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Comparative phytochemical and Isoperoxidase Studies on leaf and Leaves derived callus of *Solanum anguivi* Lam

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ABSTRACT

The present study was intended to produce a protocol for large scale production of callus and compare the phytochemical constituents and isoperoxidase profile of leaves and calli of Solanum anguivi Lam. Maximum percentage of friable callus (86.3 ± 0.94) proliferation was obtained on MS medium supplemented with 1.0 mg/l of 2, 4-D. The phytochemical study revealed that the high degree of steroids, alkaloids, phenolic compounds, flavanoids, tannins present in leaves derived calli and calli induced secondary calli. A total of 23 bands in seventeen different positions with seven activity regions (PRX1-7) were observed in the isoperoxidase enzyme system of Solanum anguivi. The leaves showed their uniqueness by the presence of following bands MW-Rf – 0.1250, 0.2125, 0.3250, 0.5000, 0.5750 and 0.6500. The primary calli expressed their individuality by the occurrence of MW-Rf 0.0875, 0.2875, 0.3625 and 0.5250. The MW-Rf 0.0750, 0.4500, 0.4875 and 0.5875 are present only in secondary calli (Calli mediated calli). We observed the genetical difference indicates the possibility for the differences due to the morphogenesis activity of the plant not because of chemical constituents.

Keywords: Callus, Isoperoxidase, Leaves, *S. anguivi*, Phytochemistry.

INTRODUCTION

Studies on plant secondary metabolites have been increasing over the last 50 years. For centuries, mankind is totally dependent on plants as source of carbohydrates, proteins and fats for food and shelter. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. Over 80% of the approximately 30,000 known natural products are of plant origin [1-3]. Biotechnology offers an opportunity to exploit the cell, tissue, organ or entire organism by growing them *in vitro* and to genetically manipulate them to get desired compounds. Since the world population is increasing rapidly, there is extreme pressure on the available cultivable land to produce food and fulfill the needs. Therefore, for other uses such as production of pharmaceuticals and chemicals from plants, the available land should be used effectively. Hence, it is appropriate to develop modern technologies leading to plant improvement for better utilization of the land to meet the requirements. Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites [4-12]. There are a number of plant cell cultures producing a higher amount of secondary metabolites than in intact plants [13-16]. However, there are still problems in the production of metabolites by cell cultures resulting from the instability of cell lines, low yields, slow growth and scale-up problems [17]. *Solanum anguivi* Lam. is a rare ethanomedicinal herb belonging to the family Solanaceae. The plant is used as therapeutic agent for various diseases. The roots are carminative and expectorant useful in coughs, cultarrhal affections, dysuria, colic, nasal ulcers, ingredient of dasamula, asthma, difficult parturition, tooth ache, cardiac disorder, worm complaints, spinal guard disorder, nervous disorder and fever. The leaves and fruits rubbed up with sugar are used as external application for itch [18, 19]. The stem, fruits, roots, flowers and leaves contain glycoalkaloids, steroidal alkaloids, solamargine and solasoline [20]. A growing demand of solamargine and solasoline from *Solanum* in pharmaceutical industry has resulted in serious reduction as a consequence of unforeseen climatic conditions, which affects the production capacity and deforestation. The major constraint in conventional propagation through seeds is the high mortality of seedlings in early stage and always the possibility of losing the mother plant during this process. *In vitro* propagation methods offer highly efficient tools for medicinal plants useful for pharmaceutical industry [21-25]. Successful development of callus induction is a prerequisite for organogenesis and somoclonal variation and for genetic transformation. Attempts have been made to achieve shoot proliferation from nodal explants of *Solanum anguivi* [26]. However, there is no literature available on callus induction and phytochemical studies on *in vitro* derived callus of *S. anguivi*. To fulfill the lacuna, the present study was intended to produce a protocol for large scale production of callus and compare the phytochemical constituents of the *in vivo* and *in vitro* tissues of *S. anguivi*. In addition the object of present study is to elucidate the isoperoxidase pattern and variation for the leaves and leaves mediated calli and secondary calli induced from primary calli of *S. anguivi*.

MATERIALS AND METHODS

The young leaves of *Solanum anguivi* Lam were collected from the green house raised plant. The leaves were washed with running tap water and surface sterilized in 0.1 (w/v) $HgCl_2$ solutions for 60 sec. After rinsing 3-4 times with sterile distilled water, leaves were cut into smaller segments (1cm) and placed horizontally on solid basal Murashige and Skoog [27] (MS)

medium supplemented with 3% sucrose, 0.6% (w/v) agar (HIMEDIA, Mumbai) and different concentration and combination of 2, 4-D, BAP, Kin, NAA and IAA. The pH of the medium was adjusted to 5.8 before autoclaving at 121^{o}C for 15min. The cultures were incubated at 25 $\pm$ 2^{o}C under cool fluorescent light (2000 lux 14 hr photoperiod). The callus cultures maintained for a period of over 10 months by periodic sub-culturing with 15 days intervals on to fresh callus proliferating medium. The experiments were repeated in triplicate and the results were recorded in the Table 1. Growth and phytochemical assays were performed at the 21st day. Consequently, the callus were harvested at the transfer age of 3 to 4 weeks, kept at above 90°C for 3 to 5 minutes at hot air oven to inactivate the enzyme activity followed by continuous drying at 50 to 60°C, till a constant weight was obtained and these callus were further exploited for extraction, phytochemical analysis. Dried *in vitro* derived callus were powdered using the electric homogenizer exhaustively extracted with ethanol, chloroform and benzene using the soxhlet apparatus for 8 hrs. The preliminary phytochemical screening was performed according to the Harborne [28] method and tabulated the results. For isoperoxidase analysis, mother plant and leaves segments derived calli were harvested and ground on ice cold mortar and pestle with 0.1 M phosphate buffer (pH 7 .0). The slurry was centrifuged at 10,000 rpm at 4^{o} 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 [29] 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) [30]. 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 was initiated from the leaves on the MS basal medium supplemented with different concentrations and combinations of 2, 4-D (Table 1). Maximum percentage of friable callus (86.3 ± 0.94) proliferation was obtained on MS medium supplemented with 1.0 mg/l of 2, 4-D. The callus obtained from leaf was white in colour on MS medium supplemented with 2, 4-D. The effect of 2, 4-D in the induction of callus was also reported by Manickam *et al*., [31] in *Withania somnifera,* Johnson *et al.,* [32] in *Rhinacanthus nasutus,* Johnson [33] in *Phyllanthus amarus.* In the present study we obtained maximum percentage of callus on MS medium supplemented with 2, 4-D. Our result was directly consonance with the Manickam *et al*. [31] observation on *Withania somnifera*. Friable and Semi-friable calli were obtained from leaves segments. Friable calli was creamy white in colour and semi-friable calli was in yellowish brown in colour. When the concentration of auxins increases the calli morphology and colour also varied. In high concentration of auxins, the callus was hard and dark yellowish brown in colour (Table - 1). The accumulation of phytochemicals in 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 the desired phytochemicals [34]. Currently, scientists are using metabolic engineering and genetic transformation techniques to improve or modify the secondary metabolic content of medicinal and aromatic plants [35].

Table-1: Effect of 2, 4 - D on Callus formation on Leaves of *Solanum anguivi* Lam

MS + 2, 4 - D (mg/l)	Mean Percentage of Callus induction ± S.E.	Friable Callus	Semi – Friable Callus	Compact Callus
0.0	-	-	-	-
0.5	68.2 ± 0.28	+++	+++	-
1.0	86.3 ± 0.94	+++	++	-
1.5	77.5 ± 0.43	++	++	-
2.0	65.3 ± 0.59	++	+	-
2.5	57.4 ± 0.56	+	+	+

+ - callus formation, - absence of callus formation; + - Low, ++ - Medium, +++ - High.

In the present study also we compared the phytochemcial constituents of the leaves, leaves derived calli and calli derived calli of *S. anguivi*. The phytochemical study revealed that the high degree of steroids, alkaloids, phenolic compounds, flavanoids, tannins present in leaves derived calli and calli induced secondary calli. Different kinds of solvents (ethanol, benzene and chloroform) were used for extraction, of which ethanol extracted solvents showed maximum values (7/11) compared to others (Table - 2).

The isoperoxidase study produced the isozymic pattern for the Leaves (M), Leaves derived primary calli (C) and calli induced secondary calli (Cs). A total of 23 bands in seventeen different positions with seven activity regions (PRX1-7) were observed in the isoperoxidase enzyme system of *Solanum anguivi*. Region one contained two bands, of which PRX 1^1 (0.0750) is present only in Secondary calli with restriction. PRX 1^2(0.0875) showed their unique presence only in leaves derived calli.

Table - 2: Phytochemical Analysis on the Leaves, Leaves derived calli and calli mediated calli of *S. anguivi*

Experiments	Leaves			Leaves derived calli			Calli derived calli		
	Et OH	Ben	Chl.	Et OH	Ben	Chl.	Et OH	Ben	Chl.
Steroids	+	+	++	++	++	+++	++	++	+++
Triterpenoids	-	-	-	-	-	-			
Reducing Sugar	+	-	+	+	-	+			
Sugars	+	+	+	+	+	+++	++	+	+++
Alkaloids	+	+	-	++	+	-	+++	+	-
Phenolic compound	+	+	+	++	++	+++	++	+++	+++
Flavanoids	+	+	-	+++	++	-	+++	+++	-
Catachin	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-
Tannins	+	+	++	++	++	+++	+++	+++	+++
Anthro quinone	-	-	-	-	-	-	-	-	-

(+) - Presence of the metabolites; (-) - Absence of the metabolites

Region two expressed two bands PRX 2^1(0.1250) is present only in leaves and PRX 2^2 (0.1875) was jointly present in leaves derived calli and secondary calli. Region three demonstrated with six bands in four different positions. PRX 3^1(0.2125) showed their unique presence in Leaves, PRX 3^2(0.2375) is commonly present in leaves derived calli and secondary calli; PRX 3^3(0.2750)

is shared by Leaves and secondary calli and PRX 3^4(0.2875) is present only in leaves derived calli. Region four illustrated with two bands (PRX 4^1 and PRX 4^2) in two different positions (0.3250 and 0.3625). PRX 4^1 expressed only in leaves and PRX 4^2 showed only in leaves derived calli. Region five expressed five bands with four different Rf values, PRX $5^{2\ \&3}$ (0.4500 and 0.4875) are unique to secondary calli; PRX 5^4 (0.5000) for leaves. PRX 5^1 (0.4125) is shared by leaves and leaves derived calli. Region six contained three bands in three different banding positions. PRX 6^1 (0.5250) is showed the restricted presence in leaves derived calli; PRX 6^2 (0.5750) for leaves, PRX 6^3 (0.5875) for secondary calli. Region 7 expressed only one band, PRX 7^1 is unique to leaves (Table – 3). The leaves showed their uniqueness by the presence of following bands MW-Rf – 0.1250, 0.2125, 0.3250, 0.5000, 0.5750 and 0.6500. The primary calli expressed their individuality by the occurrence of MW-Rf 0.0875, 0.2875, 0.3625 and 0.5250. The MW-Rf 0.0750, 0.4500, 0.4875 and 0.5875 are present only in secondary calli (Calli mediated calli) Fig. 1.

Fig. 1. Isoperoxidase profile of the *Solanum anguivi* Lam, M – Leaves; *In vitro* raised calli (C) and Calli mediated Calli (Cs)

Electrophoresis is a versatile biochemical technique to detect genetic variation within and between the species or populations. Isozyme joins with electrophoresis 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. Nowadays, molecular diagnostic techniques are functional to estimate the degree 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. Isoenzymes have proven to be reliable genetic markers in breeding and genetic studies of plant species, due Leaves (M), Leaves Derived raised Primary calli (C) and Secondary Calli (From primary) to consistency in their expression, irrespective of environmental factors.

Table 3: Isoperoxidase profile of the Leaves and Leaves derived calli of *S. anguivi*

MW- Rf	Region and Locality	Leaves (M)	Leaves Derived Primary Calli (C)	Secondary Calli (Cs)
0.0750	$PRX1^2$	-	-	+
0.0875	$PRX1^3$	-	+	-
0.1250	$PRX2^1$	+	-	-
0.1875	$PRX2^2$	-	+	+
0.2125	$PRX3^1$	+	-	-
0.2375	$PRX3^2$	-	+	+
0.2750	$PRX3^3$	+	-	+
0.2875	$PRX3^4$	-	+	-
0.3250	$PRX4^1$	+	-	-
0.3625	$PRX4^2$	-	+	-
0.4125	$PRX5^1$	+	+	-
0.4500	$PRX5^2$	-	-	+
0.4875	$PRX5^3$	-	-	+
0.5000	$PRX5^4$	+	-	-
0.5250	$PRX6^1$	-	+	-
0.5750	$PRX6^2$	+	-	-
0.5875	$PRX6^3$	-	-	+
0.6500	$PRX7^1$	+	-	-

In the present study the isoenzyme system was used to reveal the phytochemical and biochemical variation. This study was further confirmed and supplemented the observations of Agarwal and Subhan [36] while studying *Centella asiatica* (Linn.), Johnson et al. [32] in the case of *Rhinacanthus nasutus* and Johnson [33] *Phyllanthus amarus*. The growth and the differentiation in a large number of different plants are regulated by hormones. Hormonal regulation of plant peroxidase (EC.1.11.1.7) has also been the subject of intensive study [37- 38]. Peroxidase exists in multiple forms [39] and the change in isoperoxidase patterns occur after application of growth regulators in higher plants and plant tissue cultures [40].The increased peroxidase activity in slowly growing tissues and dwarf plants suggest that peroxidase may be a growth inhibiting factor in the plant due to the IAA oxidase activity of some peroxidases [41-43]. The involvement of plant peroxidase in lignifications and shoot formation were suggested by many workers [44-48]. Many physiologists observed increases in total peroxidase and isoenzymes of peroxidase activities during shoot formation. In the present study we observed that the leaves, leaves derived calli and secondary calli showed seven bands in the isoenzyme system. We are not observed any differences in the quantitative (Number of bands) profiles but the banding positions are varied with different Rf values. Our results further strengthen the previous observations. It has also been reported that significant increase in total activity and changes in isoenzyme pattern of peroxidase occurs in the process of shoot differentiation [46].

In certain regions, more than one distinct bands are resolved; these bands could represent allelic isozymes, coded by different alleles of the same gene at a locus and thus occupy that particular zone on the system. In the present study also similar kind of banding profiles are obtained except

the seventh region and all other regions are expressing the presence of multiple alleles. Preliminary phytochemical screening of *Solanum anguivi* leaves and leaves derived calli shows the presence of alkaloids, tannins, flavanoids, phenolic group and steroids and absence of anthroquinones and catachin in leaves and leaves derived calli. There is similarity in the distribution of such major chemical groups with varied degree of presence, primary and secondary calli showed high degree metabolites presence than the leaves. We observed the genetical difference indicates the possibility for the differences due to the morphogenesis activity of the plant not because of chemical constituents.

The present study has produced a valuable callus production protocol for medicinally important plant *Solanum anguivi* Lam, which thus constructed the way for the large scale production (high value compounds) with low volume of explants. In addition, the present study produced the phyochemical and biochemical marker, it will very much useful in future research dealing with pharmaceuticals and molecular plant systematic.

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