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In Vitro Zygotic Embryo Culture and Influence of TDZ on Shoot Proliferation in *Drypetes Roxburghii* (Wall.) Hurusawa

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Abstract The present study was designed to standardize the protocol for in vitro zygotic embryo culture and micropropagation of potentially economic important tree *Drypetes roxburghii*. Among all the culture media tested for embryo culture, efficient embryo germination and seedling conversion was noticed on full strength MS medium supplemented with 3% (w/v) sucrose and 3.0 μM GA_3.Among all the concentrations of TDZ tested for in vitro shoot proliferation, MS medium supplemented with 3.0 μM TDZ induced maximum number of shoots 3.8 per explant. The shoots formed in this concentration showed quite often callus formation and failed for further elongation. However maximum shoot sprouting (99.5) percentage with mean number of 1.8 shoots/explant were noticed on MS medium supplemented with 6.6 μM TDZ. Even though the number of shoots formed in this concentration are decreased increase in shoot length (4.4 cm) was noticed. The in vitro raised shoots were best rooted on medium supplemented with 2.0 μM IBA. The primary hardened plantlets transferred to the earthen pots showed 85% survival under field conditions.

Keywords: *Drypetes roxburghii,* Zygotic embryos, TDZ, Shoot proliferation

Introduction

Drypetes roxburghii (Wall.) Hurusawa is a moderate sized deciduous tree belongs to the family Euphorbiaceae (Haldar *et al*., 2013). In recent days population of this species is becoming rare due to over exploitation of its valued wood, which is very light, soft and flexible, commonly used in toy making. The toy making industries especially artisans of Nirmal toys (Andhra Pradesh) are dependent on this wood for their livelihood (Ambasta *et al*., 1992). Seeds yield oil with mustered smell which is used in diesel engine because of its fuel properties which are comparable with diesel (Halder *et al*., 2009). This species also has great medicinal importance and widely used in Thai and Indian system of medicine. The ethnobotanical importance of this species is mainly

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due to presence of huge number of bioactive secondary metabolites like triterpeneic acids such as hydroxy ketones (putranjivanonol) and hydroxy acids (putranjic acid) (Garg and Mitra, 1968), biflavones (putraflavone) (Garg and Mitra, 1971) etc.. The whole plant of *D. roxburghii* has been used in curing various ailments like fever, rheumatism, azospermia, hemorihoidsetc (Phuphathanaphong and Chayamarit, 2006; Murthy *et al*., 2013). Stem bark of *D. roxburghii* along with leaves of *Pterospermum suberifolium* was applied to join fractured bones (Kottaimuthu, 2008).

The natural population of this species is greatly depleting at an alarming rate because of largescale destruction of trees and due to very poor seed germination. Development of standardized techniques for the improvement of native species mainly forest trees is an important issue for preserving biodiversity (Ishii and Kambou, 2007). Embryo culture and micropropagation hold great attention in overcoming the difficulties encountered in propagation of plants through conventional methods (Samuel *et al*., 2009). Zygotic embryo culture plays a significant role in understanding the barriers that affect the natural seed germination along with production of large number of seedlings in vitro. Apart from this in vitro shoot proliferation of *D. roxburghii* could be suitable for large scale multiplication and for its commercial cultivation. In view of these the present study was designed to develop an efficient protocol for multiplication and conservation of *D. roxburghii* by using zygotic embryo culture.

Materials and methods

The fresh ripened fruits of *D. roxburghii* were collected from the trees growing in dry deciduous forests of Eastern Ghats of Andhra Pradesh, India in the months of October to November 2011 and 2012 and shade dried under normal room conditions. The seeds obtained from fresh dried fruits were decoated and used for in vitro zygotic embryo culture. The decoated seeds were surface sterilized under laminar air flow conditions. Initially the seeds were surface sterilized with 0.1 % (w/v) $HgCl_2$ for 5 min followed by ethyl alcohol for 30 sec and rinsed with sterile double distilled water for five times. Latter the seeds were soaked in sterile distilled water for 48 h in laminar air flow cabinet and the soaked seeds were used as a source for zygotic embryos.

In the present study different types of media like MS, $B_{5,}$WPM was used in both full and half strengths comprising with 1.5 or 3.0% (w/v) carbon source (sucrose) and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.6 to 5.8 prior to adding agar. The prepared medium was autoclaved at 121 ^{0}C and 15 psi for 15 to 20 min. All the cultures were maintained under controlled conditions of 25 ±2 ^{0}C temperature and with 16 h light/8 h dark photoperiod by

supplying 3000 Lux light intensity with cool white florescent lights (Philips, India).

To evaluate the effect of different media on embryo germination, zygotic embryos were isolated from blot dried seeds and cultured on half and full strength MS, B_5 and WPM media supplemented with 1.5 and 3% (w/v) sucrose. Along with type and strength of the media the best hormonal concentration for efficient in vitro zygotic embryo germination was also studied by using different concentrations of plant growth regulators such as BAP, NAA and GA_3. In all the treatments percentage of embryo germination and percentage of germinated embryos converted into healthy seedlings by developing shoots from cotyledonary nodes was taken as observations for selection of optimum medium and hormone for in vitro zygotic embryo culture of *D. roxburghii*.

Seedlings with two cotyledons and with shoots were used as a source of explants for in vitro shoot multiplication under the influence of TDZ treatment. The radical and hypocotyls from the seedlings were removed by cutting with the scalpel. Then the explants (shoot tips, nodes and cotyledonary nodes) of approximately 1 cm in length obtained from seedlings were used for in vitro shoot proliferation in *D. roxburghii*. The explants were cultured in test tubes containing approximately 10 ml medium. All the explants were inoculated in a vertical position to maintain polarity. They were cultured on full strength MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of TDZ. All the chemicals used for the preparation are of analytical grade. After four weeks of inoculation the number of shoots formed and shoot length were taken as observation to understand the optimum concentration of TDZ for maximum shoot proliferation of *D. roxburghii*.

The healthy shoots with 3 to 4 nodes were selected and inoculated on MS medium supplemented with different concentrations of IBA (1.0 to 5.0 µM), 3% (w/v) sucrose and 0.8% (w/v) agar for root induction. The percentage of rooting, number of roots formed for shoot and root length were calculated after four weeks of culture. The in vitro grown seedlings and micro propagated plants were washed carefully with distilled water to remove the traces of agar sticking to the roots. Then the plantlets were placed in paper cups containing autoclaved sand, soil and vermicompost in 2:1:1 ratio. The plantlets placed in paper cups were covered with polythene bags and placed in culture room to maintain relative humidity. These plantlets were irrigated with ½ strength MS medium devoid of sucrose and hormones. After 4 weeks pores were made on polythene bags to reduce humidity. Latter gradually the pore size was increased and finally the covers were removed and plantlets were transferred to earthen pots and irrigated with tap water and maintained under shade conditions. After this

the plantlets were transferred to field conditions and percentage of survival was calculated.

In all the experiments at least twenty replicates were used per treatment and the experiments were repeated thrice. In all the experiments the cultures were observed every day and the data were collected periodically and subjected to analysis of variance (ANOVA). It was carried out by using the SPSS 20 (SPSS Inc. Chicago, IL, USA). The values with $P \leq 0.05$ were considered to be statistically significant.

Results

In the present experiments mature zygotic embryos isolated from water (2 days) soaked seeds were used as explants (Fig. 1a, b). The germination of embryos started within three days. At the end of the first week radicals from the germinated embryos elongated and grew into the medium (Fig. 1c).

Cotyledonary leaves expanded above the medium showing typical sign of germination. Among the three different media tested to evaluate the best medium for optimum growth of zygotic embryos, MS medium played a significant role. Along with the medium type, strength of the medium also played a significant role in embryo germination and it was found that full strength MS medium showed significant germination percentage. Exposure of embryos excised from fresh ripened fruits of *D. roxburghii* to different concentrations of sucrose showed varied responses. Rather than the type and strength of the medium supplemented for in vitro zygotic embryo culture, incorporation of different concentrations of sucrose influenced maximum embryo germination. MS medium supplemented with 3% (w/v) sucrose proved superior in promotion of germination (95.5) along with subsequent conversion (73.8) to healthy seedlings (Table 1, Fig. 1c).

Zygotic embryos inoculated on MS medium supplemented with 3% (w/v) sucrose and different plant growth regulators showed different responses. In the present study among all the plant growth regulators tested, efficient germination of embryos was observed on MS medium supplemented with 3% (w/v) sucrose and 3.0 μM GA_3. The germination frequency was found to be 96.0% with subsequent plantlet conversion 80.3%. (Table. 2, Fig. 1d) The influence of BAP and NAA is lower when compared with GA_3. When NAA was used it was observed that decrease in zygotic embryo germination and the germinated embryos failed to convert into seedlings.

In the present study the influence of TDZ on in vitro shoot proliferation of *D. roxburghii* was evaluated. Among all the tested concentrations of TDZ, the lower concentration i.e. 3.0 μM induced maximum shoot bud initiation with 3.8 shoots/explant (Table. 3, Fig. 1e). However the shoots formed in this

concentration were stunted with quite often callus formation at the base and failed to further elongation. Whereas at higher concentration 6.6 μM TDZ maximum shoot sprouting (99.5) percentage was observed, however decline in shoot number was noticed. The mean number of shoots formed in this concentration was found to be 1.8 shoots/explant (Table. 3, Fig. 1f). However in this concentration even though the number of shoots formed was found to be less, the increase in shoot length was noticed.

Rooting of in vitro raised shoots was carried out by transferring the healthy grown shoots containing 3 to 4 nodes with green leaves on to rooting media supplemented with various concentrations of IBA. The shoots cultured on MS medium supplemented with 2.0 μM IBA showed maximum (41.5) percentage of root induction with mean number of 2.1 roots with root length 6.2 cm (Table. 4, Fig. 1g). The induction of roots was noticed after 2 to 3 weeks of inoculation and elongated within a week. The increase or decrease in concentration beyond 2.0 μM IBA resulted in further decrease in root number and root length. The plantlets with well established roots were taken out from the test tubes and placed in a paper cups containing sand, soil and vermicompost in 2:1:1 ratio (Fig. 1h). The plantlets after initial hardening showed 85% of survival rate in earthen pots contain normal garden soil.

Table 1. Effect of different types of media, strength and sucrose concentration on *in vitro* zygotic embryo culture of *Drypetes roxburghii*

Type and strength of the media	Sucrose concentration (%)	Germination (%)	Seedling conversion (%)
MS	1.5	80.5±0.9^{b}	42.9±0.6^{d}
MS	3.0	95.5±0.3^{a}	73.8±0.6^{a}
½ MS	1.5	72.9±0.7^{c}	44.5±1.2^{d}
½ MS	3.0	93.9±0.7^{a}	69.2±0.4^{b}
WPM	1.5	63.0±0.6^{d}	23.2±0.7^{f}
WPM	3.0	75.3±0.9^{c}	43.3±0.6^{d}
½ WPM	1.5	49.5±1.1^{e}	18.1±0.5^{g}
½ WPM	3.0	70.5±0.9^{c}	34.6±0.7^{e}
B_5	1.5	74.4±1.1^{c}	38.0±0.6^{e}
B_5	3.0	89.5±1.1^{b}	62.3±0.6^{b}
½ B_5	1.5	64.9±0.7^{d}	33.1±0.5^{e}
½ B_5	3.0	80.5±0.9^{b}	57.5±0.8^{c}

Date indicate mean ± S.E followed by the same letter was not significantly different by rgw Tukey - Karmer multiple comparisons test at 0.05% probability. Twenty replicates were used per treatment experiments were repeated thrice.

Table 2. Influence of plant growth regulators on in vitro zygotic embryo germination of *Drypetes roxburghii*

BAP (µM)	NAA (µM)	GA_3 (µM)	Germination (%)	Seedling conversion (%)
1.0			65.0±1.9[d]	57.5±0.8[b]
3.0			80.5±0.9[b]	49.5±0.9[c]
5.0			74.4±1.1[c]	17.8±0.7[f]
	1.0		78.3±1.9[c]	37.8±1.5[d]
	3.0		62.6±1.6[d]	-
	5.0		53.0±1.6[e]	-
		1.0	76.5±1.2[c]	23.2±0.7[e]
		3.0	96.0±2.2[a]	80.3±2.3[a]
		5.0	78.3±1.9[c]	59.4±1.3[b]

Table 3. Influence of TDZ on in vitro shoot proliferation of *Drypetes roxburghii*

TDZ (µM)	Regenerating shoots (%)	No of shoots (explants^{-1})	Shoot length (in cm)
0.1	-	-	-
0.5	72.9±0.7[b]	1.6±0.2[c]	1.0±0.2[d]
1.0	74.4±1.1[b]	2.1±0.2[b]	1.3±0.1[d]
2.0	94.9±2.9[a]	3.2±0.2[a]	1.1±0.1[d]
3.0	95.3±1.8[a]	3.8±0.3[a]	1.5±0.1[d]
4.4	96.0±2.2[a]	2.7±0.2[b]	3.7±0.1[b]
6.6	99.5±2.8[a]	1.8±0.1[c]	4.4±0.2[a]
8.8	94.8±3.2[a]	1.3±0.1[c]	3.3±0.3[b]
11.0	73.8±0.6[b]	1.0±0.1[c]	3.9±0.2[b]
13.2	63.9±0.5[c]	1.0±0.1[c]	2.6±0.3[c]

Table 4. Effect of IBA on in vitro rooting of *Drypetes roxburghii*

IBA (µM)	Regenerating roots (%)	No of roots (explants^{-1})	Root length (in cm)
1.0	30.5±1.5 [c]	1.6±0.2[c]	6.0±0.1[a]
2.0	41.5±1.5 [a]	2.1±0.3[a]	6.2±0.2[a]
3.0	36.3±2.3 [b]	1.9±0.3[b]	5.3±0.2[b]
4.0	20.0±2.6 [d]	1.4±0.3[d]	4.9±0.1[c]
5.0	17.2±2.8 [e]	1.1±0.1[e]	3.8±0.5[d]

Date indicate mean ± S.E followed by the same letter was not significantly different by rgw Tukey - Karmer multiple comparisons test at 0.05% probability. Twenty replicates were used per treatment experiments were repeated thrice.

Fig. 1. In vitro zygotic embryo culture and micropropagation of *Drypetes roxburghii*

A. Zygotic embryos isolated from water soaked seeds of *D. roxburghii*
B. Mature zygotic embryo inoculated on MS medium supplemented with 3.0% sucrose
C. Germinated embryo of *D. roxburghii* on MS medium supplemented with 3.0% sucrose
D. Healthy seedlings obtained on MS medium supplemented with 3.0% sucrose and 3.0 µM GA_3
E. Stunted shoots with quite often callus formation on MS medium supplemented with lower concentration of (3.0 µM) of TDZ
F. Well developed shoots formed on higher concentration (6.6 µM) of TDZ
G. In vitro rooting of *D. roxburghii* on MS medium supplemented with 2.0 µM IBA.
H. Acclimatized plantlets ready to transfer into field conditions

Discussion

In the present study full strength MS medium supplemented with 3% (w/v) sucrose and 3.0 µM GA_3 induced maximum germination of zygotic embryos. Previously Kalita and Sarma (2001) also reported the maximum percentage of germination in *Acampe longifolia* on MS medium. Whereas Samuel *et al*. (2009) reported that reduction of major salts to half strength improved the germination percentage of zygotic embryos in *Givotia rottleriformis*. Sucrose was found to be the good source for both carbon and energy (Taiz and Zeiger 2006).The vital role of sucrose in embryo germination was previously reported by many biologists. Germination of all (100%) the embryos on MS medium supplemented with 30 grams sucrose was reported in *Givotia rottleriformis* (Rambabu *et al*., 2006). In vitro cultured embryos of *Prunus ameniaca* showed maximum germination and significant growth on MS medium supplemented with 3% (w/v) sucrose (Yildirim *et al*., 2007). The promotion of germination of zygotic embryos depends on concentration of sucrose since sucrose was found to be the rapidly transport carbohydrate in plants (Ghorpade *et al*., 2010).

Several reports are there on GA_3 as a potent growth regulator in breaking seed dormancy and regulating seed germination in several ways (Nikam and Barmukah 2009). The significant role of GA_3 on in vitro zygotic embryo culture was reported in *Givotia rottleriformis* (Samuel *et al*., 2009). In contrast with GA_3, BAP and NAA showed lower percentage of embryo germination and plantlet conversion. The negative role of auxins and cytokinins on embryo germination was previously reported (Ghorpade *et al*., 2010). However lower concentrations of IBA and NAA favoured early shoot and root formation in zygotic embryos of *Givotia rottleriformis* (Rambabu *et al*., 2006).

The beneficial effects of TDZ on regeneration and shoot proliferation have been demonstrated for several tree species (Murthy *et al*., 1998; Thomas, 2007). However it was found that in many trees the shoots formed with the influence of TDZ are stunted, previously to overcome this problem Samuel et al. (2009) reported a two step procedure in *Givotia* in which the shoot cultures initiated on primary medium with TDZ which were transferred to secondary medium containing Kn for further elongation. TDZ is regarded as a potent growth regulator in in vitro morphogenesis for a variety of woody plants (Khurana *et al*., 2005). Khan and Anis (2012) reported that WPM medium supplemented with TDZ was found to be the best for in vitro shoot proliferation of *Salix tetrasperma*. The significant role of IBA in root induction was previously reported in various other plant species. The positive role of IBA at lower concentrations on in vitro root induction in *Salix tetrasperma* was previously reported (Khan and Anis, 2012). Whereas Samuel *et al*. (2009)

reported that half strength MS medium supplemented with 2.4 µM IBA induced maximum percentage of root induction in *Givotia*.

From our study it is clear that high frequency zygotic embryo germination and their plantlet conversion was achieved on MS medium supplemented with 3% sucrose. It was noticed that addition of GA_3 to the medium further improved the germination and plantlet conversion. In addition efficient micropropagation system was standardized that can be explored for large scale propagation of this species which will prevent the loss of natural plant recourse of *D. roxburghii*.

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References

Ambasta, S. P., Ramchandran, K., Kashyapa, K. and Chand, R. (1992). Useful plants of India. Council of Science and Industrial Research (CSIR), New Delhi.

Garg, H. S. and Mitra, C. R. (1968). *Putranjiva roxburghii* wall.-ii: triterpenes of the trunk bark. Phytochem 7:2053-2055.

Garg, H. S. and Mitra, C. R. (1971). Putraflavone, a new biflavonoid from *putranjiva roxburghii*. Phytochem 10:2787-2791.

Ghorpade, R. P., Chopra, A. and Nikam, T. D. (2010). In vitro zygotic embryo germination and propagation of an endangered *boswellia serrata* roxb., a source of boswellic acid. Physiol. Plant Molecular Biology 16:159-165.

Haldar, S. K., Ghosh, B. B. and Nag, A. (2009). Utilization of unattended *putranjiva roxburghii* non-edible oil as fuel in diesel engine. Renewable Energy 34:343-347.

Haldar, K. M., Haldar, B. and Chandra, G. (2013). Fabrication, characterization and mosquito larvicidal bioassay of silver nanoparticles synthesized from aqueous fruit extract of putranjiva, *drypetes roxburghii* (wall.). The journal Parasitology Research 112:1451-1459.

Ishii, K. and Kambou, S. (2007). In vitro culture of an african multipurpose tree species: *adansonia digitata* L. Propagation of Ornamental plants 7:62-67.

Kalita, M. and Sarma, C. M. (2001). Selection of suitable medium for in vitro germination and protocorm formation of *acampe longifolia* lindl. Plant Science 14:243-246.

Khan, M. I. and Anis, M. (2012). Modulation of in vitro morphogenesis in nodal segments of *salix tetrasperma* roxb. through the use of TDZ, different media types and culture regimes. Agroforestry Systems 86:95-103.

Khurana, P., Bhatnagar, S. and Kumari, S. (2005). Thidiazuron and woody plant tissue culture. The Journal of Plant Biology 32:1-12.

Kottaimuthu, R. (2008). Ethno botany of the valiyins of karandamalai of dindigul district, Tamil nadu, India. Ethnobot Leaflets 12:195-203.

Murthy, B. N. S., Murch, S. J. and Saxena, P. K. (1998). Thidiazuron a potent regulator of in vitro plant morphogenesis. *In vitro* Cellular and Developmental Biology 34:267-275.

Murthy, K. S. R., Reddy, M. C., Kiran, A. R. and Emmanuel, S. (2013). Ethnobotany and chemistry of *drypetes roxburghii* - a review. Journal of Tropical Medicine 14:109-113.

Nikam, T. D. and Barmukh, R. B. (2009). GA_3 enhances in vitro seed germination in *Santalum album*. Seed Science and Technology 37:276-280.

Phuphathanaphong, L. and Chayamarit, K. (2006). Flora of Thailand euphorbiaceae. Natl Herb Nederland 336:1877-1887.

Rambabu, M., Upender, M., Ujjwala, D., Ugandhar, T., Praveen, M. and Swamy, N. R. (2006). *In vitro* zygotic embryo culture of an endangered forest tree *givotia rottleriformis* and factors affecting its germination and seedling growth. *In Vitro* Cellular and Developmental Biology - Plant 42:418-421.

Samuel, K., Debashish, D., Madhumita, B., Padmaja, G., Prasad, S. R., Murthy, V. B. R. and Rao, P. S. (2009). In vitro germination and micropropagation of *givotia rottleriformis* griff. In vitro cell dev. Biologia Plantarum 5:466-473.

Taiz, L. and Zeiger, E. (2006). Plant physiology 4th edition. Sinauer Associates Inc. Publishers, Sunderland, Massachusetts pp. 222-250.

Thomas, D. T. (2007). Pretreatment in thidiazuron improves the invitro shoot induction from leaves in *curculigo orchioides* gaertn., an endangered medicinal plant. Acta Physiologiae Plantarum 29:455-461.

Yildirim, H., Tilkat, E., Onay, A. and Ozen, H. C. (2007). In vitro embryo culture of apricot, *prunus armeniaca* l. Cv. International Journal of Science and Technology 2:99-104.

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