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Full Length Research Paper

Effect of explant age, hormones on somatic embryogenesis and production of multiple shoot from cotyledonary leaf explants of *Solanum trilobatum* L.

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The present study examines the effect of explant age and various concentrations of kinetin and BAP on somatic embryogenesis and organogenesis in *Solanum trilobatum* L. MS medium fortified with 11.1 µM BAP + 13.95 µM KN produced highest frequency of embryogenesis (97.3%) and average number of multiple shoots (48.4 ± 1.33) from cotyledonary leaf explants at two opened leaves stage. The presence of BAP and/or KN was critical in increasing embryos and multiple shoots per explant with its increasing concentrations which was also supported from significant positive corrilation ($p < 0.05$). It was also observed that embryogenic and organogenic response was significantly affected by explant type in all concentrations of BAP and KN alone as well as in combinations ($P < 0.01$). Rooting of shoots occurred on half strength MS medium supplemented with 7.35 and 9.8 µM IBA respectively. The rooted plantlets were well accomplished with a survival frequency of 86.5 ± 5%. Moreover, there were no phenotypic differences observed between the *in vitro* regenerated and *in vivo* plantlets.

Key words: Somatic embryogenesis, regeneration, endogenous hormones, explant type, explant age, *Solanum trilobatum* L.

INTRODUCTION

Solanum trilobatum (Solanaceae) is an economically important and a rare perennial, medicinal herb found in tropical and subtropical regions (Kirtikar and Basu, 1975). Almost all parts of this herb are used in the treatment of several ailments in herbal systems of medicine (Purushothaman et al., 1969; Mohanan and Devi, 1998, Subramanian and Madhavan, 1983; Govindan et al., 1999; Rajkumar and Jebanesan, 2005). The presence of the active principle sobatum has increased its demand, which has lead to the indiscriminate harvesting of this medicinal plant. Hence it becomes imperative to establish a suitable protocol for *in vitro* propagation of this plant system. Earlier reports on somatic embryogenesis and shoot formation of *S. trilobatum* have used either alone or combinations of NAA or IAA and BAP or kinetin using shoot tips, leaf, stem segments, nodes and inter nodal segments as explants (Arokiasamy et al., 2002; Alagumanian et. al., 2004; Jawahar et al., 2004). According to the report of Arokiasamy et al. (2002) 5 mg/l BAP in combination with either 0.05 mg/l IAA or NAA produced optimum number (12.3 and 13.3 respectively) of shoots. Alagumanian et al. (2004) observed highest average of 45 shoots from stem explant inoculated on MS medium supplemented with 13.3 µM BAP and it was maximum of 30 - 40 shoots on combinational treatment of 2.7 µM NAA + 4.4 µM BAP concentration. Jawahar et al. (2004) found highest of 95% of regeneration and 36.33 ± 1.52 multiple shoots observed from nodal segments inoculated on 8.8 µM BAP supplemented medium, while it was 20.0 ± 1.00 on KN added medium. Since the effect of BAP and KN individually has proven very efficient in regeneration of this species (Alagumanian et al., 2004; Jawahar et al., 2004) and there is no reports on effect of explant age and production of multiple shoots from different combinational concentrations of BAP and KN, we have chosen two types of cotyledonary leaf explants to produce highest number of multiple shoots under the influence of different

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Abbreviation: **MS**, Murashige and Skoog; **BAP**, 6-benzyl adenine purine, **KN**, kinetin; **IBA**, indole-3-butyric acid; **SE**, somatic embryogenesis.

combinational concentrations of BAP and KN. This is the first report on production of highest number of multiple shoots from two stages of cotyledonary leaf explants of *S. trilobatum* L.

MATERIALS AND METHODS

Plant material and explant type

Seeds of *S. trilobatum* L. were obtained from National Seed Corporation, PUSA Campus, New Delhi, were used for present study. Seeds were treated with 1% Bavistin and 4 ppm chloramphenicol for 5 min and thoroughly washed with sterile distilled water. Disinfected seeds were sown in plastic cups containing a sterilized mixture of garden soil and vermiculate (1:1, v/v). Within one week, the seedlings assumed the walking stick shape (stage I) wherein the cotyledons did not open up. After 3 - 4 days the cotyledons opened up and seedlings assumed T-shape (stage II). Cotyledons at these two stages of seedlings were used as the explants (Figure 2A).

Somatic embryogenesis

Cotyledonary leaves were excised from the germinated seedlings and disinfected with 0.1% mercuric chloride for 5 min and then washed in three changes of sterile distilled water. Each cotyledon was cut into two segments and these were inoculated on MS (Murashige and Skoog, 1962) medium fortified with various concentrations of BAP and kinetin separately as well as in combination. The medium was adjusted to pH 5.7 to 5.8 before autoclaving and gelled with 0.8% Agar (Sigma, INDIA). The cultures were incubated at 25 ± 2°C under 16/8 photoperiod in cool white fluorescent light (40 µmol m^{-1} s^{-1}). The percentage of explants producing somatic embryos was calculated at the end of 2nd week of the inoculation and considered as frequency of initiation.

Average number of shoots

For multiple shoot production, two weeks old cultures were initially subcultured into different combinational concentrations of KN and BAP. Since 11.1 µM BAP proved to be optimum for the embryogenesis as well as shoot formation in our preliminary studies, 11.1 µM BAP and different concentrations of KN from 2.325 to 23.25 µM (with 2.325 µM interval) has been used to understand the effect of the KN for production and development of multiple shoots. Total five replicates were maintained and each replicate carries 5 segments. At the end of 4th week responded segments were transferred into pre-autoclaved culture vessels (Baby jars, 175 ml capacity, Sigma-Aldrich Co. Ltd. INDIA) closed with Magenta Caps. Cultures were incubated at 25 ± 2°C under 16/8 photoperiod in white intense fluorescent light (350 µmol m^{-2} s^{-1}). The number of shoots produced by the explants was counted at the end of sixth week after inoculation and is expressed as average.

Rooting of shoots

The shoots harvested from the media were washed with double distilled water and transferred into half strength MS medium supplemented with different concentrations of IBA from 0 – 24.5 µM with 2.45 µM interval for rooting.

In order to understand the effect of explant age and hormone concentration on somatic embryogenesis and multiple shoot production, correlations and analysis of variance have been done following Snedecor (1961).

RESULTS

Somatic embryogenesis (SE)

The initial response of explants obtained at one week after inoculation and at the end of the second week prompt somatic embryos were protruded out and appeared as green globular structures (Figures 2B and C). Explants inoculated on only KN supplemented media produced less than 30% of SEs; Type-II explants revealed a maximum of 28% in 13.95 µM KN while it was only 26% in Type-I explant at same concentration. Minimum of 2% was observed at 18.6 µM KN in Type-I explants. The response of SE is gradually increased with increasing concentration of KN up to 13.95 µM and there up on decreased (Figures 1A and B).

In BAP supplemented medium, maximum of 80 and 75% of explants showed somatic embryogenesis at 11.1 µM in Type-II and Type-I explant sources, respectively. A minimum of 5% response was noticed in Type-I explants at 2.22 µM BAP.

Of the different combinational concentrations of BAP and KN used in the present study 11.1 µM BAP + 13.95 µM KN produced highest of 97.3, 93.7 percentage of somatic embryogenesis in Type-II and Type-I explants in this order (Figures 2D and E). Response of somatic embryogenesis increased with increasing concentration of KN up to 13.95 µM concentration there upon decrease was observed. Explants inoculated on more than 22.2 µM BAP and 23.25 µM KN alone or in combinations turned brown and there is no further response even they are maintained for 6 weeks after inoculation.

Average number of shoots

Although, the production of multiple shoots at different concentrations of BAP and KN used alone as well as in combinations showed similar trend as that of somatic embryogenesis, the average number of shoots obtained from each treatment and in two types of explant was varied. Of the different hormones used, KN produced lowest number of shoots, that is, maximum of 4.6 ± 0.87 in Type-II and 4.2 ± 0.73 in Type-I explant. In combinational concentrations, explants yielded highest average of shoots than the other treatments, where the optimum of 48.4 ± 1.33 multiple shoots observed at 11.1 µM BAP + 13.95 µM KN in Type-II explants followed by 44.2 ± 1.43 in Type-I explants. When the shoot formation response on BAP supplemented media was considered, intermediary number of shoots was observed that of KN alone and in KN and BAP concentration. The maximum of 35.2 ± 2.17 multiple shoots were observed in Type-II explants and 30 ± 1.029 multiple shoots recorded in Type-I explants at 11.1 µM BAP concentration (Figure 2G).

Rooting and hardening

Well developed shoots (5 cm height) were transferred on

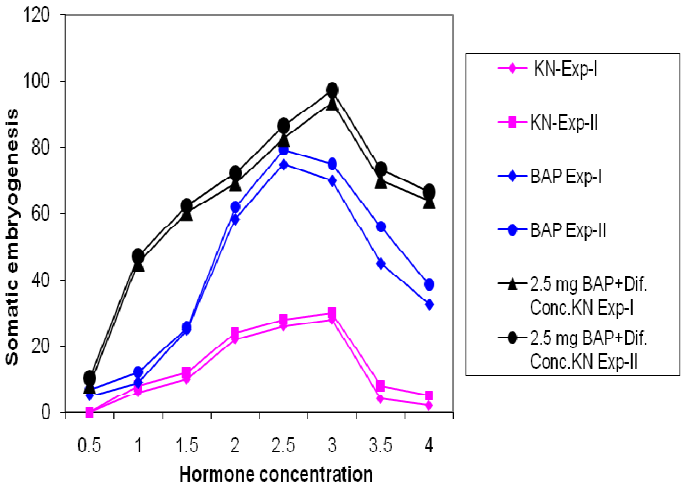

Figure 1A: Effect of explant type and BAP/KN concentrations on somatic embryogenesis.

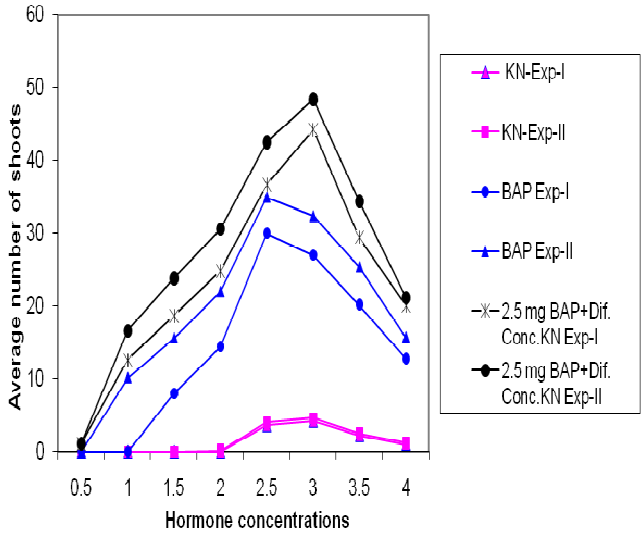

Figure 1B. Effect of explant type and BAP/KN concentrations on average number of shoots.

to rooting medium containing different concentrations (0 – 24.5 µM with 2.45 µM interval) of IBA, with the best rooting response on half strength MS medium fortified with 7.35 µM IBA (87%) followed by 9.8 µM (72%) in both types of explant sources. Plantlets at five leaves stage were transferred to (1:1) sterilized soil + vermiculate mix in growth chamber for two weeks and further they were transferred into the pots and experimental garden. Plants regenerated from two types of explants were phenol-typically normal and uniform in their growth in the green house and did not reveal any variation (Figure 2H).

Correlation analysis was carried out to find the relationship between the concentrations of KN, BAP and BAP +

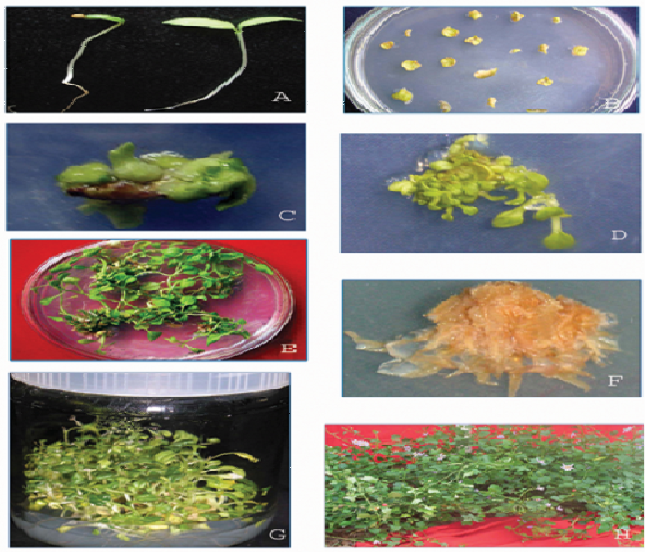

Figure 2. *In vitro* plant regeneration from two stages of explants of *Solanum trilobatum* L. A) Two stages of explants. B) Explants at initial response. C) SEs at 3rd week of inoculation. D) Multiple shoots from Type-I explant 4th week. E) Multiple shoots from Type-II explants 4th week. F) Rooty callus obtained from MS basal medium. G) Multiple shoots possiss roots on rooting medium 8th week. H) *In vitro* grown plants.

KN and response of the two explant types for SEs as well as average number of shoots. This revealed positive and significant correlation ($p < 0.05$) for all treatments except SE response in KN alone supplemented media where the correlation was positive but not significant.

The response of two explant types and effect of hormonal concentrations (both individual and in combinations) on two traits used in this study were analysed by two-way ANOVA, which revealed the presence of significant differences between the explant types and concentrations of the two cytokinins (BAP and KN) individually as well as in combinations ($p \leq 0.01$; Table 1).

DISCUSSION

To our knowledge this is the first report that systematically investigated the effect of explant type on somatic embryogenesis and formation of multiple shoots in *S. trilobatum* L. The results presented here indicate that explant age and hormonal concentration play a pivotal role and constitute the primary step in the standardization of *in vitro* regeneration protocol for this plant system. The developmental window involves the walking stick stage and the two open leaved (T-stage) explants had shown diversified response to various concentrations of the BAP and KN used. Of the two types of explants, Type-II showed better embryogenic and shooting response than the Type-I explant indicating that the matured explants produce more shoots *in vitro* than the younger

Table 1. Analysis of variance and percentage contribution of concentrations and explant on somatic embryogenesis and multiple shoot production in *Solanum trilobatum* L.

Source of variation	DF	Somatic embryogenesis		Avg. number of shoots	
		MSS	% Contribution	MSS	% Contribution
Between concentrations of KN	7	256.99	405.39*	6.65	446.17*
Between two types of explants	1	18.06	28.49*	0.14	9.43*
Between concentrations of BAP	7	1481.03	298.60*	257.53	53.01*
Between two types of explants	1	78.77	15.88*	120.72	24.85*
Between concentrations of BAP +KN	7	1381.65	5038.89*	404.11	141.22*
Between two types of explants	1	32.21	117.45*	66.99	23.41*

*P ≤ 0.01.

ones. Similar conclusion has been reported by Davies and Dale (1979), in *S. laciniatum*, and by Taha and Tijam (2002) in *S. melongena*, who found older explants to produce more shoots *in vitro* than the younger ones.

Different authors reported earlier that the use of a variety of phytohormones which included auxins (NAA and IAA) cytokinins (BAP and KN) alone as well as in combinations for the *in vitro* response of *S. trilobatum* (Arockiasamy et al., 2002; Alagumanian et al., 2004; Jawahar et al., 2004). Of these, either NAA or BAP alone or in combination have been most widely used for *in vitro* morphogenesis in *S. trilobatum* L. In contrast to these in the present study, BAP in combination with KN produced more number of shoots than other treatments. However, shoot formation response in KN alone supplemented media was considerably low; it become optimum when combined with BAP indicating the combinational role of BAP and KN on embryogenesis as well as shoot formation and its development by suppress endogenous auxins level. Moreover, the gradual increase in the average number of shoots with the increasing concentration of KN indicates the influence of endogenous auxins levels during the period of embryogenesis and shoot formation. According to our observation endogenous auxin production might be increased during the somatic embryogenesis which thwarts the formation of shoots and its elongation. Increasing concentrations of BAP along with KN gradually suppress the expression of endogenous auxins and induced highest number of shoots. This idea is further confirmed by the observation that two types of explants and somatic embryos derived from two types of explants produced numerous hairy roots and rooty callus when they are transferred onto MS basal medium (Figure 2F). This was also supported by the observation of Alagumanian et al. (2004) where BAP alone producing more shoots (that is, average of 45) than the combination with synthetic auxin NAA, where they found nearly 5 - 10 % reduction in shoot production

The rooting response was best in half strength MS medium fortified with 7.35 µM IBA in this plant system. Similar occurrence of maximum and healthy rooting response was reported in this species by earlier workers on IBA containing MS/LS medium (Arockiasamy et al., 2002; Alagumanian et al., 2004; Jawahar et al., 2004).

During the present study an interesting observation made is that the formation of somatic embryogenesis as well as regeneration of plantlets could be obtained on the same hormonal concentration (KN, BAP and KN + BAP) but at different time intervals, the former during the initial period (up to 4 weeks) of culture and the later after eight weeks. Though the exact biochemical or molecular basis of such differential response as a function of culture time could not be ascertained from the present study, it may be conjectured from the information available from earlier works that endogenous hormonal levels as well as those of polyamines might be changing with culture time and may influence the final response (Fobert and Webb, 1988; Momiyama et al., 1995; Sharma and Rajam 1995; Yadav and Rajam, 1997; Scoccianti et al., 2000). The present plant system and explant types provide a suitable experimental system to undertake detailed analysis of these biochemical factors. Apart from this, the present protocol is useful for producing highest number of plantlets without any seasonal constraints for crop improvement.

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