of the secondary metabolites (Figure 1 D-K). The results of phytochemical and antibacterial screening tests of leaves segments and leaves segment derived calli extracts of *Passiflora mollussima* in different solvents against pathogenic bacteria using diffusion techniques are depicted in TABLE 1 and 2. The ethanol extracted solvents showed the maximum bio-efficacy compared with other solvents due to the presence of more compounds such as saponins, steroids, tannins, phenolics, triterpenoids, alkaloids and flavanoids (TABLE 3). Results of the present study are found directly correlated with the observations of previous workers[2,14,17,20,21]. Both isopropanol and petroleum ether extracts were found to be ineffective of bacterial inhibition, due to the presence of less active compounds saponins, steroids and alkaloids. Observation of the present study was supported by the previous observation on *Rauvolfia tetraphylla* and *Physalis minima* and *Passiflora edulis* leaf and callus extracts[3, 20]. The mother plant and leaves segments derived calli mediated somoclonal variant ethanol extract inhibited growth of all tested bacteria (TABLE 2). Earlier observations on *Baliospermum axillare, Mimosa hamata* and *Nerium oleadaner* leaf and callus extracts showed considerable antibacterial and antimicrobial activity[14,22,23]. The present observation augments the previous phytochemical and bio-efficacy studies on cell cultures. The present study observation is strengthening the bio-efficacy studies on cell cultures. Further work is required to find out the active principle from the plant extracts and to carry out pharmaceutical studies.

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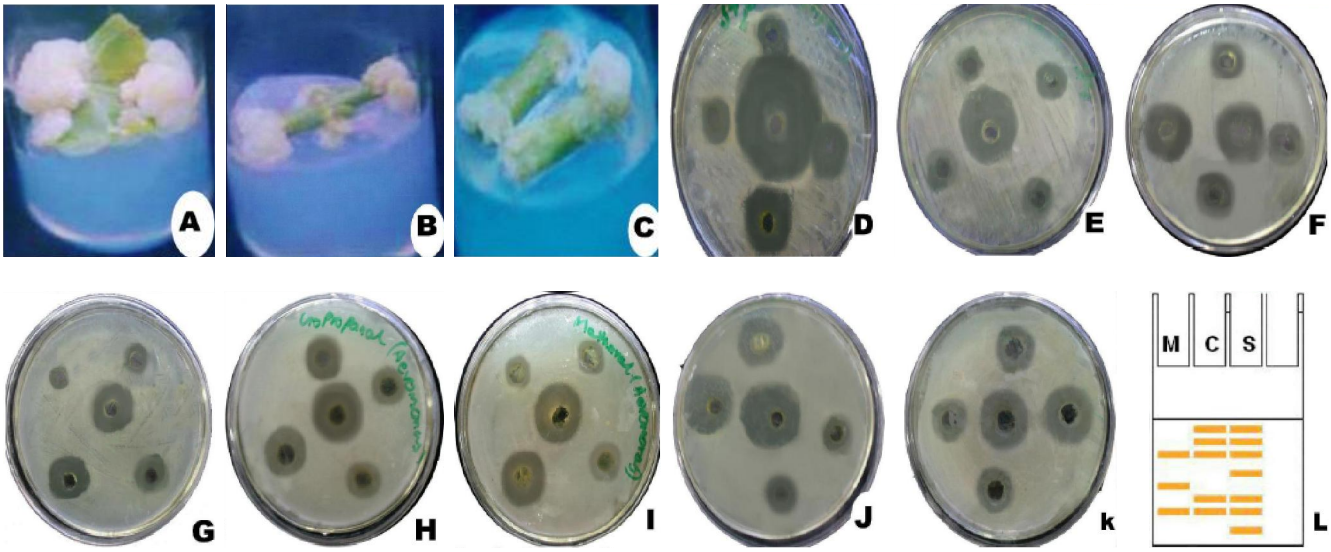

Figure 1 : Somoclonal variation studies on *Passiflora mollussima, In vitro* raised calli from the leaves segments, *In vitro* raised calli from the petiole segments, *In vitro* raised calli from the inter-nodal segments, Ethanolic extracts of *P. mollussima* against *Staphylococcus aureus,*, Ethanolic extracts of *P. mollussima* against *Pseudomonas aeruginosa,* Ethanolic extracts of *P. mollussima* against *Klebsiella aerogenes,* Ethanolic extracts of *P. mollussima* against *Serratia* sps. Isopropanol extracts of *P. mollussima* against *Aeromonas sps,* Methanolic extracts of *P. mollussima* against *Aeromonas sps,* Ethanolic extracts of *P. mollussima* against *Escherichia coli,* Ethanolic of extracts *P. mollussima* calli against *Escherichia coli,* Isoperoxidase profile of the Mother plant (M), *In vitro* raised calli (C) and Calli mediated plantlet (S)

TABLE 1 : Effect of 2, 4-D on callus formation of *Passiflora mollussima*

MS+2,4 -D(mg/l)	% of Callus formation±S.D		Type of Calli					
	Inter- node	Leaves	Inter- node			Leaves		
			F	S.F.	C	F	S. F.	C
0.0	0.0 ± 0.0	0.0 ± 0.0	-	-	-	--	-	-
0.5	58.3 ± 0.81	64.5 ± 0.62	+	+	-	+++	+++	-
1.0	75.2 ± 0.90	78.7 ± 0.51	+	++	-	+++	++	-
1.5	69.4 ± 0.38	72.3 ± 0.67	-	+++	-	++	++	-
2.0	58.6 ± 0.42	65.8 ± 0.29	-	+++	+	++	+	-
2.5	48.2 ± 0.67	56.7 ± 0.46	-	++	+	+	+	+

+ - sign indicates the callus formation, + - Low; ++ - Medium; +++ - High.

TABLE 2 : Comparative preliminary phytochemical screening of mother plants and calli mediated *Passiflora mollussima*

Experiment	Benzene		Ethanol		Chloroform		Isopropanol		Methanol		Petroleum Ether	
	M	C	M	C	M	C	M	C	M	C	M	C
Saponin	+	++	+	++	+	+	+	+	+	+	+	+
Steroid	+	++	+	++	+	+	+	+	+	+	+	++
Amino acid	+	+	+	++	+	++	-	-	+	+	-	+
Tannin	+	+	+	++	+	++	+	+	+	+	+	+
Phenolics	+	++	+	++	++	++	-	+	+	++	+	+
Sugar	-	-	+	+	-	-	-	-	-	+	-	-
Triterpene	-	-	+	+	+	++	-	-	-	+	-	-
Alkaloids	+	++	+	++	++	++	+	++	+	++	+	+
Flavanoids	+	+	+	++	++	++	-	+	++	++	-	-

The isozymic study produced the isozymic pattern for the *in vitro* induced calli (C), calli mediated plantlets (S) and mother plants (M). Multiple regions of activity were obtained for the peroxidase system (PRX1- 4). Region 1 contained three bands, $PRX1^{1to2}$, First ($PRX1^{1}$) and second ($PRX1^{2}$) bands were showed their unique presence with calli (C) and calli mediated (S) *in vitro* raised plantlet. The third band ($PRX1^{3}$) was common to all. Region 2 contained bands in two different positions. Calli mediated plantlets (S) showed the unique presence in two different positions ($PRX2^{1\&2}$). Region 3 also illustrated the variation between the calli mediated and others. $PRX3^{1}$ was so specific to the mother plants and nodal derived plants or directly regenerated plants. The second band ($PRX3^{2}$) was restricted to calli and calli mediated plantlet. Region 4 contained two bands in two different positions. The first band ($PRX4^{1}$) was common to calli; calli mediated and direct regenerated plantlets. The second band ($PRX4^{2}$) was restricted to calli mediated plants (Figure 1 L). Electrophoresis is a versatile biochemical technique to detect genetic variation within and between the species or popula-

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TABLE 3 : Antibacterial efficacy of *Passiflora mollussima*

Nature of Extract	Conc. Inμl	Whole plant-*in vivo* extract activity against the bacterial inhibition zone in mm						Callus tissue-*in vitro* extract activity against the bacterial inhibition zone in mm					
		EC	*SA*	*PA*	*KA*	*A*	*S*	*EC*	*SA*	*PA*	*KA*	*A*	*S*
Ethanol	100	3	3	0	0	5	3	5	3	0	2	5	3
	125	5	3	0	0	5	5	5	3	0	2	6	5
	150	8	3	1	1	6	8	6	3	1	3	8	8
	175	7	5	1	1	7	7	8	5	2	2	6	6
	200	8	7	1	1	7	7	8	8	2	2	6	6
	225	8	7	1	1	7	7	9	8	2	2	6	8
	250	8	8	1	1	7	7	11	9	2	2	8	8
Methanol	100	3	5	1	1	2	2	2	5	2	2	2	2
	125	3	6	1	1	3	5	2	5	2	2	3	3
	150	5	8	2	2	3	6	5	6	2	2	3	5
	175	8	8	2	2	5	6	5	7	2	2	5	6
	200	8	8	3	3	5	8	5	7	2	3	5	8
	225	8	8	5	5	5	8	8	7	3	5	5	8
	250	8	9	5	5	6	9	8	8	3	5	6	7
Chloroform	100	5	6	2	3	3	3	3	5	2	3	3	3
	125	5	6	2	3	4	3	5	6	2	3	3	3
	150	5	7	3	3	5	6	5	7	3	3	5	6
	175	8	9	5	3	5	7	6	7	3	3	5	7
	200	7	10	5	5	6	7	6	8	3	5	6	7
	225	7	10	5	5	6	7	8	8	3	5	6	7
	250	8	10	5	5	7	9	8	9	3	5	8	9
Petroleum ether	100	5	3	2	3	3	3	3	3	2	2	3	3
	125	5	3	2	3	3	3	3	3	2	2	3	3
	150	5	5	3	3	5	6	3	5	2	3	5	6
	175	8	6	5	3	5	7	8	5	2	3	5	7
	200	7	6	5	5	6	7	7	6	2	5	6	7
	225	7	6	5	5	6	7	7	6	3	5	6	7
	250	8	8	5	5	7	9	8	8	3	5	6	7
Isopropanol	100	3	2	2	2	5	3	3	2	2	2	5	3
	125	5	3	2	2	5	5	3	2	2	2	5	5
	150	8	3	2	2	6	8	6	3	2	3	6	8
	175	7	5	2	2	7	7	6	3	3	3	6	6
	200	9	7	2	2	7	7	7	6	3	3	8	8
	225	9	7	2	2	7	7	8	8	4	3	8	8
	250	10	8	2	2	7	7	8	8	4	3	8	7
Benzene	100	2	3	2	2	2	2	2	3	2	3	2	2
	125	2	3	2	2	2	2	2	3	2	3	2	2
	150	3	4	2	2	3	3	3	3	2	3	2	3
	175	5	5	2	2	3	3	5	5	2	3	2	3
	200	5	5	2	3	3	3	5	5	2	3	2	3
	225	6	6	3	3	3	4	6	5	2	3	2	3
	250	6	6	3	4	4	4	6	6	2	3	2	3

M: Mother plant; C: Calli mediated plantlets; (+) - Low; (++) - High and (-) - Absent

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tions. The genetic similarity co efficiencies provide to summarization of isoenzyme data for inter sample comparative studies and characterization. Nowadays, molecular diagnostic techniques are applied to estimate the extent of genetic diversity within and between populations. Unlike, morphological markers, biochemical markers are not prove to environmental influences and port ray the genetic relationships between plant groups. Isozymes are adaptable tool for the species diversity analysis within and between the species of the plants and animals[24-27]. In addition, most of population DNA based marker studies provides the same type of information as isozymes[28]. Isozyme provides relatively simple and inexpensive method of attaining genetic information. Most of the population, conservation and rescue projects hold the role 'the cheaper and better' because cost can be crucial point.

The present study has produced a valuable callus production and somoclonal variant protocol for the horticulturally and medicinally important plant *Passiflora mollussima*, which thus constructed the way for the large scale production of this medicinally and horticultrally potential plant. Also, the phytochemical, antibacterial activity and isozyme analysis will very much useful in future research dealing with pharmaceuticals and molecular plant systematic.

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