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Research Article

IN VITRO PROPAGATION OF *SOLANUM SURATTENSE* BURM. F BY HIGH FREQUENCY MULTIPLE SHOOT INDUCTION FROM FLORAL BUD EXPLANTS

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A B S T R A C T

An efficient and reproducible protocol has been developed for *in vitro* propagation from floral bud in *Solanum surattense* on MS medium fortified with various concentrations of cytokinins such as BAP and Kn individually and also in combination with auxins IAA(0.5mg/L)+BAP/Kn (1.0-8.0mg/L) and NAA (1.0mg/L) +BAP/Kn (1.0-8/0mg/L) for multiple shoot induction. High Multiple shoot buds/explnt (35.0 ± 0.35) proliferation was observed at NAA(0.1mg/L) and Kn (5.0mg/L) from the floral bud explants within four weeks of culture was attained. Individual shoots were aseptically excised and sub cultured in the same media for shoot elongation. The elongated shoots were transferred to (IBA) (1.0–5-0 µM) for root induction. Rooting was observed within two weeks of culture. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 96%. Plants looked healthy with no visually detectable phenotypic variations.

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. *Solanum surattense* Burm. (Solanaceae) is a perennial herb. It is usually found in India, Pakistan, Malaya, and Australia. In Bangladesh, it was found as wild herb in almost all northern parts and it was very common in the Barind region. Nowadays, this plant rarely gets in the Barind region only. The solasodine and glycosides are rich in this plant, are very common properties for anticancer (Cham, 2007). Besides, this plant is widely used as folk medicine for breathing trouble, heart diseases and pain. Some drug companies (Unani, Hamdard Laboratories, Ayurvedic) are developed in India based on plant extract and they are attracted by the people. These companies are using extract of *S. surattense* as to prepare remedy for breathing disease, as well this plant is widely planted in highland of Bangladesh. Since this herb becoming a potential medicinal plant in south Asia, more advance investigations are needed concerning modification of characteristics including rapid growth, increase essential chemicals content, disease resistant and stress tolerance in this plant. Limited reports have been published on the *in vitro* propagation as well as genetic transformation systems of *S. surattense.* Pawar *et al.* (2002) developed a technique for direct shoot organogenesis from shoot tip and leaf segments. Using nodal and shoot tip segments, a micropropagation technique also established on this plant by Rama Swamy *et al.,* (2004). Rama Swamy *et al.,* (2005a) established a protocol on plantlet regeneration through somatic embryogenesis from cotyledon and leaf explants. Callus induction and shoot organogenesis system from apical bud were also reported earlier for this plant proliferation (Prasad *et al.,* 1998). Ayodhya Ramulu. *et.al.,* (2014) reported protoplast isolation from leaf explants of *S. surattense.*

Ugandhar *et al.,* (2016) Plantlet regeneration via callus induction from leaf explants of *S. surattense.* Rama Swamy (2006) reported *Agrobacterium-mediated* genetic transformation systems using leaf explants of *S. surattense.* (Rama Swamy *et. al.,* 2005b) established streptomycin-resistant *S. surattense* plantlets using *in vitro* mutagenesis. For genetic improvement of plant, we usually use selection method as well as *in vitro* molecular breeding technique. Plant breeders showing great interest on molecular breeding technique for plant modification genetically because conventional selection method takes long time, tedious and

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occurs large variation within clones. For molecular breeding based genetic transformation, we know, efficient regeneration systems are prime requirement. Stem segments are used as important explant for genetic transformation system, described in many plant species (e.g., Rastogi and Dwivedi, 2006).

The technique of culturing young excised flower buds affords a potentially useful tool for study concerned with the control of flower morphogenesis; influences of growth regulators and nutrients without the presence of intervening vegetative tissue (Konar and Kitchlue, 1982). In several plant species flower buds have been successfully cultured through to anthesis using different growth hormones/regulators of various concentrations (Galun *et. al.*, 1962, 1963; Tepfer *et. al.,* 1963; Blake, 1966, 1969; Hicks and Sussex, 1970; Bilderback, 1971, 1972; Rastogi and Sawhney, 1986, 1988).

Floral bud culture *in vitro* offers an unique technique where by the influence of vegetative parts could be eliminated facilitating analysis of the role of nutritional, hormonal and environmental factors in successful morphogenesis (Konar and Kitchlue, 1982). Floral bud culture in vitro was reported in a number of species (Dunstan and short, 1979; Novak and Howel, 1981). In the present study an effort was made to establish a protocol for the *in vitro* propagation of *S. surattense* from the floral bud explants of field grown plant through direct adventitious shoot organogenesis.

METHODOLOGY

Plant material

S.surattense plant bearing inflorescence having 1-2 flower buds measuring (1.0-1.5 cms) were cut from plants grown in the experimental field of S.R.R.Govt. Arts & Science College Karimanagar. The shoots were trimmed carefully by removing excess leaves and washed several times in tap water. The shoots were then surface sterilized by completely submerging the shoots for 15 minutes in 5-7% (v/v) Sodium hypochlorite solution prepared in double glass distilled water and having a few drops of a wetting agent Tween 20. The flask was shaken periodically to allow complete wetting of the shoots. These were then washed 3-4 times with sterilized double glass distilled water. Again the excised inflorescences were placed in 1% (w/v) "Mericuric chloride solution for 1-2 minutes and agitated to ensure complete surface sterilization. The shoots were then transferred to another flask and washed several times with sterilized double glass distilled water to ensure complete removal of mercuric chloride solution. For the purpose of surface sterilization and washings of excised inflorescences only sterilized glasswares were used.

The shoots were then taken out and the flower buds were carefully cut in size, leaving a small portion of the stem attached to the inflorescence, with the help of a sterilized blade in a sterilized petriplate. The floral buds were then placed on a sterilized 1% agar-plate in a petridish for easy/quick handling during inoculation. All these processes of surface sterilization, washing and cutting of the plant material and inoculation were done under aseptic condition using a laminar air-flow chamber also fitted with ultraviolet light.

Culture media and Culture Conditions: These were placed on modified MS (Murashige & Skoog1962) medium (Devoid of $CaCl_2$ $2H_20$ & KI) containg 6% (w/v) sucrose and solidified with 0.8% agar and supplemented with various concentrations of cytokinks such as BAP and Kn (Table-1) individually and alo in combination with auxins viz. IAA (0.5mg/L) + BAP /Kn (1.0-8.0mg/L) (Table-2) and NAA (1.0mg/L) +BAP/Kn (1.0-8/0mg/L) (Table-3) pH 5.7- 5.8. The percentage of explants responding was evaluated after 4 weeks of culture. Responses scored were the percentage of explants with evidence of multiple shoot bud stage floral buds. All the cultures were incubated under 16/8 h. light/ dark photoperiod at 25 ± 2ºC a light intensity of 40 µ mol m-2 $^{s-1}$ was provided by cool- white florescent tubes. The cultures were transferred to fresh medium after an interval of 4 weeks.

During inoculation one flower bud was transferred onto the medium in each 250 ml flask with the help of a sterilized inoculating needle. The stem portion of the flower buds was slightly inserted into the medium. As soon as inoculation was over each flask was re-plugged with the cotton plug and capped with aluminium foil.

The regenerated plantlets were transferred to poly cups containing sterile compost and soil (1:1) mix for 3 weeks for acclimatization. Subsequently these were shifted to the greenhouse. For statistical analysis, means were based on 25 replicates for each treatment. Data were collected after 6 weeks beginning of the experiments.

RESULTS

Within the first week, most of the inoculated flower buds enlarged. After 2 weeks of culture they opened followed by the enlargement of the ovary. Adventitious shoot buds were induced from the ovary region after 3 weeks of culture. Direct shoot buds were formed from the explants on modified MS medium amended with cytokinins alone and also cytokinins in combination with auxins tested. These were developed without intervening callus phase.

Influence of cytokinins on multiple shoot bud induction: Effect of BAP/Kn on multiple shoot bud induction

The floral buds cultured on MS modified medium supplemented with (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 mg/L) BAP/Kn showed maximum percentage (62%/57%) of responding cultures at (3.0 mg/L) BAP/Kn. At higher concentration of BAP/Kn (8mg/L) the percentage of response was reduced gradually upto (30%/25%) respectively. Likewise maximum number of multiple shoot buds/explant was found at (3.0 mg/L) BAP when it was added alone to the medium, but highest percentage of responding cultures and more number of shoot buds (17.0±0.32) were recorded at (3.0 mg/L) BAP in comparison to Kn (13.0±0.25). As the concentration of cytokinin increased upto (3 mg/L) BAP/Kn the frequency of number of shoots induction was found to be decreased (12.0 ± 0.23/10.0 ± 0.17, 8.0 ± 0.27/6.0 ± 0.40 shoots at 4.0, 5.0 mg/L) BAP/Kn. At 8.0 mg/L BAP/Kn very less number of shoots (4.0 ± 0.17/3.0 ± 0.22) were developed compared to other concentrations of BAP/Kn individually (Fig. 1). (Plate-I)

Table 1 Effect of BAP and Kn on induction of high frequency multiple shootbuds from floral bud explants of *S.surattense*

Hormone cone (mg/L	Hormone	% of cultures responding	Average No of Shoot buds /explants (S.E)**
1.0	BAP	28	4.0 ± 0.42
1.0	Kn	23	5.0 ± 0.29
2.0	BAP	33	10.0 + 0.43
2.0	Kn	29	9.0 ± 0.37
3.0	BAP	62	17.0 ± 0.32
3.0	Kn	57	13.0 ± 0.25
4.0	BAP	55	12.0 ± 0.23
4.0	Kn	47	10.0 ± 0.17
5.0	BAP	51	8.0 ± 0.27
5.0	Kn	32	6.0 ± 0.40
6.0	BAP	50	9.0 ± 0.25
6.0	Kn	39	5.0 ± 0.29
8.0	BAP	30	4.0 ± 0.17
8.0	Kn	25	3.0 ± 0.22

* Data Scored at the end of 4 weeks of culture based on 20 replicates
* Mean ± Standard Error

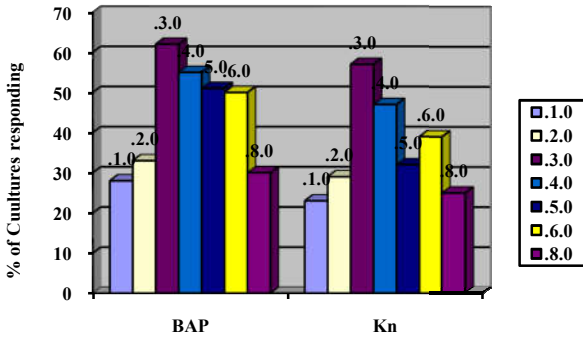

Fig-I Hormone concentration of (mg/L)

Table 2 Effect of IAA in combination with various concentration of BAP/Kn induction of high frequency multiple shoot buds from floral bud explants of S.surattense

Hormone cone IAA+BAP/Kn	Hormone	% of cultures responding	Average No of Shoot buds /explants (S.E)**
0.5+1.0	BAP	30	8.0 ± 0.32
0.5+1.0	Kn	38	6.0 ± 0.43
0.5+2.0	BAP	38	12.0 ± 0.23
0.5+2.0	Kn	42	11.0 ± 0.32
0.5+3.0	BAP	62	19.0 ± 0.35
0.5+3.0	Kn	59	15.0 ± 0.45
0.5+4.0	BAP	58	17.0 ± 0.35
0.5+4.0	Kn	50	14.0 ± 0.35
0.5+5.0	BAP	52	12.5 ± 0.27
0.5+5.0	Kn	45	11.3 ± 0.35
0.5+6.0	BAP	52	8.3 ± 0.25
0.5+6.0	Kn	38	6.3 ± 0.34
0.5+8.0	BAP	30	5.3 ± 0.25
0.5+8.0	Kn	32	4.0 ± 0.35

* Data Scored at the end of 4 weeks of culture based on 20 replicates
* Mean ± Standard Error

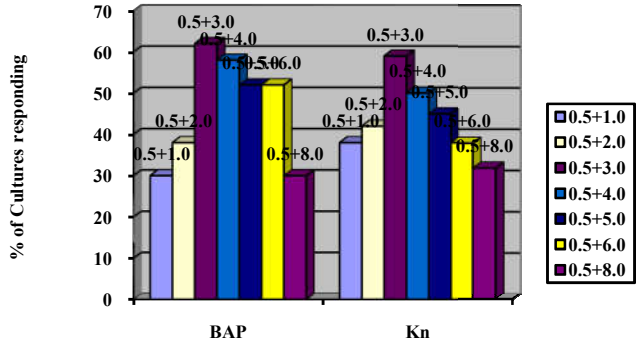

Fig-II Hormone concentration of IAA+BAP/Kn

Table 3 Effect of NAA in combination with various concentration of BAP and Kn on induction of high frequency multiple shoot buds from floral bud culture of *S.surattense*.

Hormone cone NAA+BAP	% of cultures responding	Average No of Shoot buds/explants (S.E)**
0.1+1.0	65	10.0 ± 0.32
0.1+2.0	70	15.0 ± 0.45
0.1+3.0	75	17.0 ± 0.35
0.1+4.0	80	18.0 ± 0.43
0.1+5.0	85	25.0 ± 0.43
0.1+6.0	70	20.0 ± 0.35
0.1+8.0	65	8.0 ± 0.35
NAA+Kn		
0.1+1.0	62	12.0 ± 0.35
0.1+2.0	68	18.0 ± 0.32
0.1+3.0	78	24.0 ± 0.45
0.1+4.0	85	32.0 ± 0.32
0.1+5.0	90	35.0 ± 0.35
0.1+6.0	73	20.0 ± 0.25
0.1+8.0	60	10.0±0.35

* Data Scored at the end of 4 weeks of culture based on 20 replicates
* Mean ± Standard Error

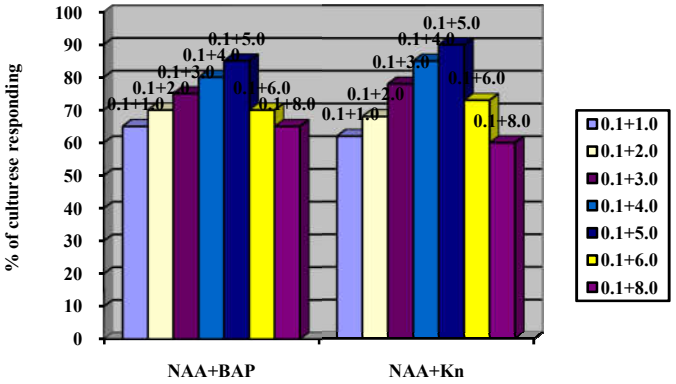

Fig-III Hormone concentration of NAA+BAP

Plate: *In Vitro* Propagation of *Solanum surattense* Burm. F by High frequency multiple Shoot Induction from Floral bud Explants a) Formation of high frequency shoot buds on MS+0.1mg/L NAA+5.0 mg/LBAP b) Induction of Multiple shoot buds on MS+0.5mg/L IAA+3.0mg/L BAP c) Formation of shoot buds on MS+3.0mg/L Kn d)High frequency multiple shoot

formation after 6 weeks i) MS+3.0mg/L ii) MS+0.5mg/L IAA+3.0mg/LBAP iii) MS+0.1mg/L NAA+ 4.0mg/Lkn e) e) *In Vitro* rooting from Micro shoots on MS+ 3.0 mg/L IBA after after six week f) Hardening of plantlets

Influence of cytokinin and auxin combination on multiple shoot bud induction

Effect of IAA and BAP/Kn on multiple shoot bud induction

Modified MS medium containing (0.5 mg/L) IAA in combination with BAP/Kn (1.0-8.0 mg/L) showed the enhanced efficiency in inducing the adventitious shoot buds from the explant as compared to media with cytokinin alone. IAA (0.5 mg/L) in combination with (3.0 mg/L) BAP/Kn produced maximum number of shoots (19.0 ± 0.35/15.0 ± 0.45) with highest responding frequency (62%/59%) compared to other concentrations of BAP/Kn. As the concentration of BAP/Kn increased in the medium showed the less response and decreased number of shoots gradually from (4.0 mg/L) BAP/Kn + (0.5mg/L) IAA combination onwards (17.0 ± 0.35/14.0 ± 0.35, 12.5 ± 0.27/11.3 ± 0.35, 8.3 ± 0.25/6.3 ± 0.34, 5.3 ± 0.25/4.0 ± 0.35 shoots' at 4.0, 5.0, 6.0 and 8.0 mg/L) BAP/Kn +(0.5 mg/L) IAA (Plate-I)(Fig-2).

Effect of NAA and BAP/Kn on multiple shoot bud induction

Direct shoot bud proliferation was also found in all the concentrations of BAP/Kn with (0.1 mg/L) NAA added to the MS modified medium. More number of adventitious shoots per explant was recorded at (0.1 mg/L) NAA + (5.0 mg/L) BAP/Kn compared to all other concentrations of cytokinins used. Low concentration of BAP/Kn induced less number of shoots/explant (10.0 ± 0.32/12.0 ± 0.35 shoots at 1.0 mg/L) but gradually the shoot bud induction was found to be increase up to (5.0 mg/L) BAP/Kn + (0.1 mg/L) NAA (15.0 ± 0.45/18.0 ± 0.32, 17.0 ± 0.35/24.0 ± 0.45, 18.0 ± 0.43/32.0 ± 0.32) shoot buds at (2.0, 3.0 and 4.0 mg/L) and at high, concentrations the shoot bud proliferation from floral bud cultures was reduced (20.0 ± 0.35/20.0 ± 0.25, 8.0 ± 0.35/10,0 ± 0.35 shoots at 6.0 and 8.0 mg/L) BAP/Kn + (0.1 mg/L NAA). High percentage (85%) of responding cultures and maximum number of shoots (25.0 ± 0.43/ 35.0 ± 0.35) were developed on MS modified medium supplemented with (0.1 mg/L) NAA + 5.0 mg/L) BAP/Kn compared to all other combinations and concentrations studied (Plate-I) (Fig-3).

Rooting of Shoot and Acclimatization of Plantlet

The microshoots were excised and individually transferred to MS medium augmented with (0.5 to 1.5 mg/L) IAA for root induction. Root initiation was profuse with 8-10 roots in the medium containing (1.0 mg/L) IAA as compared to (0.5 mg/L) (4 roots) and (1.5 mg/L (6 roots) IAA. High percentage of rooting efficiency (90%) was observed at (1.5 mg/L) IAA followed by (1.0 mg/L) (80%) and (0.5 mg/L) (70%) IAA from *in vitro* regenerated shoots. These *in vitro* regenerated plants were shifted to the greenhouse after acclimatization in the culture room.

DISCUSSION

In the present investigations, direct shoot regeneration from floral buds was obtained in all the concentrations and combinations of plant growth regulators used. The cytokinin BAP had shown superiority over Kn in all the concentrations and combinations studied for inducing multiple shoots in S. *surattense.*

The requirement of growth hormones for flower bud development under *in vitro* conditions has been demonstrated by many workers (Tepfer *et. al.,* 1963; Hicks and Sussex, 1970; Polowick and Greyson, 1982; Rastogi and Sawhney, 1986,) The present findings that flower buds can be grown to anthesis in artificial medium supplemented with a single growth hormone is in agreement with the findings of (Rastogi and Sawhney 1986, 1988). (Brulfert and Fontaine) (1967) also obtained normal flowers from excised flower buds of *Anagallis arvensis* in simple medium supplemented with IAA.

Hicks and Sussex (1970) and Rastogi and Sawhney (1986, 1988) demonstrated the essentiality of kinetin and BAP for the growth and development of floral organs to maturity. In the present study BAP or kinetin (cytokinin) could not induce formation of pollen grains in the anther. This is contradictory to the findings of earlier workers (Hicks and Sussex. 1970; Rastogi and Sawhney, 1986, 1988) who observed pollen grains development in floral buds grown in kinetin supplemented medium.

Maximum efficiency of shoots formation per explant was observed on MS modified medium comprising (0.1 mg/L) NAA and (5.0 mg/L) BAP/Kn followed by (0.5 mg/L) IAA and BAP/Kn. When auxin (IAA/NAA) was added in combination with cytokinins showed the high induction efficiency during the present studies (Plate-I).

Adventitious shoot regeneration in S. *surattense* has been reported with 58 shoots per explant from internodal explants cultures respectively (Mahabubur Rehaman *et.al.,* 2011) and also 25.8 shoots from leaf and 23.6 shoots from nodal explants (Seetharam *et.al.,* 2003). Where as during the present investigation the number of shoot buds developed directly from the explant was 35. When the same was subcultured in a conical flask containing the fresh medium, induced in thousands of shoots per explant.

About 90% of the plantlets survived onto compost soil two months after transfer and seasonal flowering observed in the *ex vitro* conditions. The detectable variation in the *in vitro* grown potted plants was not found when compared with the donor plants. Hence, the present study shows that the floral bud culture is amenable to high frequency regeneration in medicinally important herb S. *surattense,* which could be easily adopted for large scale multiplication of the species. Thus, it opens up the possibility of using this plant in genetic manipulation for introducing genes of interest using particle gun bombardment or *Agrobacterium tumefaciens* and also the established shoot regeneration technique in the present study has a potential impact for clonal propagation of elite genotypes and improvement characteristics by molecular breeding technique of *S. surattense* for plantation and utilization in the drug industries.

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