Plant Regeneration via Somatic Embryogenesis in *Solanum nigrum* (black nightshade)

ABSTRACT

Aim: To study the effect of various plant growth regulators (PGRs) for induction of somatic embryogenesis and plantlet formation from cotyledon and leaflet explants in *S.nigrum* (night shade) an important medicinal plant used in treatment of digestive problems and skin infections.

Place and Duration of study: Department of Biotechnology, Kakatiya university, Warangal. Telangana, India, 3years.

Methodology: Cotyledon (0.8 cm^2) and leaflet explants (0.8-1.0 cm^2) from 3 week and 4 week old were cultured on MS medium supplemented with 30 g/L sucrose along with different concentrations of 0.5 mg/L BAP+NAA (0.5 – 6.0 mg/L) .

Results: Maximum percentage of somatic embryogenesis was observed in cotyledon(89%) and leaf (98%) explants on MS medium augmented with 0.5mg/L BAP in combination with 2.0 mg/L NAA whereas the highest number of somatic embryos per explant (86 ± 0.19) was formed in leaflet explant.

Keywords: *Solanum nigrum*; somatic embryogenesis; acclimatization; plantlet establishment.

Conclusion: Direct somatic embryogenesis was induced from both cotyledon and leaf explants. Since it is threatened and medicinally important species *S.nigrum*, the present protocol can be used for its conservation and genetic transformation experiments.

ABBREVIATIONS: PGRs: Plant Growth Regulators; BAP: 6-Benzylamino purine; 2, 4-D: 2,4-Dichlorophenoxy acetic acid; NAA: α-Naphthalene acetic acid; IAA:Indole-3-acetic acid; GA3:Gibberelic acid; mg/L: Milligram/Liter

1. INTRODUCTION

Somatic embryogenesis provides an efficient method for plant micropropagation and conservation of the species [1,2]. The plants regenerated via somatic embryogenesis are of single cell origin with *true-to-type* and are produced in large numbers within a short period [3,4]. Somatic embryogenesis is a preferred method for rapid *in vitro* multiplication of plants, production of artificial / synthetic seeds and also for *Agrobacterium tumefaciens* mediated genetic transformation and regeneration of transgenic plants [5].

Following the initial reports of Reinert [6] and Steward et al. [7], the phenomenon of somatic embryogenesis was reported in a number of medicinal plants: *Solanum melongena* [8,9], *Tribulus terrestris* [10], *Psoralea corylifolia* [11], *S. surattense* [5] and *Senna alata* [12].

The species *Solanum nigrum* is an important ingredient in traditional Indian medicines. Infusions are used in dysentery, stomach complaints, and fever. The juice of the plant is used on ulcers and other skin diseases. The fruits are used as a tonic, laxative, appetite stimulant, and for treating asthma and "excessive thirst". Traditionally the plant was used to treat tuberculosis. It is known as *peddakasha pandla koora* in the Telangana region. The leaves are used to treat mouth ulcers that happen during winter periods. It is known as *manathakkali keerai* in Tamil Nadu and *kaage soppu* in Karnataka, and apart from its use as a home remedy for mouth ulcers, is used in cooking like spinach. In North India, the boiled extracts of leaves and berries are also used to alleviate liver-related ailments, including jaundice. In Assam, the juice from its roots is used against asthma and whooping cough. *S. nigrum* is a widely used plant in oriental medicine where it is considered to be antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic, and antipyretic. *S.nigrum* is known to contain solasodine (a steroidal glycoalkaloid that can be used to make 16-DPA progenitor); a possible commercial source could be via cultivating the hairy roots of this plant [13,14].

Inview of its medicinal importance the plant has become threatened/endangered. Hence we have developed the protocol for plant regeneration via direct somatic embryogenesis for conservation of the medicinally important species *S. nigrum*.

2. MATERIALS AND METHODS

2.1 Plant Material:

For somatic embryo induction and plantlet formation the seeds of *S.nigrum* were soaked in sterile distilled water for 24 hrs. These were sterilized with 70% (v/v) alcohol for 2-3 minutes followed by 1% (w/v) aqueous solution of sodium hypochlorite for 3-5 minutes. Later, the sterilized seeds were washed thoroughly with sterile distilled water and were germinated aseptically on MS [15] basal medium.

2.2 Culture Media and Culture Conditions:

The explants viz., cotyledon (0.8 cm^2) and leaf (0.8-1.0 cm^2) from 3 week and 4 week old axenic seedlings respectively were transferred on to MS medium containing 30 g/L sucrose along with different concentrations of NAA (0.5 – 6.0 mg/L) + 0.5 mg/L BAP.

For further proliferation the explants with somatic embryos were cultured on MS medium augmented with 0.5 mg/L BAP + 2.0 mg/L NAA.

For germination and plantlet formation, the bipolar (torpedo-shaped) stage embryos were transferred onto ½ strength MSO, MSO and MS medium fortified with different concentrations of BAP (1.0 – 3.0 mg/L) + 0.5 mg/L IAA. The pH of the medium was adjusted to 5.8 prior to addition of 0.8% (w/v) Difco-bacto agar and autoclated at 121^0C for 15-20 minutes. All the cultures were incubated under 16/8 h light / dark photoperiod at 25 ± 2^0C.

2.3 Data Analysis:

Data were recorded after 4 weeks of culture. Each experiment was repeated at least twice and 20 replicates were maintained for each experiment.

3. RESULTS:

The induction of direct somatic embryos and plantlet formation from cotyledon and leaf explants was studied on MS medium augmented with 0.5 mg/L BAP in combination with 0.5-6.0 mg/L NAA in *S. nigrum*. The results are presented in Tables 1-3 and shown in Fig.1.

3.1 Somatic embryogenesis from cotyledon explants:

The cotyledon explants were cultured on MS medium fortified with different concentrations of NAA (0.5 – 6.0 mg/L) + 0.5 mg/L BAP. Cotyledon explants were swollen after 4 days of culture and globular somatic embryos were induced directly from the explant after 10 days of culture (Fig.1 a, b). Somatic embryogenesis was induced from the cotyledon explants cultured on all the concentrations of NAA + 0.5 mg/L BAP except at 6.0 mg/L NAA in which callus was induced. High percentage (89) of somatic embryogenesis with maximum frequency number (65±0.23) of somatic embryos formation was observed at 2.0 mg/L NAA + 0.5 mg/L BAP. As the concentration of NAA in combination with BAP increased, the percentage of somatic embryo induction and as well as somatic embryo number per explant were enhanced upto 2.0 mg/L NAA (Table 1). But at high concentration of NAA + 0.5 mg/L BAP, the percentage of somatic embryogenesis was reduced.

Globular embryos were converted into bipolar embryos on all the concentrations of NAA used except at 4.0 & 6.0 mg/L NAA + 0.5 mg/L BAP. The embryo conversion was found to be dependent on the level of NAA. High percentage of bipolar / torpedo-shaped embryos formation was recorded at 2.0 mg/L NAA(Fig.1c-e). Less percentage of conversion and absence of somatic embryo conversion was observed at 3.0 & 4.0 g/L NAA respectively.

For further proliferation of somatic embryos, the explants with embryos were cultured on MS medium supplemented with 2.0 mg/L NAA + 0.5 mg/L BAP. Further maturation of somatic embryoids was absent even after 2nd subculture on the fresh medium containing the same PGRs.

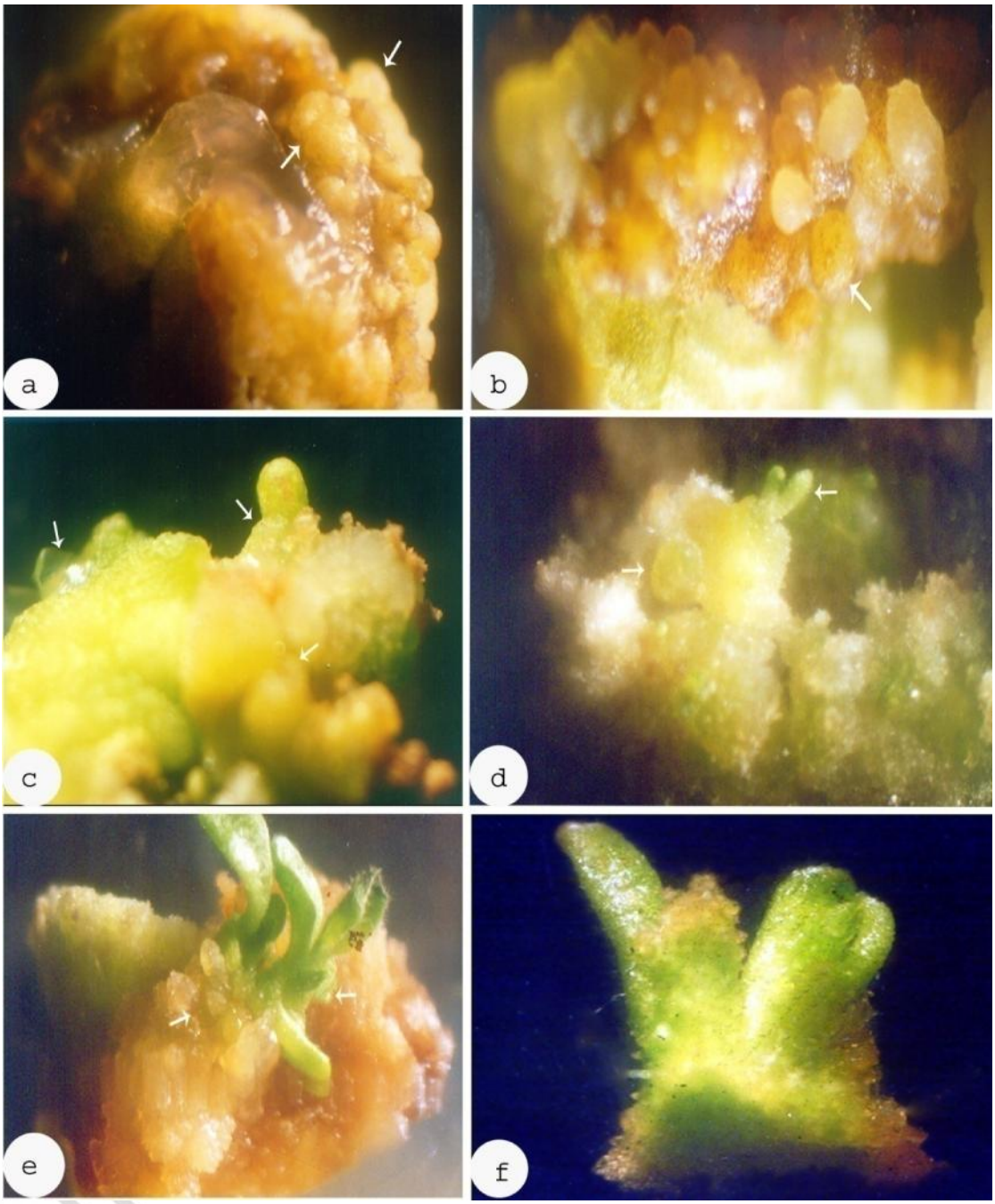


Fig.1 a-m: Induction of direct somatic embryogenesis from cotyledon(a-h) and leaf(i-j) explants of *S. nigrum*

a) Globular embryoids on MS + 0.5 mg/L BAP + 1.0 mg/L NAA; b) Many Globular embryoids on MS + 0.5 mg/L BAP+2.0 mg/L NAA; c) Globular and torpedo-shaped embryos on MS + 0.5 mg/L BAP + 2.0mg/L NAA after 1st subculture; d) Globular and heart-shaped embryoids after 1st subculture on MS +0.5 mg/L BAP +2.0 mg/L NAA; e) Various stages of embryoids (Note the cotyledonary stage embryoid) after 6 weeks of culture; f) Cotyledonary stage and torpedo-shaped embryoids.

TABLE – 1. Induction of direct somatic embryogenesis from cotyledon explants of *S.nigrum* on MS+0.5 mg/L BAP +NAA

Growth regulators (mg/L)	% of cultures with somatic embryogenesis	Average number of somatic embryos per explant (±SE)[a]	% of somatic embryos conversion into bipolar embryos
BAP + NAA			
0.5+0.5	52	27±0.01	31
0.5+1.0	63	43±0.03	48
0.5+1.5	78	59±0.19	61
0.5+2.0	89	65±0.23	73
0.5+2.5	65	43±0.09	33
0.5+3.0	61	22±0.21	12
0.5+4.0	43	13±0.11	-
0.5+6.0	Callus	-	-

[a] Mean ± standard error

3.2 Somatic embryogenesis from leaf explants:

Leaf explants of *S.nigrum* were cultured on MS medium augmented with different concentrations of NAA in combination with 0.5 mg/L BAP (Table 2). Somatic embryogenesis was initiated directly from the explant in all the concentrations of NAA except at high concentration (6.0 mg/L NAA). As in the cotyledon explant, the somatic embryogenesis was inhibited at 6.0 mg/L NAA + 0.5 mg/L BAP and callus was induced. Somatic embryoids were formed after 10 days of culture. High percentage of somatic embryogenesis was observed at 2.0 mg/L NAA followed by 1.5 mg/L NAA + 0.5 mg/L BAP. Whereas maximum frequency number of somatic embryos per explant was observed at 2.5 mg/L NAA(Fig.1i). Less number of somatic embryo induction was recorded at 4.0 mg/L NAA. As the concentration of NAA increased, there is an increase in the average number of somatic embryos development per explant upto 2.5 mg/L NAA.

The conversion of somatic embryos from globular to torpedo-shaped was found in all the concentrations of NAA tested with an exception of 4.0 mg/L NAA. Maximum percentage of bipolar embryos was recorded at 2.0 mg/L NAA + 0.5 mg/L BAP.

For further proliferation and maturation of somatic embryos, the leaf explant consisting of somatic embryos in different stages (globular to bipolar) was transferred onto fresh medium containing 2.0 mg/L NAA + 0.5 mg/L BAP. Bipolar somatic embryos did not mature further

even after 2nd subculture on the same fresh medium, But the somatic embryos number per explant was enhanced.

Individual embryos developed into distinct bipolar structures and passed through each of the typical developmental stages (globular, heart, torpedo / bipolar) after 4-6 weeks of culture. The development of somatic embryos was asynchronous. As a result, various stages of embryo development could be observed in the same cluster of embryos originated from the explants (Fig.1e).

TABLE – 2.Induction of direct somatic embryogenesis from leaf explants of *S.nigrum* on MS + 0.5 mg/L BAP + NAA

Growth regulators (mg/L)	% of cultures with somatic embryogenesis	Average number of somatic embryos per explant (±SE)[a]	% of somatic embryos conversion into bipolar embryos
BAP + NAA			
0.5+0.5	28	17±0.13	22
0.5+1.0	48	32±0.01	28
0.5+1.5	83	55±0.21	35
0.5+2.0	98	77±0.13	69
0.5+2.5	79	86±0.19	38
0.5+3.0	63	44±1.3	17
0.5+4.0	10	20±0.09	-
0.5+6.0	Callus	-	-

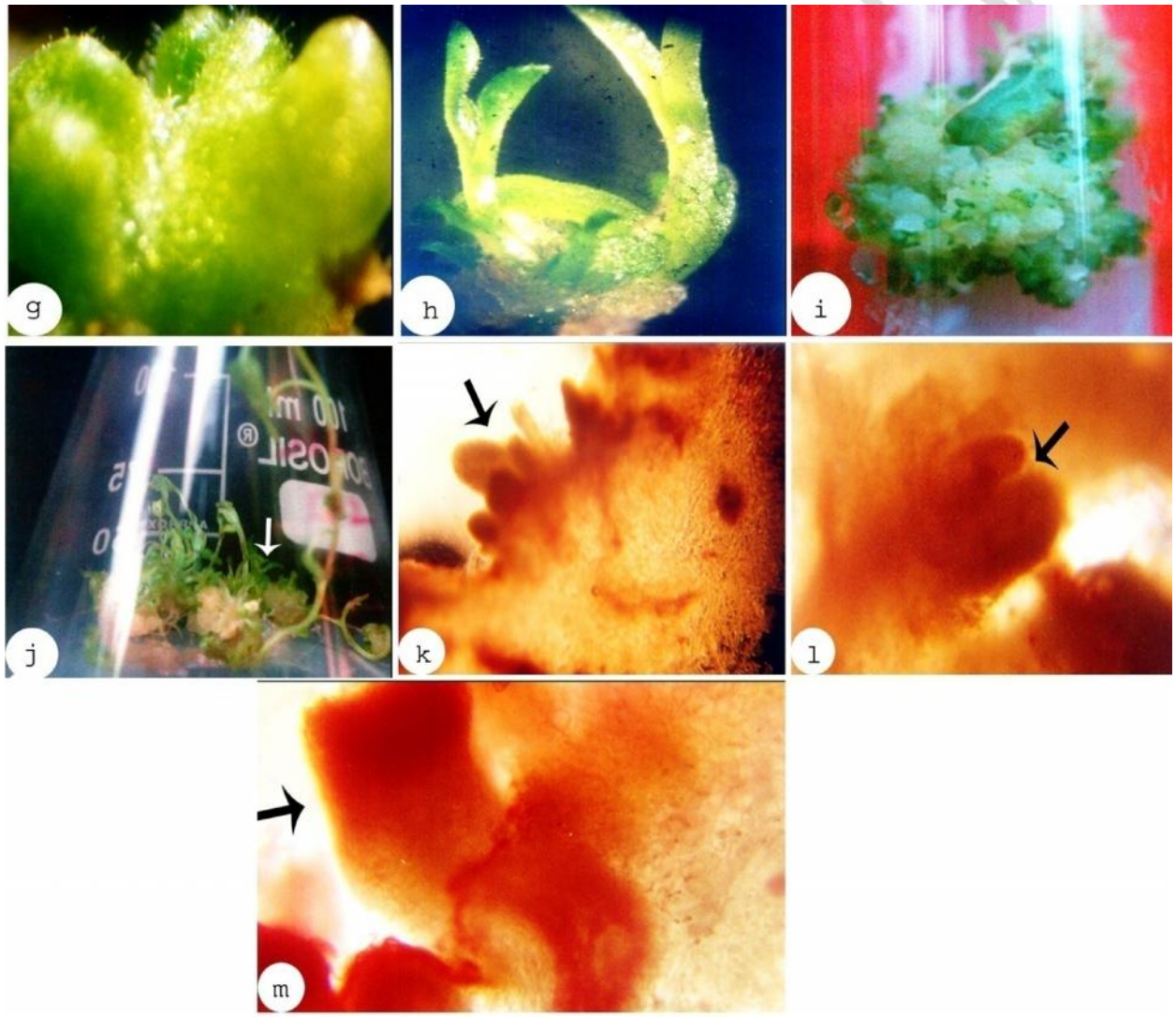
[a] Mean ± standard error

3.3 Somatic embryo germination & plantlet formation:

For germination of somatic embryos, globular, heart and torpedo-shaped embryos (a mixture) developed from cotyledon and leaf explants were transferred on to ½ strength MS medium, MS medium without growth regulators and MS medium supplemented with different concentrations of BAP in combination with 0.5 mg/L IAA (Table 3). Somatic embryos did not germinate on ½ strength MS medium and also on MS medium without PGRs.. The highest (73.8%) frequency of embryo germination was noticed on medium containing 0.5 mg/L IAA + 1.5 mg/L BAP. Whereas embryo germination frequency was reduced at high concentration of BAP.

Histological sections of embryo forming explants clearly revealed a globular-shaped embryo, a heart-shaped embryo with a notch and two cotyledons and torpedo-shaped embryo with shoot and root poles (Fig.1 k-m) upon transfer to a medium containing 0.5 mg/L IAA + 1.5 mg/L BAP, the embryos turned green with folded cotyledons, which subsequently developed into whole plantlets (Fig. 1j.)

Plantlets regenerated via somatic embryogenesis were transferred to polycups containing mixture of soil and sand in ratio of 3 : 1 with 75% survival rate. A total of 30 regenerated plants were transferred to earthenware pots from the polycups and maintained in the research field under shady conditions. These *invitro* regenerated plants via somatic embryogenesis were found similar to donar plant.



g)A group of torpedo-shaped somatic embryoids; h)Cotyledonary stage embryos on MS + 0.5 mg/L IAA + 1.5 mg/L BAP after 6 weeks of culture; i) Conversion of somatic embryoids into different stages developed from leaf explants on MS+0.5 mg/L BAP + 2.5 mg/L NAA after 1st subculture; j) Plantlet formation of somatic embroids developed from leaf explants on MS + 0.5 mg/L IAA + 1.0 mg/L BAP (Note the rooting too). k-m: Histological sections of somatic

embryogenesis showing different stages in *S.nigrum*: k) Globular, heart-shaped, torpedo-shaped embryos; l) Single heart-shaped embryo; m) Single torpedo-shaped embryo.

TABLE – 3. Effect of IAA + BAP on germination of somatic embryos in *S.nigrum*

Growth regulators (mg/L)	Germination Frequency (Mean±SE)[a]
½ MSO	-
MSO	-
IAA + BAP	
0.5+1.0	23.3+0.13
0.5+1.5	73.8+0.17
0.5+2.0	38.0+0.72
0.5+2.5	30.0+1.2
0.5+3.0	28.0+0.05

Data scored after five weeks of culture; [a] Mean ± standard error

4. DISCUSSION:

Somatic embryogenesis was induced directly from the cotyledon and leaf explants in *S.nigrum* on MS medium fortified with different concentrations of NAA (1.0 – 6.0 mg/L) in combination with 0.5 mg/L BAP except at 6.0 mg/L NAA. The present investigations showed that auxins such as NAA along with cytokinins BAP are required for inducing the somatic embryogenesis. For somatic embryogenesis the nature of PGRs and their concentration and combinations used in the culture medium play a vital role. The type of auxin or auxin in combination with cytokinin used in the induction medium can greatly influence somatic embryo frequency. The requirement of cytokinin in addition to auxin was observed in medicinal plants like *Terminalia arjuna* [16], and *Psoralea corylifolia* [11], as it was observed in the present studies. Somatic embryogenesis was induced on medium containing NAA alone in *Solanum melongena* (Matsuoka and Hinata [17, 18, 19]. Recently, [5] have also reported the essentiality of both auxin-cytokinin combination for inducing somatic embryogenesis in an endangered medicinal plant *S.surattense* a medicinal plant.

In the present investigations, leaf explants showed maximum frequency number of somatic embryos production and also conversion into bipolar embryos at 2.0 mg/L NAA + 5.0 mg/L BAP compared to cotyledon explants. Similarly it was also observed in *S.surattense* [5].

BAP induced the direct somatic embryogenesis and also the number of embryos further increased by enriching the medium with NAA in *Hippeastrum hybridum* [20]. Similar findings were also made by [21] in *Brimeura amethystina.* [11] have also observed the high frequency somatic embryogenesis in hypocotyl explants on MS

medium supplemented with NAA (1.4 μM) + BAP (2.2 μM) in *Psoralea corylifolia*, whereas somatic embryogenesis was reported on medium containing NAA alone in *Solanum melongena* [21].

Somatic embryo maturation is a critical step in somatic embryogenesis which leads to the complete plantlet formation. In the present investigation both auxin and cytokinin combination favoured the maturation and germination of somatic embryos.

This is probably because of conversion of some of the heart-shaped embryos to torpedo or cotyledonary stage embryos and their subsequent germination in the presence of IAA+BAP. Thus, a combination of IAA+BAP combination seems to be necessary for maturation and germination of bipolar somatic embryos in *S.nigrum* [22] have reported that TDZ (1.0 mg/L) in combination with GA_3 (1.0 mg/L) was found to be comparatively more effective than BA for somatic embryo maturation in *Pimpinella tirupatiensis* an endangered medicinal plant. The requirement of auxin–cytokinin combination was also reported in *S.surattense* for germination of torpedo-shaped embryos[5] as it was noted in the present investigations.

According to [23] new gene products are needed for the progression from the globular to the heart-stage and these new products are synthesized only when an exogenous auxin is removed. But, according to our observations in *S.nigrum* for induction of somatic embryos, auxins and cytokinin combination is required. At higher concentration of auxin probably the population of embryogenic cells drops due to their disruption and elongation and the embryogenic potential of the culture is lost [24]. Similarly, in the present investigation embryogenesis was inhibited at 6.0 mg/L concentration of NAA + 0.5 mg/L BAP.

Whereas [25] reported that the entire process of induction and maturation of the embryos was completed on the same MS medium containing auxins and cytokinins (2,4-D + TDZ) in *Capsicum annuum* as it was observed the requirement of both the hormones in the present investigations. Similarly, somatic embryos maturation on MS medium containing the combination of auxins (NAA) and cytokinins (BAP) was observed in *Prunus avium* [26].

Thus, Somatic embryogenesis always appeared to be dependent on the type of auxin / cytokinin / auxin + cytokinin and their concentrations in the medium. The type of growth regulator and its concentration also varies from genotype to genotype. High concentration of auxin in combination with less concentration of cytokinin induced the somatic embryogenesis and maturation of somatic embryos in *S.nigrum*. However, for germination of somatic embryos, low level of auxins and high concentration of cytokinin combination is required.

Regeneration via somatic embryogenesis is better for obtaining genetically uniform plants than through organogenesis. It is evident from the present studies that the somatic embryogenesis in this species will be useful in the conservation and improvement of this threatened medicinally important species *S.nigrum*. Somatic embryogenesis is also preferred because it allows production of plants without somaclonal variation and also used for genetic transformation [27]. These somatic

embryoids induced in *S.nigrum* can also be used for development of synseed technology for germplasm storage, conservation and also for exchange.

Thus, for induction of *in vitro* somatic embryogenesis the type of primary explant, genotype and growth regulators concentration and combinations play an important role. The protocol developed in the present investigation can be used for mass–scale propagation of *true-to-type* of *S.nigrum.*

5.CONCLUSION: In conclusion, this is the first report of a successful procedure to regenerate *S.nigrum* via somatic embryogenesis. The cotyledon explants were proved to be efficient for in vitro somatic embryogenesis compared to leaflet explants in *S.nigrum*. MS medium supplemented with higher amounts of auxins in combination with lower concentrations of cytokinins favor the induction and proliferation of somatic embryogenesis. Thus, the present reproducible regeneration protocol can be used for mass multiplication, genetic transformation, artificial seed production and cryopreservation of the important medicinal plant *S.nigrum* .

REFERENCES

1. **Ammirato, P.V.** (1987). Organization events during somatic embryogenesis. In : *Plant Tissue and Cell Culture*, (Eds.) Green, C.E., Somers, D.A., Hackett W.P. and Biesboer, D.D. Alan R. Liss, Inc. New York, pp. 57-81.
2. **Roberts, A.V., Yokoya, K., Walker, S. and Mottley, J**. (1995). Somatic embryogenesis in woody plants (eds) Jain, S. Gupta, P. and Newton, R) Kluwer Academic Publishers, The Netherlands, pp. 277-289.
3. **Ammirato, P.V.** (1983a). Embryogenesis. In : *Hand book of plant cell cultures* (eds.) Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. McMillan Publishing Co., New York, pp. 82-123.
4. **Ammirato, P.V.** (1983b). The regulation of somatic embryos development in plant cell cultures, suspension cultures technique and hormone requirements. *Bio / Technol.,* **1 :** 68-74.
5. **Rama Swamy, N., Ugandhar, T., Praveen, M., Venkataiah, P., Rambabu, M., Upender, M. and Subhash, K.** (2005). Somatic embryogenesis and plantlet regeneration from cotyledon and leaf explants of *Solanum surattense. Indian J. Biotech.,* **4:**414-418.
6. **Reinert, J.** (1958). Morphogenese and ihre, kontrolle an gewebekulturen aus carotten. *Natusuissen schaften*, **45 :** 344-345.
7. **Steward, F.C., Mepes, M.G. and Mears, K.** (1958). Growth and organized development of cultured cells organization in cultured grown from freely suspended cells. *Amer. J. Bot.,* **445 :** 705-708.
8. **Yadav, J.S. and Rajam, M.V.** (1997). Spatial distribution of free and conjugated polyamines in leaves of *Solanum melongena* L. associated with differential morphogenetic capacity : efficient somatic embryogenesis with putresine. *J. Exptl. Bot.,* **48 :** 1537-1547.
9. **Yadav, J.S. and Rajam, M.V.** (1998). Temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in egg plant. *Plant Physiol.,* **116 :** 617-625.

10. **Mohan, J.S.S., Vijayakumar, V., Aparna, V. and Vaidya, R.P.** (2000). Somatic embryogenesis and plant regeneration in *Tribulus terrestris* (L.). *Phytomorphology,* **50 :** 307-311.
11. **Sahrawat, A.K. and Chand, S.** (2001). Continuous somatic embyogenesis and plant regeneration from hypocotyl segments of *Psoralea corylifolia* Linn. An endangered and medicinally important Fabaceae plant *Curr. Sci.,* **81:** 1328-1331.
12. **Archana.P, Mahitha.B and Rama Swamy.N** (2016). Plant Regeneration via Somatic Embryogenesis in an Ethnomedicinal Plant Senna alata (L.) Roxb. 15(4): 1-8
13. **Ko Kyung-Soo** , **Heo In-Ok** , **Yang Kwan-Pal** , **Lee Woon-Jin** , **Kim Chang-Min** , **Jo Pill-Hyeong** (1991)Transformation and Hairy Root Culture in Solanum nigrum by Agrobacterium rhizogenes, 22(1): 26-32
14. **Wu XF1, Shi HP, Tsang P, Keung E.(** 2008**)** Induction and in vitro culture of hairy roots of Solanum nigrum L. var. pauciflorum Liou and its solasodine production; 41(3):183-91.
15. **Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.,* **159 :** 473-497.
16. **Kumari, N. Jaiswal, U, and , Jaiswal, V.S (**1998). Induction of somatic embryogenesis and plant regeneration from leaf callus of *Terminalia arjuna. Curr. Sci.,* **25 :** 1052-1055.
17. **Matsuoka, H. and Hinata, K.** (1979). NAA-induced organogenesis and embryogenesis in hypocotyl callus of *Solanum melongena* L. *J. Exptl. Bot.,* **30:** 363-370.
18. **Gleddie, S., Keller, W. and Setterfield, G.** (1983). Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of *Solanum melongena* (egg plant). *Can. J. Bot.,* **61 :** 656-665.
19. **Sharma, P. a nd Rajam, M.V.** (1995). Genotype, explant and position effects on organogenesis and somatic embryogenesis in egg plant (*Solanum melongena* L.). *J. Exptl. Bot.,* **46 :** 135-141.
20. **Mujib, A., Bandyopadhyay, S., Jana, B.K. and Ghosh, P.D.** (1998). Direct somatic embyogenesis and *in vitro* plant regeneration in *Hippeastrum hybridum*. *Plant Tiss. Cult.,* **8 :** 19-25.
21. **Cavallini, A.and Natali, L.** (1989). Cytological analyses of *in vitro* somatic embryogenesis in *Brimeura amathystina* Salisb. (Liliaceae). *Plant. Sci.,* **62 :** 255-261.
22. **Prakash E, Sha Valli Khan, Elusing Meru and Rao K.R** (2001). Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. & Subr; an endangered medicinal plant of Tirumala Hills. *Curr. Sci.* **81:** 1239-1242.
23. **Zimmerman, U.** (1993). Somatic embryogenesis : A model for early development in higher plants. *J. Plant Cell,* **5 :** 141-143.
24. **Bhojwani, S.S. and Razdan, M.K.** (1996). *Plant Tissue Culture Theory and Practice,* Elsevier, Amsterdam pp. 125-166.
25. **Binzel, M.I., Sankhla, N., Sangeeta Joshi and Sankhla, D.** (1996). Induction of direct somatic embryogenesis and plant regeneration in pepper (*Capsicum annuum* L.). *Plant Cell Rep.,* **15 :** 536-540.
26. **Garin, E., Grenier, E. and Granier, G.D.** (1997). Somatic embryogenesis in wild cherry (*Prunus avium*). *Plant Cell Tiss. Org. Cult.,* **48 :** 83-91.
27. **Sharp, W.R., Sondahl, M.R., Caldas, L.S. and Maraffa, S.B.** (1980) The physiology of in vitro asexual embryogenesis. Hortic. Rev. 2: 268–310.