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Production of solasodine by *Solanum laciniatum* using plant tissue culture technique

Pooja Bhatnagar, Madhurima Bhatnagar, Amarjit K Nath* & D R Sharma

Department of Biotechnology, Dr Y.S.Parmar University of Horticulture and Forestry, Solan 173 230, India

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Leaf and hypocotyl explants of 15 days old aseptically grown seedlings of *Solanum laciniatum* were cultured on MS medium supplemented with NAA (2 mg/l) and kinetin (0.5 mg/l) for callus initiation. For maintenance and proliferation of callus MS medium supplemented with 2,4-D (1 mg/l) and kinetin (0.5 mg/l) was used. The growth of the calli derived from hypocotyls increased with time of incubation and remained almost constant after 45 days. The solasodine content in callus culture was maximum after 30 days of incubation. Addition of L-arginine in the medium (50-150 mg/l) increased growth as well as chlorophyll content in the callus culture. The solasodine content also increased up to 1.2 to 1.4 times in these cultures. High frequency shoot regeneration was obtained in MS medium having BA (4 mg/l) and IBA (0.25 mg/l). For shoot multiplication, MS medium having BA (4mg/l) was used. Shoots rooted on the same medium. Organogenesis promoted solasodine accumulation in the cultures. Regenerated shoots yielded higher solasodine content than undifferentiated as well as organogenic callus. Solasodine contents in the regenerated shoots was found to be 10 times higher than the callus culture and approached towards the field grown plants. Thin layer chromatography revealed the presence of three compounds. The most predominant spot (R_f 0.789) corresponded to the reference solasodine.

Keywords: L-Arginine, Callus culture, Organogenesis, Solanum *laciniatum,* Solasodine

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Higher plants produce a variety of secondary metabolites. These compounds vary in concentration and yield with growth and development of plant. Active principle contents of these plants such as steroids, alkaloids, glycosides etc. present as secondary metabolites in medicinal plants are of great value in drug and pharmaceutical industry[1]. Synthetic steroid drugs are quiet costly and therefore, one has to depend upon some easily and cheaply available raw materials, which can be converted into different types of steroid drugs. Diosgenin (a steroid sapogenin) is considered as the best starting material for synthesis of steroid drugs. Diosgenin produced from *Dioscorea* tubers is used for synthesis of corticosteroidal drugs. It is efficiently converted into 16-dehydro pregnenolone acetate (16- DPA), which is further used for preparation of different steroid drugs. However, ruthless and injudicious exploitation of their resources have considerably depleted their population from natural habitats. Therefore, to ensure sustained supply of raw materials to steroid drug industry, alternate plant source have been considered worth exploitation[2]. Tissue cultures and the methodologies necessary for production of transgenic target plants (e.g. using *Agrobacterium* vectors, biolistic techniques or electroporation) are being considered for improving the biosynthetic potentialities in various *Solanum* species. Although, diosgenin has also been identified in other species such as *Costus speciosus, Trigonella foenumgraecum* and *Kallastroemia pubescens,* but neither of these sources have been considered economically profitable due to low diosgenin percentage[2]. Solasodine a natural analogue of diosgenin is a good substitute for diosgenin. *Solanum laciniatum* has emerged as a rich source of solasodine for commercial exploitation as it is found in sufficiently good quantity in all the aerial parts of this plant. *Solanum laciniatum* commonly known as kangaroo apple is a hardy, short, perennial plant with a natural range extending from southern tip of New Zealand (46.5°S) to as far as Adelaide (35°S). It is preferably grown in sub-temperate climatic conditions with annual rainfall ranging between 60 and 150cm. Whole plant of *Solanum laciniatum* typically contain up to 3 per cent solasodine (on a dry wt basis)[3]. Due to uncontrolled exploitation of the natural habitat and fluctuations in supply of raw materials, the production of secondary metabolites by plant cell cultures *in vitro* has proved to be a viable alternative. Cell cultures of *Solanum* species are known to harbour steroidal alkaloids[4]. Manipulating various cell culture parameters such as growth regulators, source of culture, temperature and dependence on macronutrients, alter the level of secondary metabolite production. Keeping in view the importance of solasodine as a source for pharmaceutical industries, the

*Correspondent author
Phone: 91 01792 252310
E-mail: amitnath2003@yahoo.co.in

present studies were undertaken to investigate and compare the biosynthetic potentiality of the tissue cultures of *Solanum laciniatum.*

Plant material—The healthy seeds of *Solanum laciniatum* were thoroughly washed with tap water to remove any extraneous material. They were then soaked for 24 hr. Soaked seeds were surface sterilized with bavistin (0.1 %) for 5 min followed by $HgCl_2$ (0.1%) for 2 min. The sterilized seeds were blotted on pre-sterilized blotting paper and then germinated in White's medium in dark.

Source of explants—Hypocotyl segments of 15 days old aseptically grown seedlings of *Solanum laciniatum* were used as source of explants.

Establishment of initial cultures—For establishment of initial cultures, the segments of the hypocotyls were cultured on MS medium supplemented with different concentrations of indole-3-acetic acid (IAA) + kinetin and 1-naphthalene acetic acid (NAA) + kinetin. The cultures were then incubated for the formation of callus under 16 hr photoperiod (1.5 Klux) at 25° ± 2°C. The nature and growth of callus was recorded after every 15 days.

Callus multiplication—The calli induced during the initial stages were sub- cultured on different media containing varying concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin for proliferation.

Addition of L-arginine—Various concentrations of L-arginine (50- 150 mg/l) were added in the medium for callus maintenance.

Organogenesis—Various combinations of growth regulators were tried along with control to induce organogenesis.

Shoot multiplication and plant regeneration—Multiplication of shoots was carried out by the method of enhanced release of axillary buds[5]. The shoots obtained were sub-cultured after 4 weeks in appropriate medium for further multiplication and were maintained on the same medium.

Measurement of growth and estimations of solasodine content in callus and shoots—Growth was measured as dry weight yield per flask. The tissue was dried overnight at 60°C till constant weight. Solasodine content in callus and regenerated shoots was estimated by the colorimetric technique[6] with slight modification at extraction stage. One gram dried powdered sample was extracted twice with 95% ethanol for 1 hr in a boiling water bath. The combined extracts were filtered and the filtrate was evaporated to dryness. The dried residue was dissolved in 3 ml of 1*N* hydrochloric acid and hydrolyzed for 2 hr at 100°C in a water bath. The hydrolysate was neutralized with 3 ml of 1 *N* sodium hydroxide. To this, 2 ml of acetic acid was added and final volume of 10 ml was made with distilled water. Further, aglycone was complexed with 1 ml of 0.05% aqueous methyl orange in acetate buffer (*p*H 4.7) for the colorimetric estimations. The methyl orange aglycone complex was extracted with 10 ml chloroform and its absorbance was read at 420 nm in a spectrophotometer against reagent blank. Solasodine content was expressed as mg/g dry wt of callus/organogenetic callus/elongated shoot. For the identification of aglycone, the neutralized hydrolysate was extracted in chloroform and chromatographed on thin layer plate of caustic silica gel G using chloroform:methanol (95:5) as solvent. Developed plates were kept in iodine jar and R_f value was calculated. Solasodine was identified by thin layer chromatography with authentic standard procured from Sigma-Aldrich Fine Chemicals.

Stastistical analysis—The data recorded for the different parameters were subjected to completely randomized design[7]. The statistical analysis based on mean values per treatment was made using analysis of variance technique for CRD using minimum of six replications.

Seeds treated with 0.1% bavistin for 5 min followed by sterilization with 0.1% mercuric chloride for 2 min showed significantly high per cent survival (72.17%). Seeds of *Solanum* species have been surface sterilized using various sterilants viz., chlorine water[8] and 10 per cent sodium hypochlorite[9]. *In vitro* germination in White's medium was initiated after 15 days of incubation. The per cent germination was significantly higher in White's medium (75%) and least in MS basal medium (33.33%) and the size of the *in vitro* seedlings was also longer. These findings have been also reported by other workers[8] in *Solanum khasianum.* Murashige and Skoog medium supplemented with NAA (2 mg/l) and kinetin (0.5 mg/l) was found to be the best for callusing using hypocotyl explants of 15 days old aseptically grown seedlings of *Solanum laciniatum.* Callus initiation started within 15-20 days from the cut ends of the explants. Friable callus from hypocotyls explants of *in vitro* germinated 3 weeks old seedlings of *Solanum laciniatum* was also reported by other workers[10]. Hypocotyl explants were used for callusing in *Solanum khasianum, Solanu*

dulcamara, and *Solanum malacoxylon* by earlier workers[8,9,11,18]. Callus induction in *Solanum dulcamara* using Erikson medium supplemented with NAA (1 mg/l) and kinetin (0.02 mg/l) was also reported[9]. For callus proliferation, comparatively faster growth was observed in MS medium containing 2,4-D (1.0 mg/l) and kinetin (0.5 mg/l). The calli were loose and friable. The first sub-culture was done at an interval of 5 weeks and later on at an interval of 4 weeks. The growth of callus was rapid on MS medium containing 2, 4-D (1 mg/l) and kinetin (0.5 mg/l). In *Solanum khasianum* callus was obtained using 2, 4-D (1 mg/l) and kinetin (0.25 mg/l)[8]. Friable callus of *Solanum malacoxylon* was obtained on MS medium supplemented with 2, 4-D (1 mg/l) and kinetin (0.3 mg/l) using hypocotyls, root and leaf explant[11]. On the other hand, callusing in leaf explants of *Solanum khasianum* was reported on MS medium containing 2, 4-D (3 mg/l) and kinetin (1 mg/l)[12]. The colour of the callus after sub-culturing turned creamish, there was, however, no change in the colour of the medium. The callus cultures were harvested at an interval of every 15 days. Maximum growth in callus cultures was found after 60 days. These cultures had a fresh weight of 2.339 g, which were, statistically at par with the 45 day old cultures. Maximum dry weight of 0.222 g was recorded in the 45 days old cultures, which was statistically at par with 60 days old ones (Table 1). Solasodine content (0.361 mg/g dry wt) of 30 days old callus was significantly higher and declined thereafter in 45 and 60 days old callus cultures (Table 1). The decline in solasodine content after 30 days of incubation may be as a result of senescence in these cultures. Solasodine content in callus cultures of *Solanum nigrum* grown under 16 hr photoperiod remained more or less constant throughout the incubation period of 39 days[13]. Addition of L-arginine (50-150 mg/l) in the medium for callus maintenance significantly increased the fresh and dry weight of the callus cultures. Visual observations indicated that addition of L-arginine also increased the chlorophyll content in the callus cultures. The fresh weight of the callus cultures to which L-arginine in different concentrations (50-150 mg/l) were added ranged from 2.531 to 5.122 g (Table 2).Increase in fresh and dry weight of these cultures was significantly higher than the control (1.612 g). Highest dry weight was found in the culture containing L-arginine (150 mg/l). Highest solasodine content (0.4277 mg) was recorded in the cultures growing on the medium to which L-arginine (100 mg/l) was added. The least solasodine content was found in case of control treatment (0.3004 mg) and it was significantly lower than all other treatments. Green callus produced in the presence of 2, 4-D and kinetin was cut into small pieces, approx 500-700 mg and was used for shoot regeneration. Roots appeared in the initial stages followed by shoot development. Shoot regeneration was recorded after about 3 weeks of incubation, the number of shoots per callus piece could not be counted as rosette was formed. Although, 6-benzylaminopurine (BAP) alone as well as in combination with NAA induced shoot regeneration on callus, the best results were obtained on MS medium containing BAP (2.5 mg/l) and 3-indolebutyric acid (IBA; 0.25 mg/l). *In vitro* established shoots were multiplied on MS medium supplemented with BAP (4 mg/l). Multiplication rate was 6-8 shoot per axillary bud sub-cultured. Fresh weight of the green callus with minute shoots and rootlets was 1.787 g per flask. The fresh weight of the regenerated shoots after 5 weeks of incubation was 9.093 g per flask. Dry weight of the morphogenic

Table 1—Effect of incubation periods on the growth and solasodine content of callus cultures of *Solanum laciniatum*

[Values are mean ± SD of 8 replications]

Days in culture	Fresh wt (g)	Dry wt (g)	Solasodine conten (mg/ g dry wt)
15	0.506±0.050	0.047±0.005	0.187±0.010
30	1.570±0.379	0.154±0.041	0.316±0.011
45	2.327±0.213	0.222±0.051	0.289±0.010
60	2.339±0.235	0.221±0.023	0.254±0.021
$CD_{0.05}$	0.272	0.027	0.015

Table 2—Effect of different concentrations of L-arginine on growth and solasodine content of callus cultures of *Solanum laciniatum* after 4 weeks of incubation

[Values are mean ± SD of 7 replications]

Treatment	Conc.of L-arginine (mg/l)	Fresh wt (g)	Dry wt (g)	Solasodine (mg/g dry wt)
TM_1 (Control)	—	1.612±0.398	0.155±0.042	0.300±0.008
TM_2	50	2.531±0.489	0.222±0.043	0.402±0.017
TM_3	100	3.606±0.407	0.341±0.040	0.427±0.012
TM_4	150	5.122±0.306	0.470±0.030	0.387±0.044
$CD_{(0.05)}$	—	0.483	0.046	0.030

TM_1-MS basal medium+2,4-D (1mg/l)+kinetin(0.5mg/l); TM_2-MS basal medium+2,4-D (1mg/l)+kinetin(0.5mg/l)+ L-arginine(50mg/l); TM_3-MS basal medium+2,4-D (1mg/l)+ Kinetin(0.5mg/l)+ L-arginine(100mg/l); TM_4-MS basal medium+ 2,4-D (1mg/l)+ kinetin(0.5mg/l)+ L-arginine(150mg/l)

callus was 0.133 g, whereas the shoots had a dry weight of 0.890 g/flask. Regenerated shoots showed root regeneration on the same medium used for shoot multiplication after 6 weeks. Shoots continued to grow even after root regeneration and formed new leaves. Development of roots and root hairs during initial stages of organ differentiation was reported in *Solanum khasianum*[14]. Maximum shoot induction in *Solanum laciniatum* was also reported on MS medium containing BAP (2.25 mg/l) and IBA (0.2 mg/l)[15]. In *Solanum dulcamara,* N^6-benzyladenine (BA) alone or in combinations with IAA was reported to be stimulatory for shoot regeneration[13]. Shoot induction in callus cultures of *Solanum khasianum* was reported on MS medium supplemented with BA (3 mg/l)[12]. The solasodine content in the shoots (approx 4.2 cm) was higher (3.057 mg/dry wt), while the differentiated callus had a significantly lower amount of solasodine (0.379 mg/g dry wt). The present studies indicated that the *in vitro* elongated shoots had higher solasodine content as compared to the organogenic as well as undifferentiated callus. The regenerated shoots yielded 10 times more solasodine than undifferentiated callus cultures. Young leaves from field grown *Solanum laciniatum* plants contained 4.12 mg solasodine per gram dry weight. Shoots of *Solanum laciniatum* cultured under photoautotrophic or photoheterotrophic conditions had solasodine yields considerably higher than that of the callus cultures and approached those of field-grown plants[16]. Thin layer chromatography revealed the presence of three compounds in the chloroform extract obtained from hydrolyzed callus, as well as shoots using chloroform:methanol (95:5) as the solvent system. The most predominant spot was of solasodine (R_f 0.789) and corresponded to the reference solasodine. One spot corresponding to the authentic sample of solasodine using chloroform:methanol (19:1) as solvent system was reported in *Solanum khasianum* Clarke. However, in *Solanum laciniatum* using chloroform:methanol:diethylamine (20:2:0.5) as solvent system R_f value of 0.68 was reported[16]. This difference in R_f might be due to different solvent systems used to run TLC. The present studies, thus, indicated that shoots regenerated *in vitro* from callus cultures could be used as an alternate source for the production and continuous supply of solasodine.

References

1 Shailender K, *Studies on variation in solasodine content at different growth and developmental stages of the plant in some Solanum species.* M.Sc. Thesis, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan (HP) 1993.

2 Gauniyal A K, Singh A K & Varmani, O P, Major medicinal plants as foreign exchange earner. Yojana, 31 (1991) 14.

3 Mann J D, Production of solasodine for pharmaceutical industry in Advances in agronomy, Vol 30, edited by Brady N C (Academic Press, New York) 1978, 207.

4 Khanna P, Uddin A, Sharma G L, Manot S K & Rathore A K, Isolation and characterisation of sapogenin and solasodine from *in vitro* tissue cultures of solanaceous plants. *Indian J Exp Biol*, 14 (1976) 694.

5 Murashige T, Plant cell and organ cultures as horticultural practices. Acta Hort, 78 (1977) 17.

6 Birner J, Determination of total steroid bases in *Solanum* species. *J Pharm Sci*, 58 (1969) 258.

7 Gomez K A & Gomez A A, *Statistical procedures for agricultural research*, (John Wiley and Sons, New York) 1984.

8 Chaturvedi H C, Chowdhury A R & Uddin A, Solasodine biosynthesis in seed and seedling callus of *Solanum khasianum* Clarke grown *in vitro, Indian J Exp Biol*, 17 (1979) 107.

9 Ehmke A & Eilert U, Steroidal alkaloid in tissue cultures and regenerated plants of *Solanum dulcamara, Plant Cell Rep*, 5 (1986) 31.

10 Chandler S F, Solasodine yield in callus cultures of Solanum laciniatum Ait selected for growth on hormone free medium, Ann Bot, 54 (1984) 293.

11 Suardi M L, Bernasconi S, Pelizzoni F & Racchi M L, *In vitro* cultures of *Solanum malacoxylon* Sendt: Nutritional requirement and sterol production, *Plant Cell Tissue Organ Cult*, 36 (1994) 9.

12 Bhalsing S R & Maheshwari V Z, *In vitro* culture and regeneration of Solanum khasianum and extraction of solasodine, *J Plant Biochem Biotech*, 6 (1997) 39.

13 Bhatt P N, Bhatt D P & Sussex I, Studies on some factors affecting solasodine content in tissue cultures of *Solanum nigrum*, *Physiol Plant*, 57(1983) 159.

14 Kokate, C K & Radwan S S, Steroidal glycoalkaloids in tissue cultures of *Solanum khasianum* during organogenesis, *Plant Med*, 33(1978) 301.

15 Chandler S & Dodds J, Solasodine production in rapidly proliferating tissue cultures of *Solanum laciniatum* Ait., *Plant Cell Rep*, 2 (1983) 69.

16 Indrayanto G, Erawati T & Santosa M H, Effect of L-arginine, casein hydrolysate, banana powder and sucrose on growth and solasodine production in shoot cultures of *Solanum laciniatum, Plant Cell Tissue Organ Cult*, 43 (1995) 237.

17 Conner A J, Differential solasodine accumulation in photoautotrophic and heterotrophic tissue cultures of *Solanum laciniatum, Phytochemistry*, 26(1987) 2749.

18 Malapthalk N P, Solasodine production in multiple shoot cultures derived from hairy roots of *Solanum khasianum* Clarke in *Plant biotechnology: Recent advances*, edited by Trivedi P C (Panima, New Delhi) 2000, 535.

19 Argolo A C C , Charlwood B V & Pletsch M, The regulation of solasodine production by *Agrobacterium* rhizogenes-transformed roots of *Solanum aviculare. Plant Med.* 66 (2000) 1.