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HIGH FREQUENCY PLANT REGENERATION, *IN VITRO* FLOWERING OF *SOLANUM NIGRUM* (L.) – AN IMPORTANT ANTIULCER MEDICINAL PLANT

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SUMMARY

An efficient protocol for direct shoot regeneration from axillary bud and shoot tip explants of *Solanum nigrum* is described. Healthy axillary bud, shoot tip explants were cultured on MS medium supplemented with different concentrations of BAP (0.5-3.0 mg/l) or Kn (0.5-3.0 mg/l) alone or in combination with IAA or NAA (0.5-1.0 mg/l). All the explants were responded effectively for regeneration. Good regeneration frequencies were observed in all the combinations tested. High frequency and maximum number of multiple shoots was obtained in shoot tip culture, cultured on MS medium supplemented with 2.0 mg/l BAP+0.5mg/l IAA.The present investigation also describes role of auxins on successful induction of *in vitro* flowering from axillary bud, shoot tip and young leaf explants of *Solanum nigrum*. Maximum number of *in vitro* flowers (8) were obtained in shoot tip culture induced on MS medium supplemented with 3.0 mg/l Kn+0.5 mg/l IAA, followed by 2.0 mg/l Kn+1.0 mg/l IAA, where maximum of six (6) flowers per culture was obtained from axillary bud explant. All the *in vitro* raised shoots were transferred to MS rooting medium supplemented with 0.25-1.0 mg/l IBA. The best rooting response was observed in 0.5 mg/l IBA. The well rooted plantlets were transferred to polybags containing soil+vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

Key words: Plant regeneration, *In vitro* flowering, Axillary bud, Shoot tip, Leaf, IAA/NAA

Abbreviations: BA, 6-benzyladenine; Kn, kinetin; NAA, α-naphthalene acetic acid; IAA, indole-3-acetic acid, GA_3 – gibberelic acid

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1. Introduction

Medicinal plants are the source of various alkaloids and other chemical substances essential for mankind. The exploitation of tissue culture techniques in medicinal plants is indeed desirable for their *in vitro* propagation and extraction of important chemical compounds [1]. *Solanum nigrum* (L.) commonly known as black night shade, an important medicinal plant of family solanacae. It is grown in dry parts of India up to an elevation of 2,100 mts. It is an important herbaceous medicinal plant, generally used in traditional and folklore medicines. Commonly it is used as leafy vegetable in preparing traditional dishes. The herb is antiseptic, antidysentric and diuretic used in the treatment of cardiac, skin diseases, psoriasis, herpivirus and inflammation of kidney. The leaves, stems and roots are used externally as poultice; wash etc., in the treatment of cancerous soles, boils, leucoderma and wounds [2]. The fruits and leaves have been traditionally used against various nerve disorders [3]. Root bark is laxative, useful in the treatment of neck, burning of throat, inflammation of liver and chronic fever. Berries are bitter, pungent and are useful in the disease of heart, piles and dysentery [4]. It has very important

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gastric ulcerogenic activities [5]. Extracts of the plant are analgesic, antispasmodic, anti-inflammatory and vasodilator [2].

Most important aspect of this medicinal plant is that it contains two important alkaloids solamargin and solasonine which yield solasodine [6] as glycone. Solasodine has embryogenic, teratonic as well as antifungal, antiviral and molluscidal effects [7]. Solasodine has great demand in pharmaceutical industry, owing to its demand in pharma industry the plant is extensively harvested. So it is necessary to establish an efficient protocol for *in vitro* propagation of this important herbaceous medicinal plant.

In vitro flowering bears immense importance in selective hybridization especially in plants that use pollens from rare stocks. *In vitro* flowering may help in obviating the intricate interactions present in whole plants. It also facilitates in understanding the nature of factors which influences the flowering. Reports on *in vitro* fruiting and seed set in *Solanum nigrum* are almost limited. Hence in the present study an attempt is made to standardize a protocol for successful plant regeneration, *in vitro* flowering using axillary bud, shoot tip and leaf explants in *in vitro* conditions.

2. Materials and Methods

Plant material

Healthy axillary bud and shoot tip explants of *Solanum nigrum* (L.) were collected from two-month-old seed germinated field grown plants growing in nursery of Biotechnology Department, S.V.University, Tirupati, Andhra Pradesh, India.

Surface sterilization

Explants were washed thoroughly under running tap water to remove traces of dust etc. followed by treatment with 10% teeepol or tween -20 for 5 minutes. Then the explants were sterilized in 70% alcohol for a minute, and finally with 0.01% mercuric chloride for1-2 minutes and washed 3-4 times with sterile double distilled water.

Culture Medium

The explants were inoculated on MS medium [8] containing 3% sucrose and gelled with 0.8% agar, supplemented with various concentrations of BAP, Kn alone or in combination with IAA or NAA. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121°C and 15 lbs pressure**.**

Sub culturing

The cultures were maintained by regular subculture at 4 week intervals on fresh MS medium.

Culture conditions

The growth room conditions maintained for *in vitro* cultures were 26 ± 2°C and 60-70% relative humidity, light intensity was 3000 lux with 18 hrs day light and 6 hrs dark. Each experiment was conducted at least thrice with 20 replicates per treatment.

3. Results and Discussion

Axillary bud and shoot tip explants of *Solanum nigrum* were cultured on MS medium supplemented with different concentrations of cytokinins such as BAP (05-3.0 mg/l), Kn (0.5-3.0 mg/l) alone or in combination with auxins IAA (0.1-0.5 mg/l) or NAA (0.1-0.5mg/l). After shoot initiation 1-2 weeks of further culturing is necessary for shoot proliferation and multiple shoot induction.

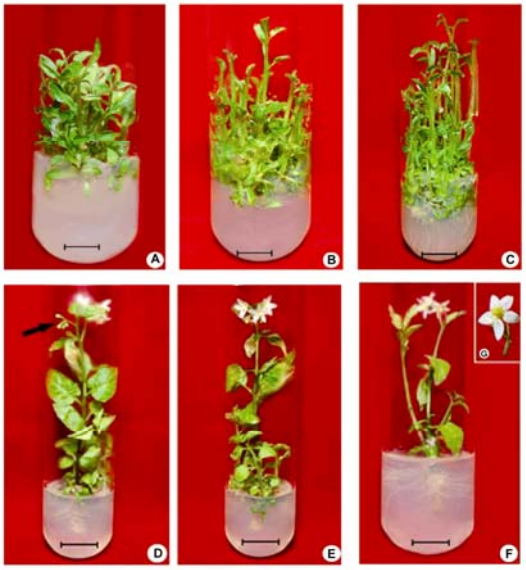
Influence of cytokinins on multiple shoot induction

Effect of BAP on multiple shoot induction

In both the explants such as axillary bud and shoot tip, multiple shoots were induced on MS medium supplemented with different concentrations of BAP (0.5-0.3mg/l). High frequency and maximum number of multiple shoots were induced on 2.0mg/l BAP. The number of multiple shoots was more in shoot tip (16.8) compare to axillary bud (8.0) respectively (Table-1; Figure-1). These findings were similar to the results reported in *Phyllanthus amarus* [9, 10]. Further increase in BAP (>2.0 mg/L) concentration inhibits the shoot formation and the shoots so formed were short and

thick. Similar findings were reported in *Phyllanthus amarus* [9].

Fig.1: Effect of different concentrations of plant growth regulators on shoot initiation, multiplication and *in vitro* flowering.



A) Shoot initiation from axillary bud explants on MS+ BA (1.0 mg/L) (bar 1 cm =1.1); B) Shoot multiplication from shoot tip explant MS+ BA (1.0 mg/L) + IAA (0.5 mg/L) (bar 1 cm = 1.0); C) Elongation of multiple shoots from shoot tip explant MS+ Kn (1.0 mg/L) + IAA (0.5 mg/L) (bar 1 cm =0.9).

***In vitro* flower bud initiation and flowering from axillary bud and shoot tip explants**

D) MS+ BAP (2.0 mg/L) + NAA (0.1 mg/L) (bar 1 cm =1.2);

E) MS+ Kn (2.0 mg/L) + IAA (1.0 mg/L) (bar 1 cm = 1.3);

F) MS+ IBA (0.5 mg/L) (bar 1 cm = 1.1);

G) Excised *in vitro* flower grown on MS+ IBA (0.5 mg/L)

Fig.2: *In vitro* rooting and hardening of *Solanum nigrum* (L.)

A) Initiation of *in vitro* roots on MS medium containing 0.5 mg/l IBA. (bar 1cm = 0.72),
B) Plantlet showing elongated root system.
C) Hardening of plantlet in polybag containing soil and vermiculite.
D) Plantlet in field conditions

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Table – 1: Effect of different concentrations of BAP, Kn singly or in combination with NAA, IAA on multiple shoot induction from axillary bud and shoot tip explants of *Solanum nigrum* (L.).

Plant Growth Regulator (mg/l)				Auxillary bud explant			Shoot tip explant		
BA	Kn	NAA	IAA	Regeneration frequency (%)	Mean no. of shoots	Mean shoot length (cm)	Regeneration frequency (%)	Mean no. of shoots	Mean shoot length (cm)
0.5	-	-	-	70	3.0±0.28	2.8±0.18	90	12.2±0.18	7.0±0.18
1.0	-	-	-	85	4.2±0.34	5.0±0.26	100	14.6±0.53	7.2±0.17
2.0	-	-	-	95	8.0±0.26	4.2±0.18	100	16.8±0.34	5.6±0.53
3.0	-	-	-	80	6.0±0.28	3.6±0.41	85	10.4±0.21	4.5±0.50
-	0.5	-	-	80	10.4±0.21	7.6±0.35	90	2.4±0.21	4.16±0.18
-	1.0	-	-	90	12.0±0.28	8.5±0.34	100	4.8±0.34	6.2±0.19
-	2.0	-	-	72	8.2±0.18	9.0±0.28	90	3.0±0.28	8.0±0.28
-	3.0	-	-	68	6.4±0.21	11.2±0.17	80	2.0±0.16	10.4±0.21
1.0	-	0.5	-	95	12.0±0.28	6.4±0.21	90	15.0±0.25	7.4±0.22
2.0	-	0.5	-	100	18.0±0.16	7.2±0.21	95	19.5±0.50	8.0±0.28
3.0	-	0.5	-	82	15.4±0.28	5.8±0.18	80	12.0±0.18	6.5±0.50
-	1.0	0.5	-	100	14.8±0.34	7.4±0.51	92	16.2±0.18	8.0±0.28
-	2.0	0.5	-	95	12.2±0.18	8.6±0.26	85	14.8±0.17	9.3±0.31
-	3.0	0.5	-	80	10.4±0.22	10.3±0.50	74	12.0±0.28	10.5±0.50
1.0	-	-	0.5	90	16.2±0.28	6.4±0.51	90	16.0±0.28	7.6±0.35
2.0	-	-	0.5	100	22.0±0.16	8.2±0.21	100	24.8±0.17	8.3±0.48
3.0	-	-	0.5	90	15.0±0.28	6.2±0.18	95	14.6±0.35	6.2±0.17
-	1.0	-	0.5	75	8.6±0.21	8.0±0.51	80	14.0±0.28	7.0±0.28
-	2.0	-	0.5	84	5.8±0.18	9.0±0.26	90	14.0±0.28	8.4±0.22
-	3.0	-	0.5	78	4.8±0.34	11.5±0.50	72	11.4±0.22	10.2±0.17

Results are mean ± S.E of 20 replicates

Effect of Kn on multiple shoot induction

Multiple shoots were also induced from axillary bud and shoot tip explants cultured on MS medium supplemented with different concentrations of Kn (0.5-3.0 mg/l). High frequency and maximum number of multiple shoots were induced on 1.0mg/l Kn. The number of multiple shoots was more in shoot tip (12.0) compare to axillary bud (4.8) respectively (Table-1). The number of multiple shoots was decreased with further increase in Kn concentration and it promotes callusing. Similar results were reported in *Gymnema silvastre* [11]. In the present study among the two cytokinins used BAP induced more number of multiple shoots (16.8), where as Kn induced maximum shoot length (11.2cm). Superiority of BAP over Kn in terms of inducing more number of multiple shoots were observed. Similar findings were reported in *Dictyospermum ovalifolium* [12].

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Influence of cytokinin and auxin combination on multiple shoot induction

It is well established that proper ratio of auxin and cytokinins is necessary for morphogenesis leading the formation of complete plantlets [13]. In the present study effect of auxins IAA or NAA (0.1-0.5 mg/l) to cytokinins BA or Kn (1.0-3.0 mg/l) were studied. Based on the results showed in (Table-1; Figure-1), high frequency and maximum number of multiple shoots were induced on MS medium supplemented with 2.0 mg/l BAP +0.5 mg/l IAA, followed by 1.0 mg/l BAP +0.5 mg/l IAA. In this combination all the explants were responded well. Maximum number of multiple shoots were reported in shoot tip (24.8) and axillary bud (22) respectively. Similar findings were reported in *Plumbago rosea* [14]. Shoots formed in this combination were healthy with broad leaves. Where as in Kn and IAA combination the number of multiple shoots were decreased comparatively with BAP and IAA combination. High frequency and maximum number of multiple shoots were recorded in 1.0 mg/l Kn + 0.5 mg/l IAA. Maximum number of multiple shoots were reported in shoot tip (14) and axillary bud (8.6) respectively.

In BAP and NAA combination multiple shoots were induced from all the two explants. High frequency and maximum number of multiple shoots were induced on 2.0 mg/l BAP + 0.5 mg/l NAA. Maximum number of multiple shoots were recorded in shoot tip (19.8) compare to axillary bud (18.0) respectively. In Kn and NAA combination the number of multiple shoots were decreased comparatively with BAP and NAA combination. High frequency and maximum number of multiple shoots were recorded in 1.0 mg/l Kn + 0.5 NAA. Maximum number of multiple shoots was recorded in shoot tip (16.2) compare to axillary bud (14.8) respectively. The obtained results are in line with earlier reports such as *Syzizium travancorium* [15], *Ancistrocladus abbreviatus* [16] and *Coleus blumei* [17]. At higher concentrations of IAA and NAA, induced lesser number of multiple shoots. In some cases at higher concentrations of IAA and NAA shoots shows stunted growth with less number of shoots. Similar findings were reported in *Plumbago rosea* [14]. Based on the above results, shoot tip is the better choice for multiple shoot regeneration in *Solanum nigrum*.

***In vitro* flowering**

In vitro flowering considered to be a complex process regulated by both internal and external factors and its induction under *in vitro* culture is extensively rare [18]. *In vitro* grown plants derived from axillary bud, shoot tip and leaf explants were sub cultured on to a fresh MS medium supplemented with BA (1.0-3.0 mg/l), Kn(1.0 – 3.0 mg/l) alone or in combination with auxins such as NAA (0.1 – 0.5 mg/l), IAA (0.5-1.0 mg/l) and GA_3 (0.5-1.0 mg/l) (Table-2; Figure-1).

The first flower induction was observed after three weeks of sub culturing on to a fresh MS medium supplemented with 1.0 mg/l BAP. Similar findings were reported in plants like *Tobacco* [19], *Bamboo* [20], *Lemna* [21], *Maize* [22], where BAP was found to promote floral bud formation, so it not only act as plant growth regulator, but it regulate to induce floral organ in regenerated plantlets as well. In Kn and IAA combination *in vitro* floral bud appears after four weeks of two successive subcultures on the same medium, with in 2-3 days floral buds are opened. High frequency and maximum number of *in vitro* flowers per culture (6.0) were reported in 2.0 mg/l Kn + 1.0 mg/l IAA. Similar findings were reported in *Withania sommifera* [23], *Ocimum basillicum* [24]. Kintzyios and Michaelakis (1999) stated that Kn inhibited the *in vitro* induction of flowers in *Chamomile* [25]. Contrary to the above statements, in the present study *in vitro* flowering was induced on MS medium using Kn and IAA in *Solanum nigrum*.

In shoot tip culture *in vitro* flower bud appeared after 4 weeks of sub culturing on to a fresh MS medium supplemented with 2.0mg/l BAP+0.1mg/l

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NAA, but in shoot tip culture high frequency and maximum number of *in vitro* flowers (8.0) were reported on MS medium supplemented with 3.0mg/lBA+0.5mg/l IAA. Similar findings were reported in Tobocco [26]. After the floral bud formation shoots at a length a of 5-6 cm with unopened floral buds were transferred to a fresh MS rooting medium supplemented with (0.5 mg/l IBA). In rooting medium floral buds are opened. They were morphologically normal, whitish yellow in colour, and a bunch of 6-8 flowers per culture were formed in shoot tip culture. Similar observations were reported that *in vitro* flowering of *Bacillicum polystachyon* [27] in IBA supplemented MS rooting medium.

In leaf culture *in vitro* flower initiation was observed after 4-5 weeks of sub culture on MS medium supplemented with 3.0mg/l BAP+1.0mg/ IAA, after flower bud initiation it takes another 2-3 weeks for opening of flower buds, only 2-4 tiny flowers per cultures were formed in leaf culture. They were morphologically normal, whitish yellow in colour. All the regenerated shoots with *in vitro* flowers were kept on the same replicative medium for another two weeks, during the period they showed flower senescence without forming any fruit. Earlier few reports on *in vitro* flowering in some plant species were reported such as *Cauliflower* [28], *Corriander* [18].

In the present study auxins such as IAA and NAA have certain role in promoting floral bud formation and flowering was clearly showed in combination with cytokinins. Sheja and Mandal [29] have also reported *in vitro* flowering and fruit formation in tomato at high levels of endogenous auxins. Emperical guidance shows that exogenous auxin could act as a principal floral inhibitor [30], but in the present investigation IAA did not inhibit *in vitro* flower formation .In the present study IAA in combination with cytokinin Kn induced *in vitro* flowers.

Table-2: Effect of different concentrations of auxins and cytokinins on *in vitro* flowering from axillary bud, shoot tip and leaf explants of *Solanum nigrum*.

Plant growth regulators (mg/l)					Explant	No. of flowers /culture
Kn	BA	NAA	IAA	GA_3		
	1.0				Axillary bud	2
1.0			0.5		Axillary bud	4
2.0			1.0		Axillary bud	6
	1.0			0.5	Axillary bud	3
	2.0			1.0	Axillary bud	5
	2.0	0.1			shoot tip	5
	3.0		0.5		shoot tip	6
3.0			0.5		shoot tip	8
	2.0		0.5		Leaf	2
3.0			1.0		Leaf	4

Observations: after 8 weeks of inoculation

Table – 3: Effect of different auxins on *in vitro* rooting

Plant growth regulators (mg/l)	**Frequency (%)**	**Mean no. of roots**	**Mean root length (cm)**
IBA			
0.25	95	26.0 ± 0.35	4.2 ± 0.18
0.5	100	38.2 ± 0.18	5.0 ± 0.28
0.75	94	28.0 ± 0.23	3.6 ± 0.35
1.0	90	18.4 ± 0.22	2.3 ± 0.31
NAA			
0.25	90	18 ± 0.23	3.4 ± 0.35
0.5	95	24.1 ± 0.43	4.5 ± 0.31
0.75	85	21 ± 0.56	3.8 ± 0.17
1.0	74	14.2 ± 0.18	2.0 ± 0.28

Results are mean ± S.E of 20 replicates

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***In vitro* rooting**

For *in vitro* root induction micro shoots derived from axillary bud and shoot tip were transferred to a fresh MS medium supplemented with different concentrations of auxins like IBA or NAA or IAA. All the micro cuttings shows rooting response against all the three auxins tested. High frequency and maximum number of roots, root length were reported in 0.5mg/l IBA (38.2), followed by 0.5mg/l NAA (24.1). Further increase in auxin concentration the frequency, number of roots, root length was decreased (Table-3; Figure-2). Similar findings were reported in case of *Acasia sinuta* [31], *Annona squamosa* [32]. Well rooted plantlets were transferred to polybags containing soil+vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

4. Conclusion

The present investigation of *in vitro* flowering may offer better understanding of nature of factors which influences *in vitro* flowering and to achieve better genetic varieties which other wise fail to produce seeds/plants of hybrid nature. Reports on *in vitro* fruiting and seed set *in Solanum nigrum* are almost scanty. Hence further investigation is highly required to explore the factors and exogenous hormonal influence on *in vitro* fruiting and seedling behavior in *Solanum nigrum.*

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