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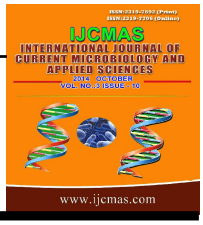

Original Research Article

In vitro Studies and *Agrobacterium* Mediated Transformation on *Solanam nigram L.*

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A B S T R A C T

In this study, in vitro culture of transformation was carried out by co-cultivation of economically important Indian medicinal plant *Solanum nigrum* L. during the infection process of the *Agrobacterium rhizogenes* transferred makes a heritable change in the DNA of the plant and the changes in the formation of a tumor by the plant. Among different explants like node of *Solanum nigrum* L. were cultured on different concentration of hormones (IAA, NAA, 2,4-D IBP and BAP). It was observed that stem explants showed better growth response like enlargement and initiation of callus than stem and node. Therefore, stem explants were used throughout the present study. Hormones play an important role in the yield of solanin. Selection of medium for optimizing the production of secondary metabolites is an important factor.

K e y w o r d s

Tissue culture, In vitro-culture, Agrobacterium rhizogenes, Secondary metabolites-Solanin.

Introduction

Plant tissue culture technique has been successfully utilized on generating genetic variability for selecting better genotypes in many crops in plants. Herbs are staging a comeback and an herbal renaissance is blooming across the world. They have been prized for their medicinal, flavoring and aromatic qualities for centuries and yet for a while they were over shadowed by the synthetic products of the modern civilization. But once having realized their serious side effects people are going back to nature with hopes of safety and security (Irfan Khan, 1999).

The *Agrobacterium* system was first used to transform plant tissue, there have been many developments since then like direct gene transfer of naked DNA to plant, a technique that has also been developed to a high degree of sophistication (Xiang et al., 2000). New emerging gene transfer technologies have enormous potential for plant improvement by introducing foreign genes in plant cells or tissues of both monocot and dicot plants conferring herbicide resistance, insect resistance, viral, bacterial and fungal resistance and genes related to increase shelf life of plan t and plant products especially secondary metabolic products.

Secondary metabolites from plants

alkaloids, flavanoids, saponins and terpenes have played a vital role in pharmaceutical, cosmetic, perfumery, drying and flavor industries. These drug, flavor, essential, oils and colors derived from plants have no apparent function in the plant primary metabolism but often play an adaptive role. Secondary metabolites are commercially feasible and as such for enhancing the in vitro production of natural products.

Due to increasing demand for herbal raw drugs accompanied by unscrupulous and unscientific collection of medicinal herbs in the wild, some of the most valuable plant species are facing extinction. Suitable agro technology has to be adapted to cultivate medicinal plant, which is in bulk demand, and modern methods of propagation inducing plant tissue culture should be evolved to multiply the medicinal plants so as to meet the demand from within and across our country (Suresh kumar et al., 1998).

The very word herbal has become the symbol of safety for these products in contrast to the synthetic ones which has become highly unsafe for human consumption once science revealed their adverse effects on human health and the environment. The revival of interest in natural drugs, especially those derived from plants, started in the last decades mainly of the widespread belief that green medicines are healthier and safer than the synthetic ones.

Materials and Methods

Plant materials

The major plant source for this study was *Solanum nigrum* Linn. (*solanum).* The plants were collected from the Herbal Garden, Departement of plant Science, Bharathidasan University, Tiruchirapalli, for tissue culture studies.

Extraction of explants

Stem and auxillary node explants were used for the present study. The young stem in the shoot apex were collected from the garden grown plant. The explants were excised with the help of sterile forceps and blade. The nodes were cut 0.5 – 1.0 cm sized segments and care was taken that each explants included the midrib portion. Auxillary shoot buds measuring 10 – 15mm in length with 2-3 stem primordial attached were also used.

Surface sterilization was done by using mercuric chloride and alcohol. The explants were treated with 0.01 % mercuric chloride for 1 to 2 minute and washed twice with sterile distilled water. Then the materials were rinsed in 70 per cent alcohol for 2 to 3 minutes and then the explants were thoroughly washed twice with sterile distilled water.

Inoculation

Before starting inoculation, culture tubes containing media, instruments like sprit lamp, sterilized forceps, scissors, Petri dishes and sterilized distilled water transferred to UV chamber and there were exposed to UV light, for 30 min implanted on the medium with abaxial surface In contact with nutrient medium.

Strain collection

Stock culture of *Agrobacterium rhizogenes* was used was used for this study, which were obtained from the Microbial Technology, Sector 39-4, Chandigarh. India. For subculture the strain was inoculated In nutrient agar medium.

Procedure

1. Grown the *Agrobaterium* in 5 ml of YEP medium overnight at 28^0C.
2. Tissue from aseptically grown sterile plants is most commonly employed. Tissues from non aseptically grown plant must be surface sterilized before use.
3. Chopped the tissue into small pieces (about 0.5 cm long).
4. Placed several pieces of plant tissue in a small Petri dish (50mm)
5. Added 50µl of an overnight grown culture of Agrobacterium.
6. Sealed the Petri plate with Para film and co cultivate bacteria with plant tissue for 2 days at 28^0C in the dark
7. Washed the plant tissue with MS medium and blotted them dry with sterilized filter papers.
8. Pre cultured the tissue for a few days on a non-selective callus inducing medium containing
9. After 5-7 days, transferred the tissue to selective medium (shoot inducing) containing 200µg/ml Kanamycin and 100µg/ml Cefotaxime.

Agrobaterium mediated transformation

In first stage, *Agrobacterium rhizogenes* co-cultivated explants were not exhibited any remarkable responses on the callus, basal and shoot induction media. In the second stage, light brown coloured callus were initiated from the cut ends of *Agrobcterium* infected explants on the media containing MS + kanamycin 200 mg/l.

Hairy roots were initiated from the adaxial side of *Agrobacterium rhizogenes* transferred stem explants after the third week of incubation period. Huge numbers of hairy roots originated from the adaxial side of the explants which was light yellowish nature. *Agrobacterium rhizogenes* transferred explants were bulged and large numbers of roots were proliferated. After five weeks it produced cottony or spongy nature of calli with few numbers of prominent roots.

Extraction of Solanine from callus (Harbone, 1973).

Alkaloids constitute the largest class of secondary plant substances. These influence the development of parasite. Solanine, the steroid alkaloid of call is modified terpenoid and is a prohibitin present in several plants. The resistance of solanaceous plants has been attributed to alkaloid.

Materials

Callus, blendor, cheesecloth, water bath at 700C, centrifuge and pH meter.

Reagents

Acetic acid 5% and Conc. NH4OH

Method

Crush the tissue in acetic acid (20mi/g tissue) in a blendor and filter the extract through cheese Centrifuge at low speed and discard the supernatant. Collect the precipitate, dry and weigh the crude solanine.

Estimation of Solanine

Materials

Crude alkaloid extract, test tubes, pipettes, colorimeter.

Reagents

90% of Ethanol, 20% and 60% of H2SO4, sample of solanine.

Method

Dissolved about 50mg of the crude solanine in a 1:1 mixture of ethanol and 20% H2SO4. To 5ml of 60% H2SO4 in a test tube, add 1ml of the alkaloid solution. After 5min, add 2-3 ml of formaldehyde reagent. Allow to stand for 3hr at room temperature and measure the absorbance at 565-570nm in a colorimeter. Calculate the amount of solanine in the crude preparation using reference solutions prepared with pure solanine treated similarly with formaldehyde in 1M H2SO4.

Result and Discussion

Different explants like node of *Solanum nigrum* L.(Fig-1) were cultured on different concentration of hormones (IAA, BAP, NAA and 2, 4-D) it was observed that only stem explants showed growth response like enlargement and initiation of callus. Therefore, stem explants of *Solanum nigrum* L. was used through the present study.

Effect of auxins on the growth of stem explant of *Solanum nigrum L.*

Stem explants cultured on MS basal medium supplemented with different concentration of IAA (0.5, 2.0 and 50 mg/l) showed varied responses. Stem explants produced callus or both callus and root in 1 and 2mg/l of IAA (Fig-2&3). Maximum callusing and highest frequency of callus forming explants was observed on medium with IAA 1mg/l (Table-1).

Out of the different concentrations of the 2,4-D (0.5 -5 mg/l) used, the maximal growth In the maximal growth in terms of fresh and dry weight was observed at 1 and 2mg/l. the lower and higher concentrations induced moderate amount of callus. These hormone proliferated callus only from the stem explants.

2,4-D induced callus at all concentrations on stem explants. Callus was initiated on the cut ends of the stem explants after 8 and 10 days respectively. Callus was brown, compact and non friable. Maximum callusing was observed on the medium supplemented with 1 and 2 mg/l of 2, 4-D. 0.5, 1 and 2 mg/l of this hormones induced callus were golden brown (Table-2,Fig-6&7).

NAA in the concentration of 0.5 – 5 mg/l was used and it was showed minimum responses than other two auxins. Among the all among the all concentrations of this hormone induced callus and shoot from the stem explants and other higher concentrations induced callus and shoot from the stem explants (Fig-8) and other higher concentrations induced minimum amount of callus (Table-3,Fig-9).

Effect of cytokinin on growth of stem explant of *Solanum nigrum L.*

The different concentrations of BAP (0.5 -5 mg/l) used, 0.5 mg/l was found to be more effective in promoting growth from the stem explants (Fig-4) and at 2mg/l minimal growth was observed. Small shoot buds originated with callus was observed on the stem explants supplemented with lower concentrations of this hormone (Table-4).

Estimation of solanin

The availability of solanin percentage in herb was 0.28%. Among the all tested hormones, only 2, 4-D yield least amount of amount of solanin. Only BAP induced callus yielded higher amount of solanin which callus nature was white and highly nodulated. Among the all factorial combination, 1 mg/l of IAA with all concentrations of BAP yielded higher amount of solanin (Table-5).

Table.1 Effect of IAA on the growth of leaf explants of *Solanum nigrum* L.

S. No.	IAA concentration (mg/l)	Growth Response (%)	Weight at harvest (mg) (Mean ± S.E)	
			Fresh Wt.	Dry Wt.
1.	0.5	55	127.5 ± 3.6	15.6 ± 0.64
2.	1.0	77	239.5 ± 2.29	27.7 ± 0.59
3.	2.0	48	106.0 ± 2.04	12.5 ± 0.8
4.	5.0	21	60.0 ± 1.94	8.9 ± 0.7

Incubation period : 6 Weeks
Initial Fresh weight : 16.3 ± 2.12 mg
Initial Dry weight : 4.5 ± 0. 9 mg

Table.2 Effect of 2, 4-D on the growth of leaf explants *Solanum nigrum* L.

S. No.	2,4-D concentration (mg/l)	Growth Response (%)	Weight at harvest (mg) (Mean ± S.E)	
			Fresh Wt.	Dry Wt
1.	0.5	64	150.2 ± 3.22	17.4 ± 0.39
2.	1.0	79	275.7 ± 4.22	28.9 ± 0. 54
3.	2.0	85	330.6 ± 3.31	37.8 ± 1.9
4.	5.0	45	117.5 ± 3.42	13.5 ± 2.8

Incubation period : 6 Weeks
Initial Fresh weight : 14.9 ± 2.6 mg
Initial Dry weight : 2.7 ± 0.3 mg

Table.3 Effect of NAA on the growth of leaf explants *Solanum nigrum* L.

S. No.	NAA concentration (mg/l)	Growth Response (%)	Weight at harvest (mg) (Mean ± S.E)	
			Fresh Wt.	Dry Wt
1.	0.5	83	58.4 ± 4.79	6.2 ± 0.6
2.	1.0	79	39.5 ±3 .48	4.1 ±0.1
3.	2.0	71	24.4 ± 2.22	3.5 ± 0..9
4.	5.0	69	19.5 ± 1.24	2.2 ± 0.4

Incubation period : 6 Weeks
Initial Fresh weight : 14.5 ±2.6 mg
Initial Dry weight : 3.2 ± 0.2 mg

Table.4 Effect of BAP on the growth of leaf explants *Solanum nigrum* L.

S. No.	BAP concentration (mg/l)	Growth Response (%)	Weight at harvest (mg) (Mean ± S.E)	
			Fresh Wt.	Dry Wt
1.	0.5	82	172.4 ± 4.5	19.3 ± 0.7
2.	1.0	77	138.6 ± 4.9	16.8 ± 0.3
3.	2.0	51	109.8 ±47.5	12.4 ± 0.5
4.	5.0	45	78.6 ± 2.3	9.9 ± 0.4

Incubation period : 6 Weeks
Initial Fresh weight : 17.8 ± 4.6 mg
Initial Dry weight : 3.4 ± 0.9 mg

Table.5 Quantitative estimation of alkaloid from *in vitro* tissues of *Solanum nigrum* L.

S.No	Media composition (mg/l)	Solanin (%)
1	Leaves of the herb	0.028
1	IAA	0.022
2	BAP	0.032
3	NAA	0.025
4	2,4-D	-
5	IAA 0.5+BAP *	0.027
6	IAA 1 + BAP *	0.032
7	IAA 2 + BAP *	0.037
8	IAA 5 + BAP *	0.019
9	NAA 0.5+BAP *	0.021
10	NAA 1 + BAP *	0.017
10	NAA 2 + BAP *	0.013
11	NAA 5 + BAP *	-

* All concentrations (0.5 – 5.0 mgl^{-1})

Table.6 Quantitative estimation of solanin from *Agrobacterium rhizogenes* induced hairy roots of ***Solanum nigrum*** L.

S.No	Age of the culture	Solanin (%)
1	Four weeks	0.36
2	Six weeks	0.52
3	Eight weeks	0.88
4	Ten weeks	0.74
5	Twelve weeks	0.42

Fig-1

Fig-2

Fig-3

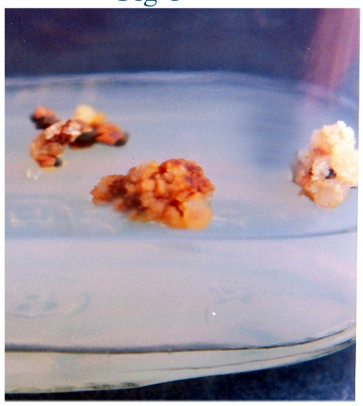

Fig-4

Fig-5

Fig-6

Fig-7

Fig-8

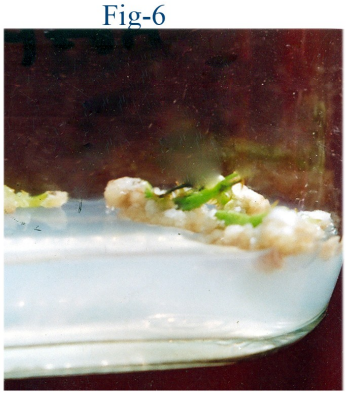

Fig-9

Habite of solanum nigrum plant (Fig-1).

IAA initiated light white callus on the explants especially IAA 0.5 mg/l (Fig.-2).

Shoot buds and roots were developed from explants on IAA 1mg/l (Fig -3), multicoloured friable callus on the media containing BAP 1mg/l (Fig.- 4), Highly nodulated white coloured callus originated from explants on BAP 0.5mg/l (Fig.-5) Maximum callusing was observed on 1 and 2 mg/l of 2, 4-D (Fig. – 6 & 7), Lower concentrations callus were friable and dark brown in colour - NAA 0.5mg/l (Fig. 8), 1 and 2mg/l of the NAA induced milky white coloured callus with prominent roots (Fig.-9).

Agrobacterium rhizogenes transformed callus tissues containing solanin percentage were varied by age of the culture period. Eight weeks old cultures yielded higher amount of solanin and after they attained decline stage (Table-6).

Among different explants like node of *Solanum nigrum* L. were cultured on different concentration of hormones (IAA, NAA, 2,4-D IBP and BAP). It was observed that stem explants showed better growth response like enlargement and initiation of callus than stem and node. Therefore, stem explants were used throughout the present study.

Stem explants of *Solanum nigrum* produced maximum callusing potential of medium supplemented with 2,4-D 0.5 mg/l which was higher than and BAP 1 mg/l. in the similar responses were recorded in Oriza sataiva (Venkatachalam et al., 2000; Kunanuvatchaidach et al., 1995); Centella asiatica (Sivakumar et al., 2006).

Explants produced single shoot from lower concentration of BAP which observations were correlated with Lingaraj Sahoo et al., (2000) findings on *Vigna unguiculata*. But higher concentrations of this hormone (BAP 5mg/l) induced single shoot from embryonic axes, shoot apices and half split embryonic axes.

In this study, transformation was carried out by co-cultivation of regenerating on an economically important Indian medicinal plant *Solanum nigrum* L. That is during the infection process, the bacterium makes a heritable change in the DNA of the plant and the changes in the formation of a tumor by the plant. Co-cultivation method was adopted by many workers for the Agrobacterium transformation studies (Akio Uchida et al., Kemel Melik Taskin et al., 2002: Zaldivar-Cruz et al., 2003).

Callus inducing ability of the four auxins on stem and noed explants of *Solanum nigrum* was in the following order 2,4-D>IAA >NAA. The preference of specific hormone and concentration of tissue explants of various plant species for callus induction is known already (Alagumanian et al., 2000; Lavanya et al., 2004; Charumathi et al., 2004; Rathore and Shekhawat, 2010; Solouki et al., 2011).

In the present study, hormones play an important role in the yield of solanin. Selection of medium for optimizing the production of secondary metabolities is an important factor. According to Yamada and Fujita (1983), there are three important factors. To be noted for successful on in vitro production of secondary metabolites.

Phyto-hormonal responses and mass multiplication were screened through the explants of *Solanum nigrum* L. Different explant used and cultured on MS medium supplemented with different concentration and combination of plant hormones like IAA, NAA, 2,4-D and BAP.

Optimum concentration of individual hormones for the growth of the stem explants was determined. It was found to be 1 mg/1 for IAA, 0.5 for NAA, 2 mg/1 for 2,4-D and 0.5 mg/1 for BAP. Higher amount of solanin was achieved on the media supplemented with IAA 1+ BAP (0.5-5 MG/1) concentration. It is suitable agro technology has to be adapted to cultivate medicinal plant, which is in bulk demand, and modern methods of propagation inducing plants to be evolved to multiply the medicinal plants so as to meet the demand from within and across our country.

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