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Micropropagation of *Thalictrum dalzellii* Hook. Through Rhizome Buds

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Summary

An efficient micropropagation protocol was developed for *Thalictrum dalzellii* an endemic, endangered plant by using rhizome buds as explants. In vitro germinated rhizome buds were used for the induction of multiple shoots on MS medium supplemented with thidiazuron (TDZ), benzylaminopurine (BAP) and Kinetin (KN). The presence of TDZ promoted a higher rate of shoot multiplication than BAP and KN. The maximum number of shoot multiplication was observed in TDZ at a concentration 0.5 mg l^{-1}. Shoot elongation was obtained when the culture transferred to MS basal medium. The maximum percentage of rooting was obtained in half strength MS medium with 0.5 mg l^{-1} IBA. Plantlets were successfully acclimatized under green house condition. The acclimatized plantlets were morphologically similar to wild plants.

Key Words: Endangered plant, Micropropagation, Rhizome buds, *Thalictrum dalzellii*

Introduction

In vitro technique have found increasing use in the conservation of threatened plants in recent years and this trend is likely to continue as more species face risk of extinction [1]. In vitro techniques have been found to be useful in the propagation of large number of endangered plants [2,3]. There is still alarming and immediate conservation measures are required to safeguard many endangered species.

The *Thalictrum* genus belongs to Ranunculaceae family and considered as extremely abundant medicinal plant source and more than 200 species are distributed worldwide [4]. *Thalictrum* plants are rich in benzylisoquinoline derived alkaloids, at least 250 have been isolated from 60 species and most of them with strong biological activities, extracts and alkaloid isomers from *Thalictrum* are known to exhibit various pharmacological activities, including antitumor, antimicrobial, antiamebic and HIV antiviral activities [4]. *Thalictrum dalzellii* Hook is a small erect herb grown in hill forests. According to Red Data Book of Indian Plants, this species is categorized under 'Indeterminate' category (endangered or extinct) [5]. Because of raising plantation crops, and destruction of natural habitat at in hill forest, *T. dalzellii* plants are being depleted rapidly, as a result, it has raised concerns about possible extinction. It is therefore imperative to develop appropriate tissue culture technique for this species. *T. dalzellii* is also endemic to Southern Peninsular India region [6].

Micropropagation technique has been used successfully for conservation of many endangered plants [2,7]. Effective in vitro propagation for *Thalictrum dalzellii* needs to be developed for conservation of this important endemic endangered plant. In vitro regeneration in *Thalictrum* genus has been scarcely reported. Samanani et al. [8] have reported the in vitro regeneration of *T. flavum* var. *glaucum* through the in vitro germinated seedlings. Though tissue culture has been reported in many Ranunculaceae members like *Aconitum heterophyllum* [9], *Cimcifuga racemosa* [10] *Coptis teeta* [11], *Delphinium malabaricum* [12], *Hydrastis canadensis* [13,14] but rhizome buds have not been used as an explants material for in vitro regeneration and there are no published reports on micropropagation of *T. dalzellii* through rhizome buds. This study was made to understand in vitro regeneration of T. dazellii through rhizome buds.

Materials and Methods

Plant material and in vitro germination of rhizome buds

The plants were collected from Bababudan Hills, Karnataka, India along with their root systems. Rhizomatous roots were separated from plants and washed in running tap water to remove the soil. The roots were again washed under running tap water with few drops of Tween-20. Rhizomatous roots were immersed in 0.3% Bavistin (Carbendizim) solution for fungicidal treatment for one hour by shaking and then rinsed with sterile water. After fungicidal treatment, the rhizomatous roots were surface sterilized with 0.1% mercuric chloride solution for 5 minutes. The roots were then rinsed three times with sterile water. By using forceps, the rhizome buds (Fig. 2A) were picked from the surface of rhizomatous roots, blot dried on sterilized blotter discs and inoculated on to the basal MS medium. To study the seasonal response of explants, rhizomatous root containing buds were collected at different seasons. The collection season was based on the life cycle of *Thalictrum dalzellii*, early stage of plant development (June), flowering (August) and fruit development stage (September).

Shoot induction, subculture and shoot elongation

An *in vitro* germinated rhizome buds were used for multiple shoot proliferation. Various plant growth regulators like, BAP, Kinetin and TDZ in different concentrations were tested individually to obtain the most suitable concentration for the proliferation of multiple shoots. The basal end of the germinated rhizome bud was trimmed and inoculated on to medium with different concentration of growth regulators. The appropriate growth regulator, which produced maximum shoots, was used for subculture. Subculture of multiple shoots was carried out at regular intervals of 4 weeks. The multiple shoot clusters were transferred to shoot elongation medium. The elongation medium was basal MS medium.

In vitro rooting

For *in vitro* root induction, individual shoots of 4-5 cm length harvested at the end of the elongation stage were used. The individual shoots were aseptically inoculated to medium containing various concentrations of auxins like IAA and IBA. During this phase, MS half- strength and MS full-strength medium with different growth regulators were evaluated to test their efficacy of *in vitro* rooting.

Hardening and acclimatization

In vitro rooted plantlets, after removing from medium washed gently under running tap water to remove the excess agar medium and were individually transferred to pots containing sterilized soil-rite. The pots were covered with transparent polythene bags and punched with hole and maintained in growth chamber at 22^{o} C. After 4 weeks the plantlets were then transferred to pots containing mixture of garden soil and farmyard. After hardening off, the plantlets were placed under greenhouse condition and watered twice a week. The plantlets were gradually acclimatized through periodic exposure to the low relative humidity of *ex vitro* condition.

Culture condition

In all cases MS medium [15] with 30 gl^{-1} sucrose was added as a source of carbon and media were gelled with 0.2% phytagel after adjusting the pH to 5.8 was used. In each experiment, the desired concentration and types of plant growth regulators were added before autoclaving and 40 ml of the respective medium dispensed to 200 ml screw capped glass culture bottles or 20 ml to culture tubes. The media were autoclaved for 20 minutes at 121^{o}C (1.06 kg/cm^{2}). Cultures were incubated in the culture room at a constant temperature of 25±2^{o}C, under white cool fluorescent light of 50 µ mol/m^{-2} photosynthetic photon flux density with 16 hour photoperiod.

Statistical analysis

Each experiment was repeated 3 times, with 20 cultures per treatment. The percentages of germination buds were recorded after 3 weeks. The mean percentage of cultures producing multiple shoots and mean number of shoots per culture were recorded after 6 weeks. The percentage of rooting and average number of roots per shoot was recorded after 4 weeks. The data were analyzed statistically by the Duncun's Multiple Range Test.

Results

Plant material and in vitro germination of rhizome buds

The indication of germination of rhizome buds was swelling up of buds and sprouting was observed after 2 weeks (Fig. 2B). It was observed that there has been correlation between the germination of rhizome buds with seasonal collection of explants. The explants collected during June showed germination percentage of >70%. (Fig.1).

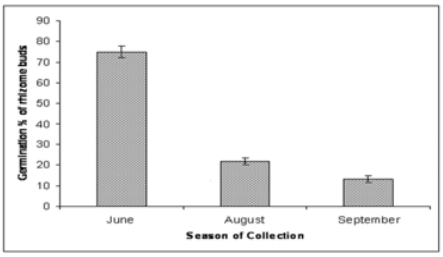

Fig.1: Seasonal response of rhizome bud germination on MS basal medium (bars indicate ± SE)

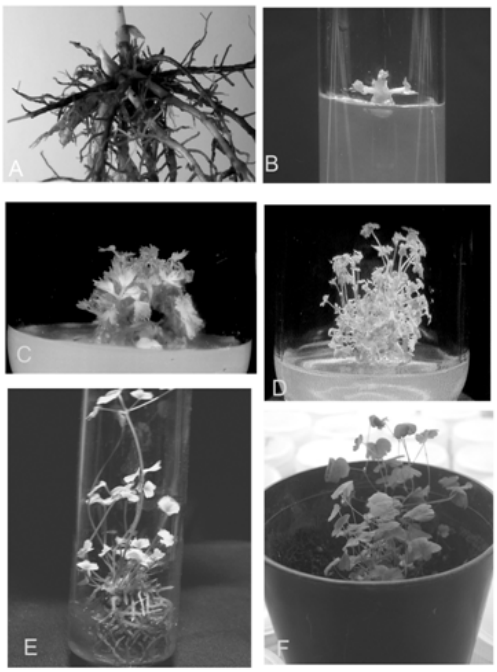

Fig. 2: Micropropagation of *T. dalzellii*
A). Rhizome buds on rhizomatous root
B). Germination of rhizome bud in MS basal medium
C). Multiple shoot induction on MS medium with 0.5 mg l^{-1} TDZ
D). Shoots elongation on MS basal medium
E). In vitro rooting in Medium containing 0.5 mg l^{-1} IBA
F). Hardened plant after 3 months

Shoot induction, subculture and shoot elongation

The effect of different growth regulators on multiple shoot induction including BAP, Kinetin and TDZ were recorded (Table 1). The highest shoot proliferation was induced with 0.5 mg l^{-1} of TDZ (Fig. 2C). BAP and kinetin also capable of inducing multiple shoots. Multiple shoots showed good elongation in MS media without growth regulators (Fig. 2D). Shoots showed elongation after 2 weeks of their transfer in to MS basal medium.

Table-1 Effect of different concentrations of cytokinins (TDZ, BAP and KN) in MS medium on the shoot multiplication of *Thalictrum dalzellii* from rhizome buds

Cytokinins	Concentration (mg l^{-1})	Percent of response (%)	Shoots/explant (mean ± SE)	Shoot length in mm (mean±SE)
control	0	43.3^{b}	1.3±0.1^{e}	19.1±0.5^{g}
TDZ	0.12	68.3^{a}	1.8±0.3^{cde}	15.8±0.2^{h}
	0.25	71.6^{a}	4.6±0.3^{b}	20.6±0.9^{f}
	0.5	75.0^{a}	7.6±0.6^{a}	20.7±0.9^{f}
	1.0	68.3^{a}	3.8±0.7^{b}	18.3±0.4^{g}
	2.0	70.0^{a}	3.3±0.3^{bcde}	15.4±0.6^{h}
BAP	0.12	70.0^{a}	1.6±0.3^{de}	25.8±0.1^{d}
	0.25	68.3^{a}	2.3±0.6^{cde}	26.6±0.3^{d}
	0.5	65.0^{a}	3.6±0.8^{bcd}	29.8±0.2^{c}
	1.0	65.0^{a}	3.0±0.5^{bcde}	23.5±0.9^{e}
	2.0	66.6^{a}	2.6±0.3^{bcde}	22.4±0.3^{ef}
KN	0.12	73.3^{a}	2.5±1.0^{cde}	33.1±0.6^{b}
	0.25	68.3^{a}	3.0±0.5^{bcde}	35.4±0.5^{a}
	0.5	66.6^{a}	3.3±0.3^{bcde}	37.3±0.5^{a}
	1.0	66.6^{a}	2.6±0.8^{bcde}	26.2±1.4^{d}
	2.0	63.3^{a}	3.6±0.6^{bcd}	26.8±0.3^{d}

Data recorded after 6 weeks
Mean of 3 replications of 20 cultures each
Means followed by the same letter were not significantly different at $P \leq 0.05$. (Duncan's Multiple Range Test).

Table-2 Effect of different concentration of auxins in different strength of MS medium for *in vitro* rooting of *Thalictrum dalzellii*

Media	Auxins	Concentration (mg l^{-1})	Percent of rooting	No. of roots/shoots (mean±SE)	Root length in cm (mean±SE)
control	0		0	0	0
Half- strength	IBA	0.25	80.0^{a}	8.3±0.1^{bc}	1.1±0.6^{c}
		0.5	75.0^{ab}	12.3±0.4^{a}	0.7±0.4^{d}
		1.0	75.0^{ab}	9.3±0.4^{b}	2.3±1.3^{b}
		2.0	70.0^{abc}	6.2±0.1^{de}	2.0±1.1^{b}
	IAA	0.25	71.6^{ab}	2.9±0.2^{gh}	1.4±0.5^{c}
		0.5	75.0^{ab}	3.1±0.3^{gh}	1.0±0.5^{c}
		1.0	68.3^{abc}	3.5±0.5^{gh}	1.7±1.0^{c}
		2.0	66.6^{abc}	3.1±0.1^{gh}	2.7±1.5^{b}
Full -strength	IBA	0.25	65.0^{bc}	7.2±0.2^{cd}	0.5±0.3^{d}
		0.5	60.0^{c}	9.5±0.5^{b}	0.9±0.5^{cd}
		1.0	60.0^{c}	7.6±0.4^{cd}	1.9±1.1^{c}
		2.0	65.0^{bc}	5.2±0.3^{ef}	2.0±1.1^{b}
	IAA	0.25	65.0^{bc}	4.4±1.4^{fg}	0.3±0.2^{d}
		0.5	65.0^{bc}	2.7±0.1^{gh}	0.4±0.2^{d}
		1.0	61.6^{bc}	2.8±0.7^{gh}	3.6±2.0^{a}
		2.0	56.6^{c}	2.2±0.3^{h}	3.6±2.0^{a}

Data recorded after 4 weeks
Mean of 3 replications of 20 cultures each
Means followed by the same letter were not significantly different at $P \leq 0.05$ (Duncan's Multiple Range Test)

In vitro rooting

The effect of different auxins on *in vitro* rooting was recorded (Table 2). The frequency of root induction in medium containing IBA was more compare to IAA, which produced highest 12 roots/shoots with 0.5 mg l^{-1} of IBA. The roots produced in IBA were thickened (Fig. 2E).

Hardening and acclimatization

Using the acclimatization protocol described, most rooted plants that were transplanted to plastic pots containing soil-rite showed normal growth. After one month, the plantlets transferred to soil continued to grow well and no apparent morphological variation was observed (Fig. 1F).

Discussion

In the present study rhizome buds were used as an explants material for *in vitro* regeneration of *T. dalzellii*.. The life cycle of *T. dalzellii* expected to have major influence in the germination of rhizome buds. Our personnel observation revealed that the life cycle of *T. dalzellii* found to be similar to another Ranunculaceae member *Hydrastis canadensis*. In species biology, the *Hydrastis canadensis* is a clonal species and aerial stem develops from the knotty underground rhizome, towards the end of the growing season a bud will be

produced on the rhizome and this bud grows in to a stem in the following year [16]. However in *T. dalzellii* many rhizome buds are formed around the rhizomatous roots at the end of growing season and one rhizome bud germinate and develops in to the plant in the next coming rainy season. The rainy season coincides with the end of June. Hence, the rhizome buds collected in the month of June are more responsive than the buds collected in month of August or September and also buds expect to have dormany. Similar result was reported with seasonal response of explants in a rare plant, *Ochreinauclea missionis*, where the plant material collected during the winter and rainy seasons gave the maximum response because the winter and rainy seasons correspond with the timing of the most active growth phase [17] and in *Hollarrhena antidysentrica*, maximum response was obtained from the beginning of May to the end of July [18]. Sharma et al. [19] reported that, the collection of explants in different season, their response and effect of different growth regulators in *Crataeva adansonii* and in *Euphorbia nivula* by Martin et al. [20].

In the shoot multiplication, TDZ has been shown to be providing an efficient stimulus for the induction of shoot regeneration. Highest rate of shoot multiplication was obtained in 0.5 mg l^{-1} of TDZ in MS medium. The role of TDZ in shoot multiplication and induction of roots reported in several plants [21] and to be effective in herbaceous plants [22]. Shoot proliferation was high in medium containing TDZ, this was in agreement with reports of other plants like *Scutullaria baicalensis* [22] and *Artemisia judaica* [23]. In the *in vitro* regeneration of *Thalictrum flavum* ssp. *glaucum*, shoot multiplication rate was high in Kinetin at 4 mg/l^{-1} compare to BAP and zeatin in B5 medium [8]. Our results are consistent with the results of Rai [24], who gained same results in *Nothapodytes foetida,* the highest rate of shoot multiplication in MS medium containing 2.2 µM (0.5 mg l^{-1}) of TDZ compare to that of BAP or KN at similar concentration or more. In present study, the shoots developed on TDZ containing medium failed to elongate. This inhibition of shoot elongation is a common problem with TDZ and may be consistent with its super optimal cytokinin activity, where as the presence of phenyl group in TDZ may be the possible cause of shoot fasciation [25].

Rooting of *in vitro* regenerated shoots was obtained in both half-strength and full strength MS supplemented with IBA and IAA. IBA was found to be effective for root induction compare to IAA. When cultured on half strength MS medium fortified with 0.5 mg l^{-1} of IBA, each shoot produced average of 10-12 roots. The effectiveness of IBA in rooting has been reported in *Thapsia garganica* [26], *Calophyllum apetalum* [27] and *Orthosiphon spiralis* [28]. Half-strength MS with IBA showed good number of roots compare to full-strength MS medium. Reducing MS salt concentration to half-strength was found to be more effective than full-strength in terms of frequency of root induction. The promontory effect of reducing MS salt concentration has been carried out for in vitro rooting of *Hemidesmus indicus* [29].

Regenerated plantlets were transferred to plastic containers filled with soil-rite. During first week the potted plantlets were covered with polythene bags to provide high humidity. Plantlets were subsequently transferred to larger pots and gradually acclimated to outdoor conditions. This micropropagation protocol provides a successful and rapid propagation technique for conservation of this endemic endangered plant. The availability of *Thalictrum dalzellii* restricted to particular season from their natural habitat. By using this technique plants can be produced throughout the year without seasonal constraints and can be used for future conservation needs.

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