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Callus culture and Morphogenetic Reposes of a Medicinally Important Plant *Butea monosperma*.

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ABSTRACT

An *in vitro* protocol was developed for callus production and regeneration of *Butea monosperma* of family fabaceae which is widely used in traditional medicine around the world. It is a common plant of medicinal value and distributed throughout India and is known as Flame of forest, palash, mutthuga, bijasneha, dhak, khakara, chichra. Different explants (seeds and nodal segments) used in the present investigation responded differently to various concentration and combinations of hormones. Nodal segments were founds to be more suitable explants than seeds of this plant. *In vitro* generation of callus was initiated from young nodal segments on MS medium supplemented with different concentrations of phytohormones. The callus initiation took place within 15-24 days of inoculation of nodal segments. The unorganized mass of callus tissues thus developed was hard and blackish. It was maintained on MS media by frequent subculturings after every 4-6 weeks. It grew profusely after a number of subculturings and possessed high capacity of growth. It was observed that BAP (2.5 mg/L) in combination with NAA (0.5 mg/L) was best for callus induction and establishment. Kn did not proved to be beneficial for producing callus in the present investigation. Nodal segments inoculated in the media supplemented with BAP: NAA: IAA (1:1: 0.2mgL^{-1}) gave long multiple shoot . These shoots were rooted in media without any growth regulators. Profuse rooting was seen in *in vitro* regenerated shoots grown in the media supplemented with IBA (3.0mgL^{-1}) and when NAA also added in the media IBA: NAA (2: 0.2mgL^{-1}), rooting from *in vitro* shoots were observed. The results accomplished were found to be useful in developing a complete *in vitro* regeneration protocol towards the mass production of *Butea monosperma*, which may provide a basis for further genetic improvement that may prove its use as an alternative medicinal resource in commercial applications.

Keywords: *Butea monosperma***,** Multiple shoots, Callus cultures, Growth regulators

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INTRODUCTION

Butea monosperma is a common plant of family fabaceae and is known as Flame of forest and is locally called as palas, palash, mutthuga, bijasneha, dhak, khakara, chichra. It is extensibly used in Ayurveda, Unani and Homeopathic medicines and commonly it is used as tonic, astringent, aphrodisiac and diuretics. It is reported to possess antifertility, aphrodisiac and analgesic activities. Flowers are useful in diarrhoea, astringent, diuretic, depurative, tonic, leprosy, skin diseases,gout, thirst, burning sensation. The stem bark is useful in indigenous medicine for the treatmentof dyspepsia, diarrhoea, dysentery, ulcer, sore throat and snake bite. Besides medicinal uses it is also having the economic use such as leaves are used for making platters, cups and bowls and bark fibres are used for making cordage.

Plant tissue culture technique is now a well-established technology to improve the quality and quantity of useful plants. Torrey (1966)[1] reported various ways of regeneration of plants either by somatic embryogenesis or through adventitious shoots. The presence of auxins and cytokinin in the culture medium regulates various aspects of dedifferentiation and differentiation[2] at cellular levels. Generally, auxins have been used for callus induction and proliferation, and both cytokinins and auxins were required for redifferentiation of callus into organized cell[3,4] .

Genetic instability being the drawback of callus, the regenerated plants are not uniform, hence, the correct initial explants should be chosen to raise callus. An important milestone in the study of morphogenesis in tissue culture is the role of auxin and cytokinin interaction. Auxins are concerned with the physiological activity leading to cell enlargement. A number of plant tissues *in vitro* synthesize growth regulators in sub-optimal amounts and therefore, require addition of external phytohormones[5-6] . Cytokinins are involved indirectly in a wide variety of biochemical activities of physiological functions leading to most heterogeneous, histological and morphological results. They play an important role in inducing chlorophyll formation and shoot induction in plant tissue culture[7] . Skoog and Miller (1957)[8] reported that high levels of auxins promoted callus initiation and establishment in tobacco plant. The observations show that high auxins : low cytokinin ratio favors callus and root induction. Therein it has been shown, that as we cannot gauge the endogenous levels of these two compounds, the relative concentration for obtaining the desired end results is largely a matter of trial and error in each case as has been reported by many workers[9-17]. *Butea monosperma* Linn. is an important medicinal plant but very less work has been reported on this plant specially in tissue culture[18-19] . The present investigation on tissue culture of *Butea monosperma* was carried out *in vitro* keeping in view

raising callus cultures from nodal segements of *Butea monosperma* under defined nutritional, hormonal and cultural conditions and to develop plantlets in vitro.

MATERIALS AND METHOD

In the present investigation seeds and nodal segments of *B. monosperma* were used as explants on MS medium[20] . The different concentrations of phytohormones (2,4-D, NAA, BAP and KN) were supplemented to MS media in isolation or combination with each other Seeds were surface sterilized with mercuric chloride ($HgCl_2$) solution (0.1%; w/v) for 2 min and subsequently rinsed thrice with sterile distilled water where as nodal segments were treated with antibiotic (Ciprofloxacin, 250mgL^{-1}) prior to inoculation in order to remove any kind of microbial interactions. Surface sterilization and inoculation of seeds and nodal segments were done in a Laminar flow hood fitted with ultraviolet light. Before starting the inoculation work, slab of Laminar flow was cleaned with rectified spirit and culture vessels containing autoclaved media, petri dishes, and spirit lamps, cotton and other things required were kept on the slab of transfer chamber. A day before inoculation of work, transfer chamber was fumigated with fumes obtained by heating formic acid and potassium permanganate ($KMnO_4$). The forceps, scalpels, needles; scissors were kept in a glass tube column containing rectified spirit. UV light was used for an hour to sterilize the chamber. Seeds as well as nodal segments were inoculated in the flasks containing culture medium aseptically. Cultured flasks were incubated in culture chamber. The temperature of chamber was maintained at 25± 1^0 C using air conditioner and light intensity (1200 lux) was provided from fluorescent tubes (40 watt) and incandescent bulbs (40 watts). A photoperiod of 16h light was provided. . Callus cultures thus established were maintained on MS medium indefinitely by frequent subculturings in fresh medium by transferring small pieces of the same to fresh agar medium with same concentration of growth hormone. The cultures were observed and examined every week and final morphogenetic data were recorded.

Nodal explants obtained from 25 days old aseptically germinated seedlings of *B. monosperma* were used as explants for tissue culture studies. Nodes were cultured on MS medium supplement with BAP (1.0-5.0 mg l^{-1}) or Kn (1.0-5.0 mg l^{-1}), either individually or in combination with NAA and IAA (0.1, 0.5, and 1.0 mg l^{-1}). Cultures were subcultured onto fresh media for multiplication of shoot bud after 4 weeks upto maximum of 6 subculturings. The frequency of explants producing shoots, number of shoots per explant and shoot length were recorded after every 2 weeks of culture. The elongated shoots (>3.0 cm length) with fully expanded leaves were transferred to rooting media consisted of MS medium supplemented with various concentrations

of IBA and NAA (0.5, 1.0 and 2.0 mg l^{-1}). The duration for rooting was recorded for various treatments. The observations on the root induction, number of roots and average length were taken after 4 weeks of culture. The plantlets with well-developed roots were carefully removed from the culture tubes and washed with water to remove agar sticking to the roots. The rooted plantlets were then transferred to plastic pots containing autoclaved soil and vermiculate (1:1) in culture room.

In vitro grown plantlets were ready for transfer from aseptic culture to the field. These were prepared for further growth, hardening and acclimatization. Plantlets were removed from the parental cultures and transferred to the reduced salt concentration eliminating vitamins and growth regulators. The plantlets were then removed from cultures and were thoroughly washed with sterile water to remove agar. They were than transferred to small preautoclaved earthen pots containing soil: vermiculture (3:1) mixture. The plantlets were watered with sterile a distilled water having 1:1 solution of ammonium nitrate and potassium nitrate and covered with polythene bags to maintain humidity. These pots were kept at 25 ± 1^0 C and 16 hr. light and then plants were steady and acclimatized, they were transferred to the field.

RESULTS AND DISCUSSION

In vitro generation of callus was initiated from young nodal segments on MS medium supplemented with different concentrations of phytohormones. The callus initiation took place within 15-24 days of inoculation of nodal segments. Nodal segments were founds to be more suitable explants than seeds of this plant. The unorganized mass of callus tissues (non-differentiating calli) thus developed was hard and blackish and thus maintained by frequent subculturings after every 4-6 weeks. It was observed that BAP (2.5 mg/L) in combination with NAA (0.5 mg/L) was best for callus induction and establishment . Kn did not proved to be beneficial for producing callus. The callus so produced was compact and blackish in color. At the beginning of culture period it was necessary to subculture after every 15 days, otherwise the calli turned dark and growth ceased. Thereafter subculturing was done after every 4 to 6 weeks. The darkening of callus was probably due to the production and oxidation of phenolic compounds released by tissue with the age of callus. It grew profusely and possessed high capacity of growth after frequent subculturings of 4-6 weeks time intervals. The growth indices (GI) of the callus were subsequently calculated after every 2 weeks of transfer age**.** The maximum GI (2.97; based on dry weight) was found in tissue grown at the transfer age of 6 weeks (42 days) which decreased subsequently in 8 weeks old tissue.

Multiple Shooting and Rooting

Variety of auxins and cytokinins concentrations gave effect on shoot production with or without callus. The lower auxin concentration enhanced multiple shoots production. Nodal segments inoculated in the media supplemented with BAP: NAA: IAA (1:1: 0.2mgL^{-1}) gave long multiple shoot . These shoots were rooted in media without any growth regulators. Profuse rooting was seen in *in vitro* regenerated shoots grown in the media supplemented with IBA (3.0mgL^{-1}) and when NAA also added in the media IBA: NAA (2: 0.2mgL^{-1}), rooting from *in vitro* shoots were observed . After successful root development, *in vitro* grown rooted plantlets were taken out from the culture vessels, without causing any damage to the delicate root system. These were then gently washed with sterile distilled water to remove every trace of media sticking to roots. Plantlets were then transferred to small plastic pots having a mixture of sterile vermiculite and soil in the ratio of 1:3. Immediately after transplantation, the pots were kept in growth chamber for 15 days at 26±2^{0}C and 2000 lux light intensity for acclimatization. In order to maintain high humidity, the pots were covered with inverted glass beakers. These plantlets were irrigated two times daily with sterilized water containing 1:1 solution of ammonium and potassium nitrate. After 2 days of transplantation, wilting and yellowing started in leaves of 35 % plantlet. After 1 week wilting increased in remaining plantlet and almost 48 % plants were wilted in next 1 week, remaining plantlets continued to grow for 20 days. After 20 days plantlet were transferred to soil where they continued to grow normally. In all about 65% plantlets were successfully survived and transferred in soil.

There is no uniform and clear definition of growth of plant cell cultures and dry weight or fresh weight methods have been in use for determining GI, because of its preciseness, accuracy in observing variation[21]. Several workers have established the unorganized static cultures of different plants on different medium and observed the sigmoid growth pattern of the callus culture[22]. In the present study a sigmoid pattern of growth curve was observed in *B. monosperma*. The maximum growth index was achieved at the 6th week of subculture indicating the exponential growth phase. Minimum growth index was observed at 2nd week of subculture. An increase in GI after supplementation of various growth regulators finds support from the observations that the growth of tissue, some time depends upon the culture medium and also controlled by the environmental and biological factors like pH, dose and combinations of growth regulators used[23]. Recently, Ratnaprabha et al., (2017)[19] reported micropropagation in *B. monosperma* through cotyledonary nodes of mature seeds. Among various cytokinins tested, high frequency of direct shoot regeneration was induced on Murashige and Skoog (MS) medium

supplemented with benzylaminopurine, which found to be more effective and showed an optimal response at 2 mg/L with a maximum number of 8.35 ± 0.32 multiple shoots per explant. In the present study also multiple shoots were observed in media supplemented with different doses of cytokinins. The observations of present investigation are in agreement with Thorpe and Patel (1984)[24] that tissue or organs used as source of explants can also be determinant for the success of plant tissue.

CONCLUSION

Nodal segments were founds to be more suitable explants than seeds for the callus initiation of *B. monosperma.* BAP (2.5 mg/L) in combination with NAA (0.5 mg/L) was best for callus induction and establishment whereas Kn did not proved to be beneficial for producing callus in this plant. Proliferation of multiple shoot initiates when Murashige and Skoog's (MS) medium is supplemented with BAP:NAA:IAA (1:1:02MG/L^{-1}) after 3-4 weeks of culture and rooting of shoots initiates when the medium is supplemented with IBA:NAA (2:02mg/L^{-1}). In all about 65% plantlets were successfully survived and transferred in soil.

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