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MICROPROPAGATION OF ADULT TREE OF *PTEROCARPUS MARSUPIUM* ROXB. USING NODAL EXPLANTS

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Abstract: Attempts were made for *in vitro* propagation of *Pterocarpus marsupium* Roxb., belonging to family Fabaceae, an economically important multipurpose tree. The tree is scared with noval antidiabetic properties. The tree shows poor seed germination capacity (30%) due to hard seed coat and conventional vegetative regeneration methods are a complete failure. Therefore, the propagation of this tree by tissue culture techniques is an urgent need and well justified. Nodal segments containing axillary bud from 10 years old tree of *P. marsupium* were evaluated for axillary shoot proliferation on Murashige and Skoog's (MS) basal medium fortified with BAP (6–benzylaminopurine) and kinetin (Kn) singly or in combinations with auxins at different concentrations. The best shoot proliferation was obtained with 13.95 µM Kn + additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM Ammonium sulphate and 217 µM Adenine sulphate) in MS medium where 64.44% of the axillary buds responded with development of (2.51±0.10) shoots. Multiplication of *in vitro* shoots were achieved on MS Medium supplemented with Kn (9.30 µM) + NAA (0.54 µM) and additives. Half strength MS medium supplemented with 4.92 µM IBA induced *in vitro* rooting of *in vitro* shoots. *In vitro* regenerated plantlets with well developed roots were successfully hardened in a greenhouse.

Keywords: acclimatization, Fabaceae, *in vitro*, recalcitrant, tissue culture

Introduction

Pterocarpus marsupium Roxb. is a deciduous tree, commonly called as Indian Kino tree or malabar kino, belonging to the family Fabaceae. It is a medium to large sized tree reaching the height upto 15-20 meter with dark brown to grey bark having swallow cracks. The bark excudes a red gummy substance called 'Gum Kino' when injured. Leaves are compound and imparipinate. Flowers are yellow in terminal panicles. Fruit is circular winged pod. Seed is convex and bony. Tree flowers and fruits in the month of March to June. *P. marsupium* is distributed in deciduous forest throughout the India [VARGHESE, 1996]. It is a multipurpose leguminous tree. Heart wood is astringent, bitter, acrid, cooling, anti-inflammatory, depurative, haemostatic, revulsive and anthelmintic. The paste of seed and wood is useful in diabetic anaemia [TRIVEDI, 2006].The paste of heartwood is useful in body pain and diabeties. Wood of this tree is useful in making the waterglasses of diabetic [REDDY & al. 2008]. Due to overexploitation of the tree for its various useful applications coupled with low germinability, *Pterocarpus marsupium* has been included in the list of depleted plant species [CHOUDHARI & SARKAR, 2002].

Tissue culture method has been proved to be a promising technique for conservation and rapid multiplication of several forest rare woody species. However,

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member of Fabaceae have been found difficult to culture *in vitro* due to their recalcitrant nature [JHA & al. 2004]. *In vitro* regeneration protocols have been reported in *P. marsupium* using different explant sources including cotyledonary nodes, nodes and hypocotyl [CHAND & SINGH, 2004; TIWARI & al. 2004; ANIS & al. 2005; HUSAIN & al. 2007, 2008, 2010]. But till date, no report is available for *in vitro* regeneration of *Pterocarpus marsupium* from nodal segments. The present work is taken with aim to establish aseptic cultures of mature tree of *Pterocarpus marsupium* from nodal segments and to develop micropropagation protocol.

Materials and methods

Source of plant material and explant preparation

The plant material was collected from AFRI nursery, Jodhpur. Nodal segments containing axillary bud were collected and pretreated with Tween-80 for 5 min. and rinsed with distilled water. After that they were treated with 0.1% bavistin (w/v) solution and 0.05% streptomycin solution for 7 min. and thoroughly rinsed with distilled water. Later the explants were surface sterilized with 0.1% $HgCl_2$ for 7 min. and rinsed with autoclaved distilled water for three–four times.

Basal nutrient medium for shoot proliferation and multiplication

The surface – sterilized explants were inoculated on MS medium with various concentrations of BAP (2.22, 4.44, 8.86, 13.32 and 17.76 µM) or Kn (2.32, 4.65, 9.30, 13.95 and 18.50 µM) for shoot proliferation. MS medium with sucrose (3%) were used throughout the experiment. Additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM ammonium sulphate and 217 µM Adenine sulphate) were also used in the MS medium. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCL and the medium was solidified with 0.8% agar. The medium was then sterilized by autoclaving for 20 min. at 121 ºC. The percentage of explants responding to shoot proliferation, the number of shoots per explants and length of the shoots was recorded and evaluated after 4 weeks. One shoot or 2 shoots together were subcultured on MS medium supplemented with different concentrations of cytokinins (BAP and Kn) alone or in combinations with auxins for *in vitro* shoot multiplication.

Culture conditions

The cultures were maintained at 26 ± 2 °C under 16 h light photoperiod with light intensity of 1600 lux, obtained by cool white fluorescent tubes of 40 watts [Philips, India].

In vitro root induction of micropropagated shoots

Healthy shoots with 2-3cm length were used for *in vitro* root induction. Various concentrations of auxins IBA (0.49, 1.23, 2.46, 4.92, 7.32 µM) and NAA (0.27, 0.54, 1.34, 2.69, 5.37 µM) were studied for *in vitro* rooting.

Hardening and acclimatization

In vitro rooted plantlets were removed from culture vessels and washed with distilled water to remove adhered traces of nutrient agar. Plants were carefully transferred to bottles containing autoclaved soilrite moistened with ½ MS medium without organics. After rooted plantlets established in soilrite containing capped bottles, caps were gradually loosened and finally removed within 2 weeks. After 3-4 weeks, the *in vitro* rooted plantlets

were transferred into polybags containing mixture of farmyard manure, soil and sand (1:1:1).

Experimental design, data collection and statistical analysis

MS medium without hormone was treated as control in all experiments. All experiments were repeated three times. Observations were recorded after 4 weeks of interval. The results are expressed as mean ± SE of three experiments. The data was analyzed statistically using SPSS version 17 and significant difference between means were assessed by Duncan's multiple range test (DMRT) at $P = 0.05$.

Results

Axillary shoot proliferation

Mature nodal explants of *P. marsupium* (Fig. 2A) were inoculated on MS medium supplemented with different concentrations of cytokinins, BAP and Kn for axillary shoot proliferation. In 4 weeks axillary bud break was obtained (Fig. 2B). The percentage bud break response, average number of axillary proliferated formed per explants and average shoot length all varied considerably with the type and concentrations of growth regulator used in MS medium (Tab. 1).

Tab. 1. Effect of various cytokinins (BAP or Kn) in MS medium on *in vitro* axillary shoot proliferation of *Pterocarpus marsupium*

BAP(µM)	Kn (µM)	Percentage shoot proliferation	Mean shoot number	Mean shoot length (cm)
0.0	-	0.00	0.00	0.00
2.22	-	26.66	1.16 ± 0.11^{a}	0.71 ± 0.02^{a}
4.44	-	31.10	1.57 ± 0.20^{b}	0.79 ± 0.06^{a}
8.86	-	46.66	1.90 ± 0.19^{b}	1.10 ± 0.03^{b}
13.32	-	37.77	1.29 ± 0.14^{a}	1.08 ± 0.01^{b}
17.76	-	31.10	1.07 ± 0.71^{a}	0.77 ± 0.03^{a}
-	2.32	35.55	1.12 ± 0.08^{a}	0.90 ± 0.03^{a}
-	4.65	44.44	1.52 ± 0.11^{b}	1.03 ± 0.03^{b}
-	9.30	51.10	2.13 ± 0.14^{c}	1.19 ± 0.02^{c}
-	13.95	64.44	2.51 ± 0.10^{d}	1.47 ± 0.02^{d}
-	18.59	51.10	1.91 ± 0.13^{c}	1.26 ± 0.016^{c}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Cytokinins played a significant role in inducing shoot proliferation from axillary bud. Of the two cytokinins used, Kn was found to be more effective than BAP for shoot proliferation from axillary bud. At lower concentration of Kn (2.32-9.30 µM), the bud break response was less. Increased concentration of Kn (13.95 µM), increased the response percentage and number of proliferated shoots. Whereas higher concentration of Kn beyond 13.95 µM resulted in decreased percentage bud break response. MS medium supplemented with 13.95 µM Kn, with additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM Ammonium sulphate and 217 µM Adenine sulphate) was found to be optimal for maximum bud break response of 64.44% with 2.51 ± 0.10 numbers of axillary shoots.

Nodal segments containing axillary buds were cultured on MS medium supplemented with different concentration of BAP. Bud break response of 46.66% was obtained at 8.86 µM concentration of BAP supplemented MS medium. Response percentage as well as number of axillary shoot proliferated per explant was 1.16 ± 0.11 at 2.22 µM, which increased to 1.90 ± 0.19 shoots per explants at 13.32 µM. Further increase in BAP concentration beyond 13.32 µM resulted in decreased bud break response.

Nutrient medium plays a vital role in propagation through tissue culture. Three basal media (MS, WPM, and B_5) were tested to assess the effect on axillary bud proliferation. Results exhibited that MS medium was best for bud break response (64.44%) as compared to WPM (48.88%) and B_5 (39.99%). The number of shoots proliferated (2.51) and their length (1.47) found best on MS medium supplemented with 13.95 µM Kn (Fig. 1).

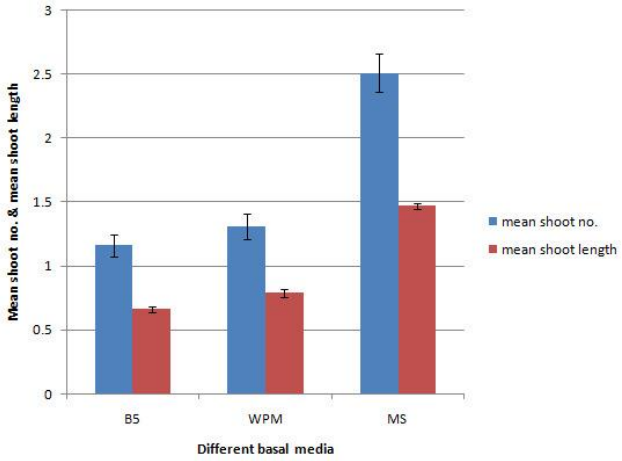

Fig 1. Effect of different basal media on shoot proliferation from nodal explants of *Pterocarpus marsupium*

In vitro shoot multiplication

The proliferated *in vitro* axillary shoots were excised from mother explants and subcultured on MS medium supplemented with cytokinins for establishment of cultures and multiplication of *in vitro* shoots. Response of *in vitro* shoot multiplication varied with cytokinin type and its concentration used in the medium (Tab. 2) BAP at 4.44 µM concentration found to be optimal, which gave an average of 4.82 *in vitro* shoots. Increased BAP concentration (13.32 µM) resulted decreased multiplication potential and only 4.17 *in vitro* shoots were obtained. Length of regenerated shoots also decreased with increase of BAP concentration. Regenerated shoots had an average length of 1.58 cm on MS medium supplemented with 4.44 µM BAP, which is decline to 1.31 cm at 13.32 µM BAP level.

On kinetin supplemented MS medium, 9.30 µM Kn gave optimal response for *in vitro* shoot multiplication 4.92 shoots per explants were developed (Fig. 2C). There was a decrease in number of shoots decreased at concentrations lower and higher than 9.30 µM Kn. At 2.32 µM Kn, average 4.62 shoots per explants were developed whereas 4.16 shoots per explants developed at 13.95 µM Kn. Average length of regenerated shoots on 2.32 µM

Kn supplemented medium was 1.32 cm which increased to 1.70 cm at 9.30 µM Kn and then declined to 1.31 cm at 13.95 µM Kn.

Tab. 2. Effect of various types of cytokinins (BAP, Kn) in MS medium on *in vitro* shoot multiplication of *Pterocarpus marsupium*

BAP(µM)	Kn (µM)	Mean shoot number	Mean shoot length (cm)
2.22	-	4.27 ± 0.09^{a}	1.32 ± 0.02^{a}
4.44	-	4.82 ± 0.11^{b}	1.58 ± 0.02^{c}
8.86	-	4.62 ± 0.12^{ab}	1.50 ± 0.02^{b}
13.32	-	4.17 ± 0.07^{a}	1.31 ± 0.01^{a}
-	2.32	4.62 ± 0.12^{ab}	1.32 ± 0.02^{a}
-	4.65	4.34 ± 0.11^{a}	1.54 ± 0.02^{c}
-	9.30	4.92 ± 0.12^{b}	1.70 ± 0.03^{b}
-	13.95	4.16 ± 0.06^{a}	1.31 ± 0.01^{a}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Cytokinin alone did not influence *in vitro* shoot multiplication in *P. marsupium*. Auxins with cytokinin enhanced *in vitro* shoot multiplication. Therefore, cytokinin-auxin interaction was also studied for *in vitro* shoot multiplication (Tab. 3). Kn (4.65, 9.30, 13.95 µM) was used in combination with NAA (0.27, 0.54, 1.34 µM). It was observed that Kn in combination with NAA increased *in vitro* shoot multiplication response. An average number of 4.16 shoots per explant was obtained at 13.95 µM Kn alone, while addition of NAA with Kn in medium increased *in vitro* shoot multiplication. The maximum 6.21 shoots was observed on 9.30 µM Kn + 0.54 µM NAA supplemented with MS medium.

Tab. 3. Effect of cytokinin-auxin interaction (Kn + NAA) in MS medium on *in vitro* shoot multiplication of *Pterocarpus marsupium*

Kn(µM)	NAA(µM)	Mean shoot number	Mean shoot length (cm)
4.65	0.27	4.27 ± 0.08^{ab}	1.41 ± 0.02^{abc}
	0.54	5.00 ± 0.10^{e}	1.59 ± 0.02^{d}
	1.34	4.66 ± 0.09^{cd}	1.47 ± 0.02^{c}
9.30	0.27	4.86 ± 0.11^{de}	1.59 ± 0.03^{d}
	0.54	6.21 ± 0.11^{f}	1.67 ± 0.01^{e}
	1.34	4.55 ± 0.12^{bc}	1.38 ± 0.02^{ab}
13.95	0.27	3.44 ± 0.09^{bc}	1.44 ± 0.02^{bc}
	0.54	4.27 ± 0.07^{ab}	1.39 ± 0.01^{ab}
	1.34	4.11 ± 0.05^{a}	1.35 ± 0.01^{a}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

***In vitro* rooting**

The *in vitro* produced shoots were capable of inducing roots when cultured on half strength MS medium containing auxins (Tab. 4). Two auxins were tried for *in vitro* rooting. IBA had pronounced effect on *in vitro* rooting than NAA. On medium supplemented with 4.92 µM IBA, 42% rooting was observed. Any increase and decrease of 4.92 µM IBA

levels reduced the rooting percentage. *In vitro* roots were also induced when the *in vitro* shoots were cultured in NAA supplemented half strength MS medium, where rooting response of 28.88% was obtained on 2.69 µM NAA (Fig. 2D).

Tab. 4. Effect of different concentration of IBA and NAA on *in vitro* rooting of *Pterocarpus marsupium*

IBA (µM)	NAA (µM)	Rooting %	Mean root number	Mean root length
0.00	-	0.00	0.00	0.00
0.49	-	0.00	0.00	0.00
1.23	-	28.88	1.38±0.01[a]	0.58±0.05[a]
2.46	-	35.00	1.57±0.02[a]	0.82±0.06[a]
4.92	-	42.22	2.14±0.17[b]	1.24±0.06[b]
7.32	-	31.10	1.82±0.12[a]	0.76±0.05[a]
	0.27	0.00	0.00	0.00
	0.54	19.99	1.00±0.00[a]	0.50±0.04[a]
	1.34	26.66	1.19±0.08[a]	0.62±0.03[a]
	2.69	28.88	1.29±0.14[a]	0.94±0.05[a]
	5.37	26.66	1.25±0.13[a]	0.71±0.02[a]

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Hardening and acclimatization

The *in vitro* raised plantlets were successfully acclimatized first under culture room conditions and then in the green house. The four weeks old plantlets were transferred in screw cap glass bottles containing 1/3 volume of autoclaved soilrite. These plantlets were nurtured with half strength MS medium (without organics) twice a week and were kept for four weeks in culture room. Then these bottles containing plantlets were transferred to mist chamber. Plants were then transferred to polybags containing sand: soil: FYM in 1:1:1 proportion and were kept in the mist chamber (Fig. 2 E&F). Under the mist chamber the plants started to harden and were shifted to shade house conditions for further acclimatization.

Fig 2. Micropropagation of *Pterocarpus marsupium* from nodal explants (A-F) **A**. Nodal segments of *P. marsupium.* **B**. Axillary shoot proliferation from nodal segment containing axillary bud of *P. marsupium* on MS medium supplemented with Kn (13.95 µM) + additives. **C**. *In vitro* shoot multiplication on MS medium supplemented with Kn (9.30 µM), NAA (0.54 µM) and additives. **D**. Induction of rooting from microshoots of *P. marsupium* on ½ MS medium supplemented with IBA (4.92 µM). **E & F**. Hardening of *in vitro* raised plantlets.

Disscusion

In the present investigation, micropropagation protocol of *Pterocarpus marsupium* from nodal explants derived from about 10 years old tree was established. During the present investigation, effect of two cytokinins, BAP and Kn were studied for axillary bud proliferation of *P. marsupium*. Axillary bud proliferation was more on Kn as compared to medium supplemented with BAP. This result is contrasting with reports on leguminous species *Dalbergia sissoo* Roxb. and *Macrotyloma uniflorum* [ARYA & al. 2013; BISHT & al. 2013] where BAP gave maximum shoot proliferation.

The effect of different basal media (MS, WPM, B_5) on axillary shoot proliferation was also tested. During present investigation it was observed that MS medium was better for axillary bud proliferation compared to other media (WPM and B_5).The findings are in agreement with earlier reports on many woody tree species including *Swartzia madegascariensis* and *Lagerstromia parviflora* [BERGER & SCHAFFER, 1995; TIWARI & al. 2002]. Whereas, WARAKAGODA & SUBSINGHE (2013) suggested that B5 medium was superior to MS for plant regeneration of *Pterocarpus santalinus*.

Shoot multiplication is the major criterion for successful micropropagation. *In vitro* shoot multiplication is affected by numerous factors, such as physiological status of plant material, culture medium and culture environment. Cytokinins are essential for *in vitro* shoot multiplication. In the present investigation two cytokinins, BAP and Kn were studied for *in vitro* shoot multiplication. The inclusion of cytokinin and auxin in the culture media stimulated the *in vitro* multiplication and growth of shoots in several plant species [GEORGE, 1993]. Interaction of cytokinin-auxin was investigated for *in vitro* shoot multiplication and different combinations of Kn and NAA were tried. Results illustrated maximum shoot numbers (6.22) on MS medium supplemented with 9.30 µM Kn + 0.54 µM NAA + additives. The results substantiate with earlier findings of several workers, where the addition of low level of auxin with cytokinin promoted shoots in *Acacia catechu*, *Eucalyptus grandis* and *Lagerstromia parviflora* [KAUR & al. 1998; CID & al. 1999; TIWARI & al. 2002]. Higher concentrations of auxins resulted in callus formation at the base of shoots, which is undesirable feature for *in vitro* shoot multiplication. Additives have also been reported to improve the multiplication rate and length of the shoots when added in combination with kinetin [KHAN & al. 2014]. The promotive role of additives in shoot multiplication has been reported in different woody species namely, *Tectona grandis*, *Bauhinia vahlli* and *Melia azedarach* [DEVI & al. 1994; DHAR & UPRETI, 1999; HUSAIN & ANIS, 2009].

As a woody perennial, *P. marsupium* is difficult to root. The ability of plant tissues to form roots depends on interaction of many endogenous and exogenous factors. A varied effect of auxins IBA, NAA was observed by incorporating them in MS medium at different concentrations. Our observation on root induction in *in vitro* shoots of *P. marsupium* reveals that IBA is more effective than any other auxin on root induction. Effect of IBA for *in vitro* rooting has also been reported in leguminous species like *Acacia auriculiformis* [RANGA RAO & PRASAD, 1991] and *Prosopis tamurago* [NANDWANI & RAMAWAT, 1992].

Conclusions

To conclude, the present communication describes an *in vitro* propagation protocol for *P. marsupium* using nodal explants. The protocol outlined above offers *in vitro* propagation and conservation of this economically important multipurpose tree and would facilitate its use for future tree improvement programme using genetic transformation technology.

Abbreviations: Kn – Kinetin; NAA – α Naphthelene acetic acid; IBA – Indole butyric acid; min. – Minutes; h – hour.

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