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Regeneration of Plants from Nodal and Internodal Segment Cultures of *Ephedra gerardiana* using Thidiazuron

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Abstract

Ephedra gerardiana is an important medicinal plant used as Soma in Indian Ayurvedic system of medicine and as Traditional Chinese Medicine since several thousand years. Excessive use of this plant has led to decline in its natural population. Present report describes the use of TDZ in MS for induction of somatic embryos and shoot buds in internodal and nodal segment cultures of *E. gerardiana*. Somatic embryos were obtained from internodal segment culture onto MS + 0.5 to 1 μM TDZ. In case of nodal segment culture, lower concentrations of TDZ induced only shoot buds in 63.33 - 88.88 per cent cultures. Onto higher concentrations, all cultures showed callus induction with shoot bud formation. Shoot buds elongated and rooted onto one fourth strength of MS having 20 μM IBA. Rooted shoots were transferred to plastic pots.

Introduction

Ephedra (Joint fir) is an interesting genus of the seed bearing non flowering plants belonging to the highly evolved order of Gymnosperm, Ephedrales (Bhatnagar and Moitra 1996). Events such as double fertilization, advanced anatomical features and entemophyllous pollination in a few species, further add to the antiquity of this plant. Ancient Indian Ayurvedic literature reveals *Ephedra gerardiana* as the Soma plant and in China; it is big used as TCM (Traditional Chinese Medicine) for more than 5000 years and is popularly known as Ma Huang. There are about 50 species of *Ephedra* (Stevenson 1993, Price 1996, Caveney et al. 2001) spreading world-wide, in Europe, temperate Asia, South America and Afghanistan to Bhutan (2400 - 5000 m) adapted to semiarid and desert environment. These are widely distributed in both Eastern as well as Western Hemisphere. *E. gerardiana* has been used for more than 5,000 years in

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China and India to treat conditions such as cold, fever, flu, headache, asthma, wheezing, and nasal congestion. It has also been an ingredient in many dietary supplements and used for weight loss, increased energy, and enhanced atheletic performance. *Ephedra* and its alkaloids are effective bronchodilators in the treatment of mild to moderate asthma and hay fever. Ephedrine is a potent bronchodilator that, in appropriate doses, can be administered safely along with therapeutic doses of theophylline without the fear of progressive tolerance or toxicity. Excessive use of powder of *E. gerardiana* as a weight loosing component and efficiency enhancement drug has also been reported. Therefore, this plant has been listed as an endangered species due to over-exploitation.

Thidiazuron (TDZ) is a substituted phenylurea (N-phenyl-1, 2, 3 thidiazol-5-phenylurea) inducing high rate of regeneration and axilliary shoot proliferation in several plant species (Fiola et al. 1990, Malik and Saxena 1992). TDZ is more biologically active than BAP or zeatin, and lower concentrations are needed in tissue culture, especially for micropropagation. Earlier somatic embryogenesis and regeneration of plantlets were reported in *Ephedra foliata* using auxin and cytokinins other than TDZ (Dhiman et al. 2010). TDZ is as or more effective in most of the species in which it has been tested, particularly in woody species (Lu 1993). Thidiazuron is being selected for micropropagation of a wide array of woody species because of its tremendous ability to stimulate shoot proliferation. In many cases, explants normally cultured on amino purine cytokinins can grow much faster when transferred to a medium containing TDZ. The concentration at which TDZ is most effective is 1 - 1000 times lower than other plant growth regulators. Overall, relatively low concentration of TDZ has the capacity to induce multiple axillary shoots to proliferate. At higher concentrations, TDZ is a very powerful agent that may result in the generation of the callus and the formation of adventitious shoots and embryo. Therefore TDZ may be the most potent of the diphenyl ureas that have been evaluated for use in plant tissue cultures (Mok et al. 1982, 1987). Most of the studies carried in gymnosperm tissue culture using TDZ as plant growth regulator are mainly carried out onto conifer species (Mithila et al. 2003, Tang and Newton 2005, Faisal and Anis 2006, Kim et al. 2009). The present report describes the role of TDZ in internodal and nodal segment culture of *Ephedra gerardiana* to study its application for micropropagation of this endangered species.

Materials and Methods

Plants of *Ephedra gerardiana* (Gymsoperm, Ephedrales) were collected from natural habitat of Chakrata forest division in the Uttarakhand state and were grown in pots in Botanical Garden of the Institute. Nodal and Internodal

segments were collected from these plants. About 1.0 - 2.0 cm long segments were taken from plants and these were also initially rinsed with 90% ethyl alcohol followed by washing with tap water. Explant was treated with 0.2% solution (v/v) of Tween 20 (Commercial Polyoxyethylene sorbitan monolaurate; S. D. Fine-Chem. Ltd., Mumbai) and kept under running tap water for 30 min. The cuttings were sterilized with 0.1% mercuric chloride solution (w/v) for three min and then repeatedly washed with sterile distilled water. At the time of inoculation, both ends of nodal and internodal segments were trimmed with the help of a scalpel under aseptic conditions. Both the ends of the internodal segments were also trimmed and a longitudinal slit was made before inoculation in these explants.

Shoots regenerated directly or indirectly through callus were of variable length. Callus pieces containing shoots or directly regenerated shoots were transferred onto basal medium for further elongation. The shoots grew further and attained a length of 3 - 8 cm onto basal medium. Rooting of excised shoots was achieved *in vitro*. For this, two auxins *viz.*, IBA and IAA were used. None of the IAA containing medium initiated roots in present investigation. The IBA only proved to be the rooting induction hormone. MS with its major salts reduced to half and one fourth strength was tested for rooting of the *in vitro* multiplied shoots of *E. gerardiana*. Shoots rooted best on one fourth strength of MS and 20 μM IBA. For transplantation, 6 - 10 cm long plantlets, 20 - 25 days after rooting, were transferred to plastic pots containing autoclaved coarse sand and garden soil in equal proportions and irrigated with sterile tap water. Initially the plants were covered with a small plastic chamber for 20 - 25 days for acclimatization. Explants were cultured onto MS having various plant growth regulators. For each treatment, a minimum of 24 cultures were raised and each experiment was repeated at least twice. The cultures were examined periodically and the morphological changes noted on the basis of visual observations. Results are expressed as per cent cultures responded and number of somatic embryos differentiated per culture. The number was counted under a stereoscopic binocular microscope. Standard error was calculated in experiments dealing with the number of somatic embryos regenerated.

Results and Discussion

Internodal segments were cultured *in vitro* to study their regeneration potential. Results obtained on different plant growth regulators are described below.

Various concentrations of TDZ were also tried in MS to study morphogenic potential of internodal explants. On to lower concentrations of TDZ (0.5 and 1.0μM), all cultures initiated callusing after 2 weeks of initial culture (Fig. 1A).

The callus yielded embryoid like structure in 10 - 33.33% cultures on to these media (Table 1). When TDZ concentrations were increased, the percentage response remained same. However, the degree of callusing was decreased. The callus was initiated from longitudinally injured surface of the explant after 2 weeks of culture on to medium containing 0.5 μM TDZ. Callus was also proliferated from cut end of the explants within next 4 - 5 weeks. The greenish white callus yielded embryoid like structure in only 10% of cultures on to this medium after further 4 weeks (Fig. 1 B, C). The embryoid like structures obtained from embryogenic callus turned into somatic embryos after 2 weeks of transfer on to basal medium (Fig. 1D).

Table 1. Effect of various concentrations of TDZ in internodal segment culture of *Ephedra gerardiana*.

TDZ (μM)	% of culture showing callusing	Degree of callusing	% of culture showing embryoid
0.5	100	+++	10
1.0	100	++	33.33
1.5	100	++	0.0
2.0	100	++	0.0
2.5	100	+	0.0
5.0	100	+	0.0

When internodal explants were cultured on to 1 μM TDZ containing medium, all cultures produced callusing from the explant. The explants yielded shoot buds directly from one end after 5 weeks of culture. The callus produced from cut ends, however, initiated somatic embryos in 33.33% of cultures after 4 - 5 weeks of culture. The callus bearing embryos were transferred on to basal medium where they germinated in to young plantlets. Higher concentrations of TDZ (1.5 - 5μM) produced only callusing without any organogenic response.

Various concentrations of thidiazuron were also used in basal medium to study regeneration potential of nodal explants (Table 2). When TDZ was used with basal medium, the explant showed initially an increase in size of scaly leaves present at the nodes followed by induction of shoot buds.

At lower concentrations of TDZ (0.5 - 2.5 μM), shoot buds produced directly from node without callus (Fig. 2E). When nodal segments were cultured on to medium containing 0.5 μM TDZ, explant showed enlargement of scaly leaves to nearly twice to their original size within 4 - 5 days of culture followed by shoot bud induction within 15 - 20 days. Percentage of cultures showing shoot bud induction was 63.66. After 6 weeks of culture, the maximum number of shoot buds was 4 and average number of shoot buds was 2.42 on to this concentration

(Fig. 1F). On to 1 μM TDZ concentration 87.5% cultures showed on an average of 2.85 shoot buds per culture. The maximum number of shoot bud was 4 on to this concentration.

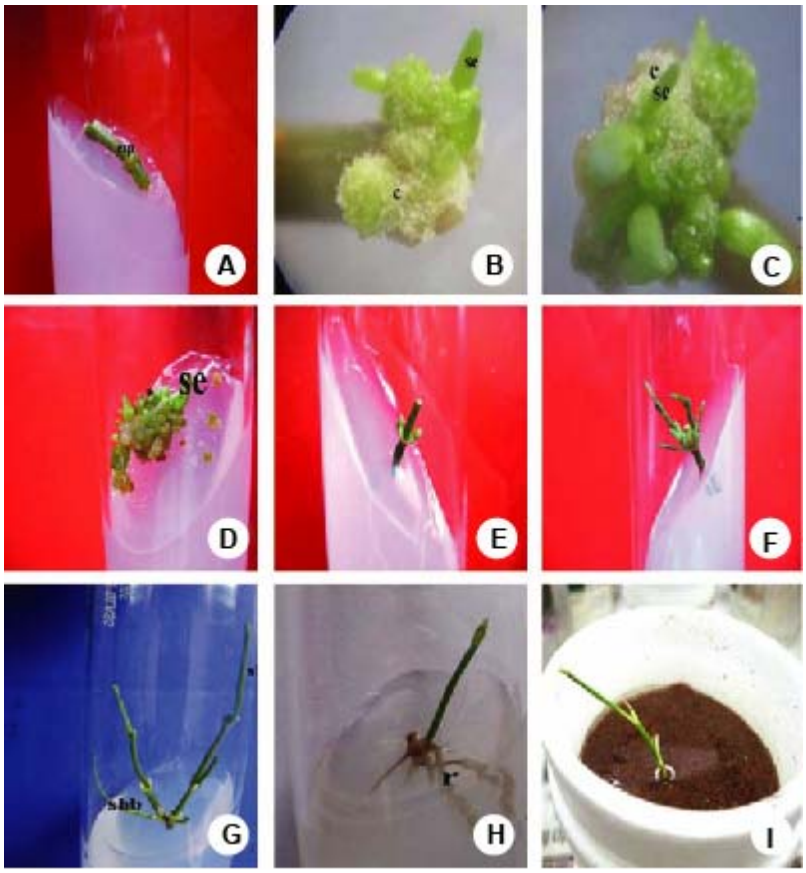

Fig. 1. Somatic embryogenesis, shoot bud formation and plant regeneration in *Ephedra gerardiana*. A. Internodal explant showing callus initiation on to MS + 1 μM TDZ after 2 weeks of culture. B, C. Embryoid formation from callus obtained on to MS + 0.5 μM TDZ after 4 weeks. D. Somatic embryos obtained from same as C after 2 weeks of transfer onto basal medium. E. Induction of shoot buds from nodal segment cultured on to MS + 0.5 μM TDZ after 1 week of culture. F. Same as E, after 6 weeks. G. Elongation of shoot on to MS after 3 weeks of transfer. H. Rooting of shoots on to one fourth MS + 20 μM IBA after 2 weeks. I. Transplanted plants in plastic pots.

At slight increased TDZ concentration (1.5 μM), percentage of cultures showing shoot buds was 88.88 (Table 2). By the end of 8 weeks, maximum number of shoot buds was 5 and average number of shoot buds was 2.62 on to this medium. At 2.5 μM TDZ, a few cultures exhibited callusing (8.3%). All

cultures showed shoot bud induction on to this medium within 10 - 15 days of culture. Average number of shoot buds was 2.14 with a maximum number of 5.

Table 2. Effect of different concentrations of TDZ in nodal segment culture of *Ephedra gerardiana*.

TDZ (μM)	% of cultures showing callusing	Average no. of shoot buds	Max. no. of shoot buds	% of culture showing shoot buds
0.5	0.0	2.42 ± 1.21	2	63.63
1.0	0.0	2.85 ± 1.17	4	87.50
1.5	0.0	2.62 ± 0.99	5	88.88
2.5	8.33	2.14 ± 1.24	5	100.00
5.0	45.45	2.72 ± 1.48	5	100.00
8.0	50.00	3.00 ± 1.61	7	100.00
10.0	66.66	6.00 ± 2.12	12	100.00
15.0	73.33	1.60 ± 0.49	2	83.33
20.0	100	1.70 ± 0.45	2	41.66

At higher concentrations of TDZ (5 to 20 μM), swelling of the node was followed by compact callusing in 45 to 100% cultures. Shoot buds were also produced from compact callus at these concentrations. However, there was a decrease in percentage of cultures responding as there was increase in TDZ concentrations from 8 to 20 μM. On to 5 μM TDZ containing medium, 45.45% cultures showed callusing within 4 - 5 weeks of culture. Explant showed induction of shoot buds after 10-12 days of culture either from the callus or directly without callus formation. All cultures showed shoot buds on to this concentration. After 6 weeks of culture, maximum number of shoot buds was 5 and average number of shoot buds was 2.72. An increase in TDZ concentration to 8 μM resulted into callusing in 50% of cultures from swollen base of the node. On to this concentration, all cultures showed induction of shoot buds. Maximum number of shoot buds was 7 and average number of shoots per explants was 3.0 on to this concentration. At further increase in concentration of TDZ to 10 μM, percentage of callusing was very high (66.66%) from the swollen base of node. Within two weeks of culture, induction of shoot buds occurred in all cultures. Maximum number of shoot buds was 12 and average number was 6 on to this medium. On to 15 μM TDZ containing medium, the growth of callus was very good. Majority cultures (73.33%) showed shoot bud induction, however maximum number of shoot buds was 2 and average number of shoot buds was 1.6 on to this concentration. At further high concentration (20 μM) of TDZ, all cultures exhibited callusing however, percentage of cultures showing shoot bud

induction was decreased to 41.66%. Maximum number of shoot buds was 2 and the average number was 1.7 on to this medium.

Shoots regenerated directly or indirectly through callus were of variable length. Callus pieces containing shoots or directly regenerated shoots were transferred on to basal medium for further elongation. The shoots grew further and attained a length of 3 - 8 cm on to basal medium (Fig. 1G). Rooting of excised shoots was achieved *in vitro*. For this, two auxins *viz.,* IBA and IAA were used. None of the IAA containing medium initiated roots in present investigation. The IBA only proved to be the rooting induction hormone. MS with its major salts reduced to half and one fourth strength was tested for rooting of the *in vitro* multiplied shoots of *E. gerardiana*. Shoots rooted best on one fourth strength of MS and 20 μM IBA (Fig. 1H). The IAA in the medium could not elicit regeneration of roots in *in vitro* raised shoots. The IBA only proved to be the root induction hormone. One fourth strength of MS was tested best for rooting. *In vitro* rooted plantlets were transferred to plastic pot containing autoclaved coarse sand and garden soil in equal proportion (Fig. 1I). Plants were hardened in a small plastic chamber before acclimatization.

Thidiazuron has been reported to induce axillary shoot proliferation in several woody species, e.g. maple hybrid (Kerns and Meyer 1986), apple (Van Nieuwkerk et al. 1986), azalea (Briggs et al. 1988), silver maple (Preece et al. 1988), pear (Singh and Bhatia 1988) and peach (Zimmerman and Scorza 1992). Ellis et al. (1991) compared role of BAP, zeatin and TDZ for adventitious bud formation from *Picea glauca* embryos and studied that TDZ was sufficient to induce bud formation, although the number of shoot buds induced with TDZ was less than either BAP or zeatin. With white spruce, TDZ induced adventitious buds formation in the absence of another endogenous cytokinin and with WP medium, was able to stimulate bud formation above the level normally induced when added with sub-optimal levels of BAP. However, TDZ had little effect on bud formation when added with higher levels of either zeatin or BAP. Several cytokinins have been used for initiation of embryogenic suspensor mass in *Abies* sp. (Kim *et al.* 2009). With mature zygotic embryos of *A. nordmannana,* among the four cytokinins tested (BAP, Kn, 2iP, and TDZ), TDZ was the most effective cytokinin, but it was not significantly different from BAP (Norgaard and Krogstrup 1991). In addition, Salajova et al. (1996) reported that BAP was the most efficient ESM in hybrid firs. It is evident that addition of cytokinin to medium is critical for ESM initiation in *Abies* sp. On the other hand, in *A. fraseri*, BAP and TDZ were found to be equally efficient and better than 2iP (Guevin et al. 1994). Tang and Newton (2005) observed a high average number of adventitious shoot in *Pinus strobus* on to medium containing 6 μM TDZ.

In the present investigation, in case of nodal segment culture, lower concentration of TDZ (0.5 - 2.5 μM) yielded shoot buds directly in 63.63 - 88.88% of cultures. 2.5 - 8.0 μM TDZ supplemented medium yielded shoot buds in all cultures with or without callus formation. Onto still higher concentrations of TDZ (10 - 20 μM), all cultures produced callus with shoot bud formation. In case of Internodal segment culture, TDZ (0.5 - 5μM) resulted into callusing in all the cultures. However, callus found on to lower concentrations (0.5 and 1 μM TDZ) produced embryoid formation. Moreover, the explants onto these concentrations also produced shoot buds directly from one end. This clearly indicates that higher concentrations of TDZ are responsible for only callusing or indirect organogenesis and lower concentration for direct organogenesis in *E. gerardiana*.

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