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REGULAR ARTICLE

EFFICIENT PLANT REGENERATION THROUGH LEAF, *INVITRO* FLOWERING AND BIOCHEMICAL STUDIES DURING MORPHOGENESIS OF *SOLANUM NIGRUM* L.

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SUMMARY

In this investigation, Murashige and Skoog (MS) medium when supplemented with 6-benzylamino purine (BAP) or Kinetin produced high frequency of in vitro shoot directly from in vitro raised *Solanum nigrum* leaves without any callusing stage. Among the plant growth regulators (PGRs) the best response was noted at 2.0 mgl^{-1} BAP and 1.5 mgl^{-1} kinetin. Shoot formation and multiplication were more pronounced on dorsal leaf surface (0.5-1.5 cm^2) compared to ventral. Biochemical studies including soluble protein, sugar and amino acid were carried out in order to know the differences in reserves of some biochemical entities. Nearly all parameters were high in samples derived from dorsal leaf, which was grown in MS augmented with 2.0 mgl^{-1} BAP or 1.5 mgl^{-1} kinetin. The present investigation also describes first successful induction of in vitro flowering in *S. nigrum*, which can augment selective hybridization. Finally, the in vitro raised shoots were treated with various concentrations of auxins for obtaining roots, the rooted plantlets were transplanted with 100% survival in field conditions.

Keywords: Plant Regeneration, *in vitro* flowering, Biochemical Studies, leaf explant, *Solanum nigrum*.

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1. Introduction

The *Solanum nigrum*, commonly known as black nightshade, an important medicinal plant of family Solanaceae. It is grown in dry parts of India up to an elevation of 21,00m. The hexaploid *Solanum* however, occur mostly in temperate parts and very rarely in warmer regions [1]. The fruits and the leaves of *S. nigrum* have been traditionally used against various nerve disorders [2]. Besides, the plant is used as

febrifuge, antidiarrhoeal treatment, hydrophobia and for eye diseases [3]; it is also valued with gastric ulcerogenic activities [4].

The primary attraction of this plant is that it contains two important alkaloids solamargin and solasonine, which yield solasodine [5]. A number of *Solanum* sps. have been propagated through in vitro processes [6] in which leaf, nodal part have been used for callus induction and regeneration purposes [7, 8]. Although callus has proved better for the synthesis of alkaloids in several cases [9], poor yield, growth and recalcitrant callus often limit production of plants in masses. Thus, other alternative and fast regeneration route is absolutely necessary in order to produce cultures as medicinal raw materials. The advantage of direct regeneration is that it reduces the possibility of somaclonal variation, noted in plants regenerated from cultured cells and tissues like callus or suspension [10, 11, 12].

In vitro flowering bears immense importance in selective hybridization especially in plants that use pollens from rare stocks. It also facilitates the understanding of physiology of flowering as it largely depends upon the level of interaction of exo- and endogenously applied phytohormones, sugars, minerals, phenolics etc. [13, 14]). The present article describes a fast in vitro direct regeneration method involving leaf discs as explant. The leaf size, positioning of explant, requirement of PGRs (BAP/Kinetin) have been studied and discussed. An account of biochemical analysis and the induction of in vitro flowering have also been made to explore the possibility of in vitro hybridization.

2. Material and Methods

Explants and culture conditions

The leaf explant was categorized into several groups according to their size (0.5, 1.0 and 1.5 cm2) excised from in vitro grown Solanum plants (previously described, Bhat et al. [15]. The excised intact leaves (section cut perpendicular to the mid vein) with the adaxial (i,e the rough dorsal side) and abaxial (i,e the smooth ventral side) surface of the leaves were separately tested by placing on solid medium for their organogenic response. Two cytokinins i.e BAP and kinetin of the same concentrations (0.25, 0.50, 1.0, 1.5 and 2.0 mgl-1) were used individually for each explant in MS [16] containing 3% sucrose. The pH of the medium was pre adjusted to 5.8 before sterilization (121 0C for 20 min). Inoculated cultures were kept at 25 ± 2 0C under 12 h light daily (100 µmol m-2 s-1 PFD). Statistical analysis were performed using analysis of variance according to the Duncan's multiple range test in which each of the experiments was conducted at least twice with 5 replicates per treatment.

Rooting and acclimatization

After 8 weeks on shoot multiplication medium, the shoots were excised and cultured for rooting. The rooting medium contained MS amended with different concentrations of auxins like IBA, IAA and NAA. Hardening of the rooted shoots was made by transferring plantlets from MS to half strength MS and finally to the medium without organics. The plantlets were maintained for 2 weeks on the above medium, later transferred to pots containing soil rite, covered with perforated transparent polythene bags, kept at culture room for 10 days with same level of light and temperature, as mentioned earlier. After10 days of incubation the polythene bags were removed and the pots were kept at same cultural condition. After one week, the plants were transferred to the field conditions.

In vitro Flowering

In vitro grown plantlets, kept in MS added with either BAP or kinetin showed flowering by producing one or more normal looking flowers.

Biochemical analysis

Various biochemical parameters such as chlorophyll a, b, total chlorophyll, carotenoid, soluble sugar, amino acid and protein were conducted. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were estimated according to the method of Hiscox and Israelstam [17]. The absorbance was recorded at 480, 510, 645 and 663 nm with a Backman DU 640 B spectrophotometer (Fullerton, USA).

The soluble protein content was estimated following Bradford's method [18] where 1.0 g of leaf sample was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.5) at 4 ^{0}C using pre chilled mortar and pestle. The homogenate was centrifuged at 5,000 rpm for 10 min at 4 ^{0}C, the supernatant was mixed with equal volume of chilled 10% Trichloro acetic acid, again centrifuged at 3,300 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 1 ml of 0.1 N NaOH after washing with acetone. To 1 ml of aliquot, 5 ml Bradford's reagent was added and mixed by vortex. Absorbance was measured at 595 nm on a spectrophotometer (Backman DU 640 B, Fullerton, USA). Protein content was calculated using the standard curve of bovine serum albumin and expressed in mg g^{-1} fr.wt.

The soluble sugar was estimated by the method of Dey [19], 0.1g chopped fresh materials were kept in 10 ml of alcohol for 1h at 600C in incubator. The extract was decanted into a 25ml volumetric flask and the residue re-extracted. Final volume was made up to 25ml by adding alcohol. One ml aliquot was transferred to a test tube and 1.0ml of 5% phenol was added to it and mixed thoroughly, 5ml of analytical grade sulphuric acid was added, mixed thoroughly by vertical agitation with a glass rod. Absorbance was recorded at 485nm on Perkin Elemer UV/VIS Spectrometer Lambda Bio 20 (Switzerland). The corresponding concentration was determined against a standard curve prepared by using a glucose solution. Lee and Takahashi's method [20] was used for the estimation of soluble amino acids. Tissue materials of 0.5g were ground in 5.0ml of absolute ethanol with the help of mortar and pestle, transferred to the centrifuge tubes. It was centrifuged at 5000 rpm for 10 min at 40C. The supernatant was taken and alcohol was evaporated by incubating sample at 800C for 1h in a water bath. Pellet was dissolved in 10ml of 0.5M citrate buffer (pH 5.5). To 0.5ml aliquot, 1.2ml of 55% glycerol and 0.5ml of 10% ninhydrin solution was added. The mixture was boiled for 20 min and volume was made up to 6ml by adding citrate buffer. Absorbance was recorded at 570 nm on UV-vis Spectrophotometer (Model DU 640 B, Beckman, USA). Calibration curve was prepared from Glycine (Sigma) of different concentrations to calculate the amino acid content in different samples. The concentration was expressed in mg g^{-1} fw.

3. Results

Leaf size, positioning and effect of PGRs on production of micro shoots

After week incubation the excised leaf started to produce small protuberances from wounded regions that immediately differentiated into micro shoots. The number and the length of shoots increased with time. It is clear that the shoot forming ability was noticed in both dorsal and ventral surfaces.

Influence of Dorsal surface: In the first set of experiments, the leaves of dorsal surface were cultured on medium added with BAP or Kinetin. Among the tested concentrations, maximum number of shoots/leaf was observed in MS supplemented with 1.5 mgl^{-1} kinetin, it produced

shoots of 19.6 within 4 weeks of culture when 1.5 cm^2 leaf discs was cultivated (plate 1A). Poor response was observed on MS, added with 0.25 mgl^{-1} kinetin, it produced only 2.3 shoots after 4 weeks (Table 1); BAP on the other hand, at 0.5 mgl^{-1} also showed fairly good response while producing shoots.

Ventral surface: The leaves with ventral surface were also similarly cultivated on MS with earlier described levels of PGRs. A significant increase i.e. 13 shoots / leaf (1.5 cm2 leaf disc) was observed when MS was supplemented with 1.0 mgl^{-1} kinetin, which after 8 weeks produced a maximum of 20.3 shoots (data not shown). The minimum number of shoots emerged from ventral surface of leaf was 3.0 after 4 weeks of culture (Table 1; plate 1B). Although both ventral and dorsal surfaces were equally responsive (100%), the numbers of shoots were more in dorsally placed leaf discs.

Variation was also noted in shoot length when leaf discs of 0.5, 1.0 and 1.5 cm^2 were cultured on different concentrations of cytokinins. Shoot length increased with enhanced level of kinetin in the medium. The maximum shoot length was noted in dorsally placed leaf discs on medium amended with 1.5 mgl^{-1} kinetin, it showed an average shoot length of 2.9 cm within 4 weeks of culture, while MS fortified with 0.25 mgl^{-1} kinetin exhibited poor growth (Table 2).

As regards to shoots originated from ventral leaf surface, the maximum length was 2.1 cm in 4 weeks old culture on medium augmented with 1.5 mgl^{-1} kinetin. Growth was poor in MS + 0.25 mgl^{-1} kinetin, produced shoots attained a maximum height of 1.1 cm only after 4 weeks of culture. Shoots originated from dorsal side grew fast and showed maximum shoot length compared to shoots derived from ventral surface.

Table 1. Effects of plant growth regulators, explant size and explant position on production of micro shoots from leaf, data were scored after 4 weeks. * Values are the mean of five individual readings. Mean within each column with same superscript are not significantly difference at > 0.05 according to the Duncan Multiple RangeTest (DMRT).

	Number of shoots / explant (leaf)					
MS+PGR	Dorsal surface Length (cm2)			Ventral surface Length (cm2)		
(mg l^{-1})	0.5	1.0	1.5	0.5	1.0	1.5
MS+ BA						
0.25	*2.6 e	3.6 e	9.0 c	1.3 d	2.6 d	8.0 d
0.5	6.3 c	5.3 d	5.3 de	2.6 c	4.6c	4.3e
1.0	3.3 e	4.3 e	4.3 e	1.6 d	3.0 d	4.6e
1.5	3.0 e	3.6 e	6.0d	2.6d	4.3 c	4.0 ef
2.0	4.6 d	6.6 c	11.3b	4.6 c	7.3b	10.0 c
MS+ Kin						
0.25	1.6 f	1.3 f	2.3 f	1.0 d	0.0 e	3.0f
0.5	7.3 c	5.3 d	8.0c	5.0 b	4.3c	4.3 d
1.0	8.6b	9.0 b	12.6b	7.0 a	9.3 a	13.0a
1.5	9.6 a	12.0 a	19.6 a	4.7b	7.0 b	11.6 bc
2.0	8.0 c	9.3b	12.3 b	5.3 b	5.3c	12.6b

Table 2. Influence of plant growth regulators on shoot length, derived from dorsal or ventrally positioned leaf of different explant sizes, data were scored after 4 weeks.

Shoot length (cm)							
MS+ PGR (mg l^{-1})	Dorsal surface Length (cm2)				Ventral surface Length (cm^2)		
	0.5	1.0	1.5		0.5	1.0	1.5
MS + BAP							
0.25	*1.4b	1.5b	1.3b		1.2b	1.2b	1.2ab
0.5	1.9b	1.4b	1.9a		1.3b	1.3b	1.5ab
1.0	1.3b	1.5b	1.6b		1.2b	1.1b	1.3ab
1.5	1.4b	1.3b	1.4b		1.1b	1.4b	1.3ab
2.0	1.4b	1.7b	1.4b		1.3b	1.5b	1.2ab
MS +Kin							
0.25	1.2b	1.1b	1.3b		1.0b	0.0c	1.1b
0.5	1.5b	1.5b	1.4b		1.2b	1.3b	1.7ab
1.0	1.8b	1.4b	1.4b		1.2b	1.3b	1.7ab
1.5	3.0a	3.4a	2.9a		2.8a	2.7a	2.1a
2.0	1.4b	1.6b	1.4b		1.7b	1.6b	1.5ab

* Values are the mean of five individual readings. Mean within each column with same superscript are not significantly difference at > 0.05 according to the Duncan Multiple RangeTest (DMRT).

Table 3. Root induction from 3 weeks old in vitro raised micro shoots, MS was added with different concentrations of various auxins.

MS + PGR (mgl^{-1})	No. of roots / plant.	Average root length (cm.)
IBA (0.25)	* 15.2a	4.5a
IBA (0.50)	14.3a	4.5a
IBA (1.00)	6.5d	4.1a
IBA (2.00)	3.2g	3.5ab
IAA (0.25)	15.2a	2.6b
IAA (0.50)	9.0c	2.9b
IAA (1.00)	7.5d	2.2b
IAA (2.00)	6.4de	2.1bc
NAA (0.25)	12.0b	3.4ab
NAA (0.50)	5.0ef	2.5bc
NAA (1.00)	4.4f	2.1bc
NAA (2.00)	3.2g	1.1c

* Values are the mean of three replicates. Mean with in each column with same superscript are not significantly difference at > 0.05 according to the Duncan Multiple RangeTest(DMRT).

Leaf length had a strong influence in producing shoots. Table 1 shows the different categories of leaf sizes and their responses. The shoot bud formation was noticed both on large and small leaves, 1.5 cm^2 leaf disc appeared to produce more influence, followed by 0.5, surprisingly 1.0 cm2 leaf disc was least responsive. In conclusion, the dorsally placed leaf disc was more responsive compared to ventrally positioned leaf and kinetin was more promotive for shoot growth compared to BAP.

Biochemical analysis of micro shoots as influenced by BAP (2 mgl^{-1}) or Kinetin (1.5 mgl^{-1})

The micro shoots originated from dorsal and ventrally positioned leaf were analysed biochemically as to know the differences of behaviour of two different sources of origin. Fig.1a depicts the biochemical reserves of shoots cultivated in medium added with BAP. It is evident that all the biochemical contents were high in shoots typically developed from dorsally positioned leaf. The content of soluble protein was pretty high (9.96 mg g^{-1} fr. wt.) in dorsal derived shoots. Soluble amino acid and soluble sugar level also followed the same trend, the contents were high (6.14 and 15.27 mg g^{-1} fr. wt. respectively) in dorsal derived shoot compared to shoots derived ventrally. The photosynthetic pigments level were high too, in dorsal leaves (i.e. chlorophyll 'a' was 1.30, chlorophyll 'b' 0.58, total chlorophyll 1.85 and carotenoid contents 0.68 mg gm-1 fr.wt) compared to shoots of ventral origin (Fig. 1b).

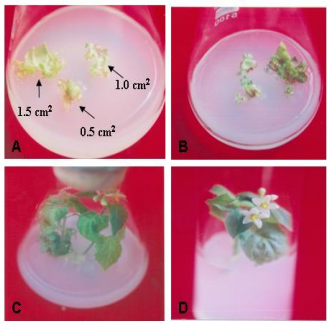

Plate 1: Effect of various concentrations of plant growth regulators on shoot induction, multiplication and *in vitro* flowering
(A): shoot multiplication on MS Medium added with 1.5 mg l^{-1} kinetin from dorsal surface
(B): shoot multiplication on MS Medium added with 1.5 mg l^{-1} kinetin from ventral surface
(C,D): *in vitro* bud and flowering, MS medium added with 0.5 mg l^{-1} BAP

The biochemical parameters did show changes, if not significantly, when the same micro shoots were cultivated in medium added with kinetin. Shoots derived dorsally showed maximum soluble protein (10.36 mg g^{-1} fr.wt. after 12 weeks), than shoots (9.93 mg/g fr.wt.) derived ventrally (Fig. 2a), so were soluble amino acid and sugar. Kinetin added cultures also showed improvement in pigment level as the dorsal leaf derived regenerants showed maximum chlorophyll 'a' 1.58, chlorophyll 'b' 0.60, total chlorophyll 2.18 and carotenoid 0.68 mg g^{-1} fr.wt. Ventrally derived shoot leaves showed lesser level of pigments (Fig. 2b).

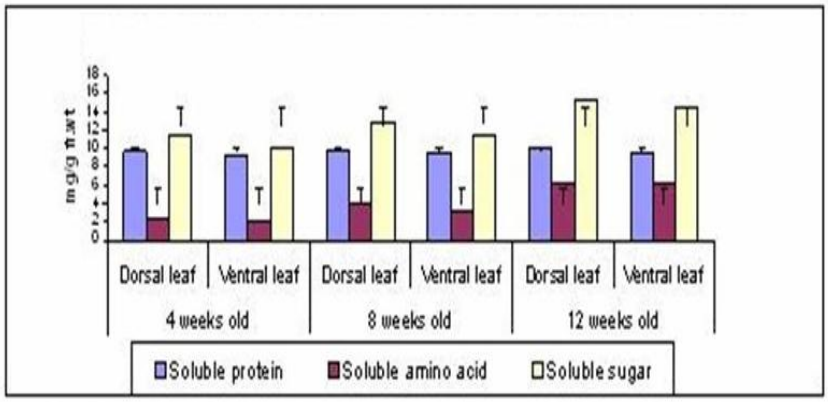

Fig. 1(a): Biochemical profiles of shoots regenerated from dorsal and ventral leaf cultured on MS + 2.0 mg/l BAP. The values (Mean ± SD) are based on five individual readings.

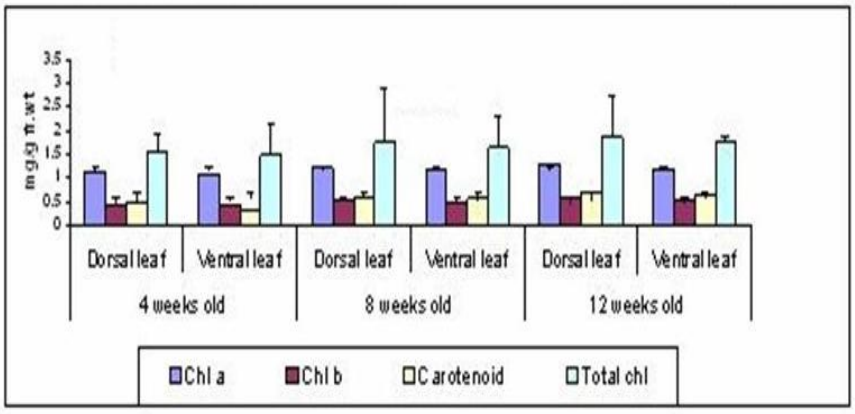

Fig. 1(b): Pigment concentrations of regenerants derived from dorsal and ventral leaf cultured on MS + 2.0 mg/l BAP. The values (Mean ± SD) are based on five individual readings.

In vitro flowering

Micro shoots, grown under in vitro situation (MS + 0.5 mg/l BAP or 0.5 mg/l kinetin) with earlier described physical conditions for a long period produced healthy in vitro flowers. The plants retained the flowers for over 2 weeks or more, but showed premature flower-senescence without setting any fruit (plate 1,C-D).

Root induction and acclimatization

The in vitro raised shoots were transferred individually to MS added with various auxins at 0.25 - 2 mgl^{-1}. Although rooting was observed in all the cultures (100%), maximum number of roots/plant i.e. 15.2 were observed at 0.25 mgl^{-1} IBA with average root length of 4.5 cm, the response was poor at 2.0 mgl^{-1} NAA, which produced 3.2 roots / plant with average root length of 1.1 cm (Table 3).

The rooted plantlets were subjected to hardening and successfully transplanted to soil rite, transferred to pots which were kept at 30 ± 2 0C in plant growth chamber. The plantlets grew well and attained 15 cm height within 2 weeks period. The morphological characteristics of in vitro raised plant like leaf size, shape and plant height were very similar to field grown plants (Plate 2A, B).

Plate 2: Hardening and Acclimatization
(A): Transplanted plant in soilrite
(B): Transplanted plant in field conditions

4. Discussion

In this study we noted direct shoot formation on leaves in *S. nigrum*. The various sizes of leaf, the positioning or orientation (dorsal or ventral), was studied during shoot production in response to different PGRs application. Green juvenile leaves of 1.5 cm^2 were efficient to induce more number of shoots compared to leaves of smaller sizes. Larger leaf sizes with higher nutrient uptake provided by their exposed surfaces may probably the reason to produce

more number of shoots compared to small sized leaves. Shoot bud formation ability was mostly noted on leaf margin compared to other regions; this study further demonstrated that dorsal surface was more responsive compared to ventral side. The explanation of differential explant's behaviour in showing organogenetic responses is not known clearly, varied physiological gradient including the level of endogenous PGRs existing in different parts of the leaf are probably involved for such responses [21, 22]. Similarly, the orientation of explant on nutrient medium seems important as it affects auxin transport and nutrients uptake in cultured tissues [12]. We also noted that kinetin was very active PGR and relatively higher level (1.5 mgl-1) showed more shoot multiplication and growth compared to another used PGR, BAP, which is in contrast to several other earlier observations where BAP's role on shoot production has been described [16]. Therefore, leaf surfaces and PGRs regulate in inducing shoots in numbers in S. nigrum. These results are in agreement with previous study [23] where plant regeneration was noted to be pretty high and fast in comparison to many other plant species [24, 25, 26]. The protein, amino acid, sugar and other biochemical reserves were noted to be high during shoot morphogenesis; the level was however, affected if not significantly by the PGRs added to the medium. In this study we also observed in vitro flowering when regenerated plants were kept on MS and added with 0.5 mgl-1 BAP or 0.5 mgl^{-1} kinetin but the shoots exhibited premature flower senescence without setting any fruit or seed. BAP was found to promote flower buds in plans like tobacco [27], maize [28], so it not only acts as PGR, but it regulates to induce floral organ in regenerated plantlets as well. This is a rare process and has enough promises to plants including medicinals where traditional breeding methods have several limitations. There are earlier a few reports of plant species, which developed in vitro flowering, for example in citrus [29], cauliflower [30], maize [28], coriander [31], bamboo [32] etc. Thus, the present observation of in vitro flowering may offer possibility to achieve varieties that otherwise fail to produce seeds/ plants of hybrid nature.

References

[1] Anonymous, 1995. Wealth of India, Vol.9, CSIR, New Delhi, India.

[2] Perez, G.R.M., L.A. Perez., D.L.M. Garcia and M.H. Sossa, 1998. Neuropharmacological activity of *Solanum nigrum* fruit, J. Ethnopharmcol. 62: 43-48.

[3] Kumar, V.P., S. Shashidhara, M.M. Kumar and B.Y. Sridhara, 2001. Cytoprotective role of *Solanum nigrum* against gentamicine induced kidney cell (ver cell) damage in vitro. Fitoterpia. 72: 481- 486

[4] Akhtar, M.S and M. Munir, (1989) Evaluation of the gastric antiulcerogenic effects of *Solanum nigrum, Brasica oleracea* and *Ocimum basilicum* in rats. J. Ethano Phrmacol. 27: 163-176.

[5] Anonymous, 1972. Wealth of India, Vol. 9, CSIR, New Delhi, India.

[6] Chandler, S.F and J.H. Dodds, 1983. Solasodine production in rapidly proliferating tissue cultures of *S. laciniatum* Ait. Plant Cell Rep. 2: 69-72.

[7] Shahzad, A., H. Hasan and S.A. Siddiqui, 1999. Callus induction and regeneration in *Solanum nigrum* L. in vitro. Phytomorphology. 49: 215-220

[8] Bhat, M.A., A. Junaid., A. Mujib and Mahmooduzzafar, 2004. Biotechnological studies to enhance the mass propagation of *Solanum nigrum* L., an anti-inflammatory herb. In Role of Biotechnology in Medicinal

and Aromatic Plants. Vol. XI, Eds., Khan I.A and A. Khanum. Ukaaz Publications, Hyderabad, pp. 107-113.

[9] Datta, A and P.S. Srivastava, 1997. Variation in vinblastine production by *Cathranthus roseus* during in vivo and in vitro differentiation. Phytochemistry 46: 135-137.

[10] Misra, P and S.K. Datta, 2001. Direct differentiation of shoot buds in leaf segments of white marigold (*Tagetes erecta* L.). In vitro Cell Dev. Biol. Plant. 37: 466–470.

[11] Dayal, S., M. Lavanya, D. Prathibha and K.K. Sharma, 2003. An efficient protocol for shoot regeneration and genetic transformation of pigeonpea (*Cajanus cajan* L.) using leaf explants. Plant Cell Reports 21: 1072–1079.

[12] Mujib, A., 2005. In vitro regeneration of sandal (*Santalum album* L.) from leaves. Turk. J. Bot. 29: 63–67.

[13] Tanimoto, S and H. Harada, 1981a. Chemical factors controlling flower bud formation of *Torenia* stem segments cultured in vitro I. Effect of mineral salts and sugars. Plant Cell Physiol. 22: 523-541.

[14] Tanimoto, S., 1981b. Chemicals factors controlling flower bud formation of *Torenia* stem segments cultured in vitro II. Effect of growth regulators. Ibid. 22: 543-550.

[15] Bhat. M.A., A. Sayeed., A. Junaid., A. Mujib., Mahmooduzzafar, 2008. Salinity stress enhanced production of solasodine in *Solanum nigrum* L. Chem. Pharm Bull. 56: 17-21.

[16] Murashige, T and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

[17] Hiscox, J.D and G.F. Israelstam, 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57: 1332-1334.

[18] Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principe of protein-dye binding. Analyst. Biochem. 72: 248-259.

[19] Dey, P.M., 1990. Oligosachaides, In Method in Plant Biochemistry, Vol. 2, Carbohydrates, Eds., P.M. Dey. Academic Press London pp. 189-218.

[20] Lee, Yand T. Takahashi, 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. 14: 71–77.

[21] Wernicke, W and L. Milkovits, 1986. Development gradient in wheat leaves. Responses of leaf segments in different genotypes cultured in vitro. J. Plant Physiol. 115: 49-58.

[22] Mujib, A., S. Bandyopadhyay., B.K. Jana and P.D. Gosh, 1996. Growth regulator involvement and somatic embryogenesis in *Crinum asiaticum*. Ind. J. Plant Physiol. 1: 84-86.

[23] Hassanien, M.A., 1999. A method for induction of callus formation and plant regeneration of Salix sufsaf. *Solanum tuberosum* and *Mililotus indicus*. Biol. Plant. 42: 314-347.

[24] Binding, H., 1985. Regeneration of plants. In: Fowke LC, Constable FC (eds.) Plant protoplasts, CRC Press, Boca Raton, pp21-37

[25] Binding, H., M. Zuba, J. Radnick, G. Mordhorst and R. Kollmann, 1988a. Protoplast cell fusion. J. Plant Physiol., 133: 409-413.

[26] Binding, H., E. Ghorschen., J. Jorgesen., G. Krumbiegel-Schroeren, H.Q. Ling, J. Rudnick., A. Sauer., M. Zuba and G. Mordhorst, 1988b. Protoplast culture in agarose media with particular emphasis to streaky culture lenses. Bot. Acta. 101: 233-239.

[27] Smulders, M.J.M., E.J.W. Visser., A.F. Croes

and G.J. Wullems, 1990. The dose of NAA determines flower bud regeneration in tobacco explant at a large range of concentration. Planta. 180: 410-415.

[28] Mandal, A.B., A. Maiti and R. Elanchezhian, 2000. In vitro flowering in maize (*Zea mays* L.). Asia Pacific J. Mol. Biol. Biotechnol. 8: 81-83.

[29] Moss, G.I., 1969. Influence of temperature and photoperiod of flower induction and inflorescence development in sweet orange. (*Citrus sinensis* L. Osbeck). J. Hort. Sci. 44: 311-320.

[30] Kumar, V.A., A. Kumar and J. Kumar, 1995. In vitro flowering and pod formation in cauliflower (*B. oleracea* var. botrytis). Curr. Sci. 69: 543-545.

[31] Stephen, R and N. Jayabalan, 1998. In vitro flowering and seed setting formation of coriander (Coriandrum sativum L.). Curr. Sci. 74: 195-197.

[32] Nadgauda, R.S., V.A. Parasharami and A.F. Mascarenhas, 1990. Precocious flowering and seedling behavior in tissue-cultured Bamboos. Nature 344: 335-336.