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IN VITRO PROPAGATION AND FIELD ESTABLISHMENT OF *HARDWICKIA BINATA* ROXB. AND ASSESSMENT POLYMORPHISM THROUGH MOLECULAR MARKERS

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Abstract: *Hardwickia binata* Roxb. is a leguminous tree of great economic importance. Yet a protocol for its *in vitro* propagation that is capable of taking the regenerated plants from lab to land does not exist. The plant is quite recalcitrant and has poor natural regeneration. Macropropagation techniques are also not standardized. Problem of leaching of phenolic compounds in culture condition, premature leaf fall during multiplication phase and callusing during rooting were sever problems encountered during the course of development of the protocol. These problems were addressed using various treatments and a working protocol for *in vitro* propagation of *H. binata* from axillary bud of the seedling nodal segments was perfected. The plantlets were hardened through a systematic two stage hardening procedure and were eventually transferred to experimental field. Here we report the development of an *in vitro* propagation protocol for *H. binata*, leading to successful establishment of plants thus obtained, in open field condition.

Keywords: micropropagation, shoot multiplication, rooting, acclimatization, RAPD

Introduction

Hardwickia binata Roxb. is a leguminous tree of great economic importance. It serves as a source of very heavy and durable timber, fiber, fuel and high quality fodder for the cattle. *H. binata*, is also an important component of several agroforestry systems of arid and semi-arid regions. However the tree suffers from various problems among which poor seed setting, low seed germination rate and lack of proper macropropagation methods are the most important ones. *In vitro* propagation of this marvelous nitrogen fixing tree species will help in production of quality planting material on a large scale throughout the year without depending on seed viability, germination and season. Woody plants, including leguminous trees appear to be recalcitrant to *in vitro* culture and plant regeneration [ZHAO & al. 1990]. Only a few reports of work on *in vitro* propagation of *H. binata* are available. ANURADHA & al. (2000) reported *in vitro* propagation using mesocotyls, shoot tips and axillary buds from 15 days old seedlings as source of explants. Direct somatic embryogenesis of *H. binata* with mean number of 18.3 somatic embryos per explant from semimature zygotic embryos was reported by CHAND & SINGH (2001). *In vitro* somatic embryogenesis was also reported by DAS & al. (1995) and DAS (2011). However, complete plants could not be obtained from somatic embryos.

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Here we report development of an *in vitro* propagation protocol of *H. binata* and analysis of genetic uniformity of tissue culture raised plants through RAPD (Random Amplified Polymorphic DNA) markers.

Materials and methods

Plant material and explants source: All explants of *H. binata* were collected from a healthy mother tree (Fig. 1) growing in and around AFRI (Arid Forest Research Institute), Jodhpur. For optimization of surface sterilization procedure for hard tissues (mature nodal segments and mature seeds) all explants were first washed in running tap water for 5-10 min to remove surface contaminants. They were then washed with detergent (Tween 80) for 7-10 minutes followed by rinsing with distilled water 4-5 times. The explants were immersed and agitated constantly in freshly prepared Streptomycin (0.5 g/l00 ml) and Bavistin solution (0.1 g/100 ml of distilled water) for 10-15 minutes followed by rinsing with distilled water. The explants were then treated with 5–20% NaOCl (Sodium hypochlorite) solution at various concentrations and different time duration.

Fig. 1. *Hardwickia binata* Roxb. **A.** Mother plant growing at AFRI Campus, Jodhpur (India); **B.** Close-up of a stem nodal segment with pinnate bifoliate leaved. The stem nodal segment was used as explants.

Leaching of phenolics and its control: The injury caused during the excision of explants, induces the cells to leach out phenolic compounds which are readily oxidised to produce quinones and cause discoloration. In case of *H. binata*, leaching occurs from seed coat. In order to control this problem cold treatment and antioxidant treatment was given for different time periods prior to inoculation. Seeds were treated with antioxidants – ascorbic acid (250 ppm), citric acid (100 ppm) for 10 minutes each. Cold water treatment was given for 40 minutes.

Bud break: For *in vitro* shoot induction nodal segments, apical buds, tender shoot from cut end of trunk of mature trees as well as stem nodal segments, apical buds and cotyledonary nodes from *in vitro* raised seedlings were inoculated on MS medium supplemented with 2.0 mg L^{-1} BAP (6-Benzylaminopurine). Mature nodal segments and apical buds from selected trees were inoculated on MS [MURASHIGE & SKOOG, 1962] medium supplemented with different cytokinins – BAP (0.5 - 5.0 mg L^{-1}), Kn (0.5 - 5.0 mg L^{-1}), 2ip (2-isopentenyl adenine) (0.5 - 5.0 mg L^{-1}) and TDZ (Thidiazuron) (0.1 - 2.0 mg L^{-1}), combination of BAP (0.5 - 5.0 mg L^{-1}) and NAA (1-Naphthylacetic acid) (0.5 - 5.0 mg L^{-1}) and combinations of BAP (1.0 - 4.0 mg L^{-1}) and Kn (Kinetin) (1.0 - 4.0 mg L^{-1}). To study the effect of different additives (glutamine and thiamine HCl), MS medium + 2.0 mg/l BAP was used along with glutamine (50.0 mg L^{-1}) and thiamine HCl (10.0 mg L^{-1}) alone and in combination. Seeds were germinated on liquid MS medium on filter paper bridges.

Shoot multiplication: The *in vitro* induced shoots obtained in above experiments were used for achieving further multiplication through axillary bud proliferation or through repeated subculturing. Effect of cytokinins used singly and in combination on multiplication from seedling derived nodal segments was seen. Among the additives tested, glutamine and thiamine HCl were used alone and in combinations.

Control of in vitro leaf fall: *In vitro* leaf fall was a persistent problem encountered during multiplication of micro-shoots of *H. binata*. In order to control the problem of leaf fall, silver nitrate (0.15 mg L^{-1}) and silver thiosulphate (0.15 mg L^{-1}) were used separately in MS medium along with 2.0 mg L^{-1} BAP, 50.0 mg L^{-1} glutamine and 10.0 mg L^{-1} thiamine HCl.

Rooting: Root induction in *H. binata* was studied on the basis of various parameters, which include plant growth regulators, strength of salts, concentration of sucrose, pulse treatment and nutrient media.

Acclimatization: This was a two stage process and carried out as follows:

i. *In vitro* hardening: Rooted plantlets were first of all transferred to jam bottles containing autoclaved soilrite and kept in culture room. They were irrigated weekly with ½ MS, gradually the caps were loosened and finally opened to expose them to decreasing humidity.
ii. *Ex vitro* hardening: *In vitro* hardened plantlets were shifted to polyhouse after 20 – 25 days. They were kept for around 1 month and then transferred to poly-bags containing soil: soilrite: manure (1:1:1) in polyhouse conditions. Hardened plants of *H. binata* were thereafter transferred to pots after two months. The field hardened plants were finally transferred to open-field condition.

The complete protocol for *in vitro* plant propagation for *Hardwickia binata* is pictorially represented (Fig. 2).

Evaluation of genetic fidelity of tissue culture raised plantlets: In order to test the polymorphism in tissue culture raised plants from seedling explants, DNA fingerprinting

using RAPD markers was carried out. Polymorphic RAPD markers were selected from the recommended marker list for leguminous tree species suggested by GOMEZ & al. (2011).

Data analysis: Each treatment consisted of 10 explants and all experiments were repeated thrice. The results were analyzed statistically using SPSS ver. 10 (SPSS Inc., Chicago, IL, USA). The results are expressed as the means ± SE of three experiments.

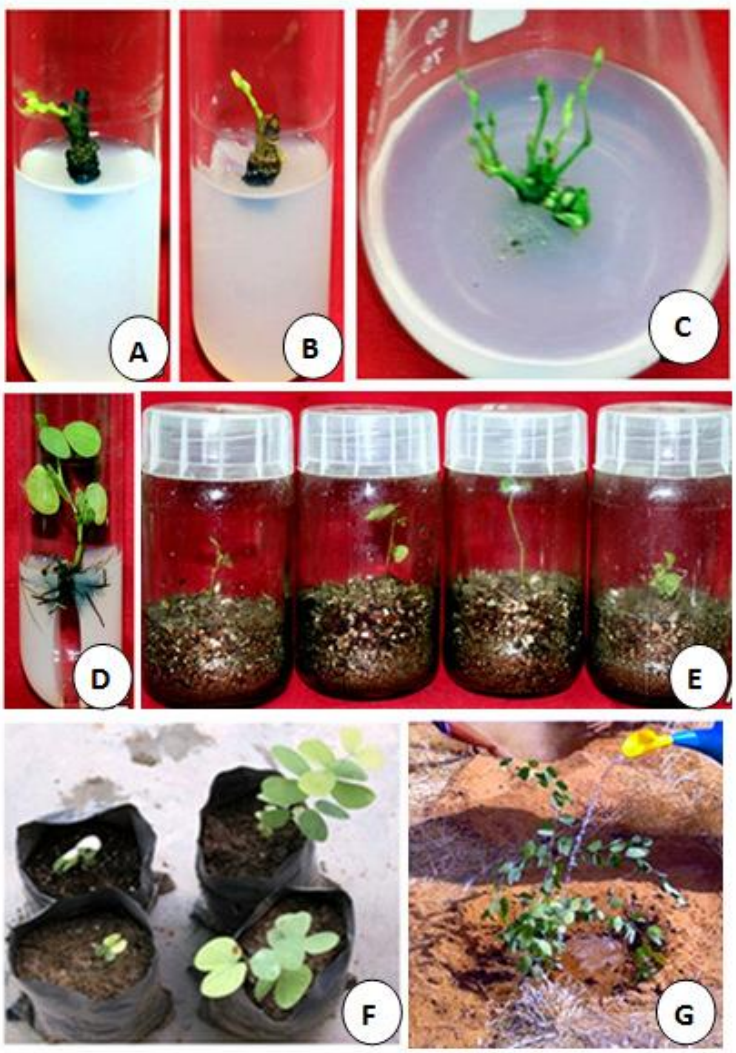

Fig. 2. *In vitro* propagation of *H. binata*. **A.** & **B.** Axillary bud-break; **C.** Shoot multiplication; **D.** *In vitro* rooting; **E.** *In vitro* hardening (acclimatization); **F.** Plantlets in poly – bags ready for field transfer; **G.** Tissue culture raised plant growing in field condition

Results and discussion

Surface sterilization of various explants of Hardwickia binata: For mature nodal segments, it was observed that 10% sodium hypochlorite was most effective at 9 minutes exposure showing only 1.67% contamination. For soft explants namely apical buds, immature seeds, young leaves, and mature leaves, it was observed that a treatment of 20% sodium hypochlorite for six minutes was most effective with only 3.33% contamination.

Effect of antioxidants on leaching of phenolics from seeds of H. binata: The percentage of leaching was observed to be 63% and 50% with the use of ascorbic acid and citric acid respectively. Cold water treatment was not found to have any positive role in reducing the extent of leaching. In the present work repeated subculturing was found most successful in reducing leaching.

In vitro shoot induction in H. binata: A maximum of 82% bud break was observed in case of stem nodal segments from *in vitro* raised seedlings, 69% in apical part from *in vitro* raised seedlings and 29% on cotyledonary nodes of seedlings. Thirteen percent bud break was observed in tender shoot from cut end of trunk (Tab. 1). A maximum of only 11% bud break was observed on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l NAA. Maximum bud induction (11%) was observed on medium with both the additives (glutamine and thiamine HCl) in it.

Tab. 1. Screening of explants for bud break response on MS medium supplemented with BAP (2 mg L^{-1}), sucrose (3%) solidified with agar (0.8%) at pH 5.8. Bold figure represents best response

Explants	Bud Break	Bud Break percentage (%)
Source: Mature tree		
• Mature nodal segments	–	–
• Apical bud	–	–
• Tender shoots arising from cut end of trunk from mature tree	+	13.33
Source: *In vitro* raised seedlings		
• Nodal segments	+	**82.22**
• Apical bud	+	68.89
• Cotyledonary node	+	28.89

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; observations were recorded after 4 weeks of inoculation.
– = no response, + = showed response.

Shoot multiplication: When *in vitro* raised nodal segments were transferred on MS media supplemented with BAP (2.0 mg L^{-1}), kinetin (2.0 mg L^{-1}) and combination of BAP (2.0 mg L^{-1}) and kinetin (2.0 mg L^{-1}), highest shoot multiplication was achieved on MS media supplemented with 2.0 mg L^{-1} BAP with 2.5 shoots per explant and mean shoot length of 3.1 cm (Tab. 2). When different media (MS, WP and B5) supplemented with BAP (2.0 mg L^{-1}), glutamine (50.0 mg L^{-1}) thiamine HCl (10 Mg L^{-1}) and silver nitrate (0.1 mg L^{-1}) were tested for shoot multiplication, MS medium proved to be the best providing mean number of 3.46 shoots per explant and mean shoot length of 3.86 cm followed by woody plant medium and B_5 medium.

Tab. 2. Effect of cytokinins alone and in combinations supplemented in MS medium having sucrose (3%) solidified with agar (0.8%) at a pH of 5.8 on multiplication from seedling derived nodal segments

Hormone concentration (mg L^{-1})		Number of shoots per explant (Mean ± SE)	Shoot Length in cm. (Mean ± SE)	Percentage of explants showing callusing (%)
BAP	Kn			
0.0	0.0	1.03 ± 0.03^{a}	0.72 ± 0.13^{a}	11.11
2.0	**0.0**	$\mathbf{2.50 \pm 0.33^{b}}$	$\mathbf{3.10 \pm 0.45^{c}}$	**22.22**
0.0	2.0	2.12 ± 0.19^{b}	1.89 ± 0.19^{b}	26.67
2.0	2.0	2.15 ± 0.16^{b}	2.29 ± 0.42^{bc}	28.89

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 15 explants, repeated thrice; C = Control. Observations were recorded after 4 weeks of inoculation.

In vitro rooting of microshoots and acclimatization: Media supplemented with 4.0 mg L^{-1} IBA and 4.0 mg L^{-1} IAA resulted into 36% and 20% rooting respectively. Mean root number (2.22) and mean root length (1.30 cm) was observed to be highest on 4.0 mgL^{-1} IBA (Tab. 3). To achieve reduction of intermittent callus formation at root shoot junction, experiments were conducted using ½ MS media + 4.0 mg L^{-1} IBA supplemented with different percentages of sucrose (1.0, 2.0 and 3.0 %). Out of these 2% sucrose proved to be the best with 40% root induction. Percentage of callus formation was reduced by 50% (Tab. 4). Survival rate of 50% was observed during *ex vitro* hardening. Fully acclimatized plants were transferred to field in August, 2012 and out of six plants transferred to field conditions five are surviving and are in a healthy condition.

Tab. 3. Effect of auxins on rooting when supplemented in MS medium. Figures in bold indicate best performance for the particular auxin treatment

Hormone (mg L^{-1})			Percentage of explants showing rooting	No. of roots/shoot (Mean ± SE)	Shoot Length in cm. (Mean ± SE)	Percentage of explants showing callusing
IBA	IAA	NAA				
0.0 (C)	0.0 (C)	0.0 (C)	0.0	0.0	0.0	0.0
1.0	0.0	0.0	10.00	1.33 ± 0.88^{ab}	0.69 ± 0.35^{ab}	**53.33**
2.0	0.0	0.0	0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	83.33
3.0	0.0	0.0	13.33	1.50 ± 0.50^{ab}	1.16 ± 0.24^{ab}	80.00
4.0	0.0	0.0	**36.67**	$\mathbf{2.22 \pm 0.40^{b}}$	$\mathbf{1.30 \pm 0.18^{b}}$	80.00
5.0	0.0	0.0	23.33	1.94 ± 0.24^{b}	1.18 ± 0.23^{ab}	83.33
0.0	1.0	0.0	0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	**46.67**
0.0	2.0	0.0	6.67	1.00 ± 0.58^{a}	0.32 ± 0.16^{a}	63.33
0.0	3.0	0.0	10.00	0.83 ± 0.44^{a}	0.47 ± 0.26^{a}	76.67
0.0	4.0	0.0	**20.00**	0.67 ± 0.33^{a}	0.40 ± 0.23^{a}	76.67
0.0	5.0	0.0	16.67	$\mathbf{1.44 \pm 0.44^{a}}$	$\mathbf{0.50 \pm 0.16^{a}}$	83.33
0.0	0.0	1.0	3.33	0.33 ± 0.33^{a}	0.17 ± 0.16^{a}	**46.67**
0.0	0.0	2.0	6.67	1.00 ± 0.58^{ab}	0.70 ± 0.39^{ab}	60.00
0.0	0.0	3.0	13.33	1.22 ± 0.62^{ab}	0.66 ± 0.33^{ab}	80.00
0.0	0.0	4.0	**20.00**	2.06 ± 0.34^{b}	$\mathbf{1.24 \pm 0.06^{b}}$	80.00
0.0	0.0	5.0	23.33	$\mathbf{2.17 \pm 0.44^{b}}$	1.05 ± 0.09^{b}	90.00

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; C = Control. Observations were recorded after 6 weeks of inoculation.

Tab. 4. Effect of different concentrations of sucrose on rooting with supplemented in ½ strength MS media having IBA (4 mg L^{-1}), and solidified with agar (0.8%) at a pH of 5.8. Figures in bold indicate best performance for the particular sucrose percentage.

Sucrose percentage (w/v)	Percentage of explants showing rooting (%)	No. of roots/shoot (Mean ± SE)	Root Length in cm. (Mean ± SE)	Percentage of explants showing callusing (%)
0.0 (C)	3.33	0.33 ± 0.33^{a}	0.33 ± 0.33^{a}	33.33
1.0	10.00	1.00 ± 0.58^{ab}	0.82 ± 0.42^{a}	**43.33**
2.0	**40.00**	2.11 ± 0.11^{b}	1.11 ± 0.02^{a}	50.00
3.0	33.33	**2.26 ± 0.14^{b}**	**1.16 ± 0.02^{a}**	83.33

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; C = Control. Observations were recorded after 6 weeks of inoculation.

Silver nitrate proved to be better than silver thiosulphate in controlling leaf fall to 20% with 3.8 shoots per explant.

Selection of Molecular markers and analysis of polymorphism: RAPD analysis was performed based on earlier screening report by GOMEZ & al. (2011) for leguminous tree species *Acacia nilotica*, *Adenanthera pavonina*, *Prosopis juliflora*, *Pithecellobium dulce*, *Clitoria ternatea* and *Pongamia pinnata*, from which highly polymorphic primers were selected. These four highly polymorphic random and arbitrary 10-base primers [(5'AAGGCGGCAG (OPI-06), 5'TGCCCAGCCT (OPI-18), 5'TGGGCGTCAA (OPL-02) and 5'AGGTTGCAGG (OPL- 16)] were used for genetic fidelity test. The banding patterns from micropropagated plants through seedling material were slightly polymorphic (Fig. 3). The variations from the mother plant were due to the reason that *in vitro* seedlings were the source material for micropropagation.

Conclusions

Hardwickia binata is an important tree species that has nitrogen fixing capability, and hence also an important part of agro-forestry system. However due to its poor natural regeneration, and not-so-well developed propagation protocols, the present work was carried out leading to the development of a working protocol for *in vitro* mass propagation of the species (Fig. 4). Moreover, the DNA fingerprinting study using RAPD markers has also been carried out to establish the molecular polymorphism in the regenerated plants.

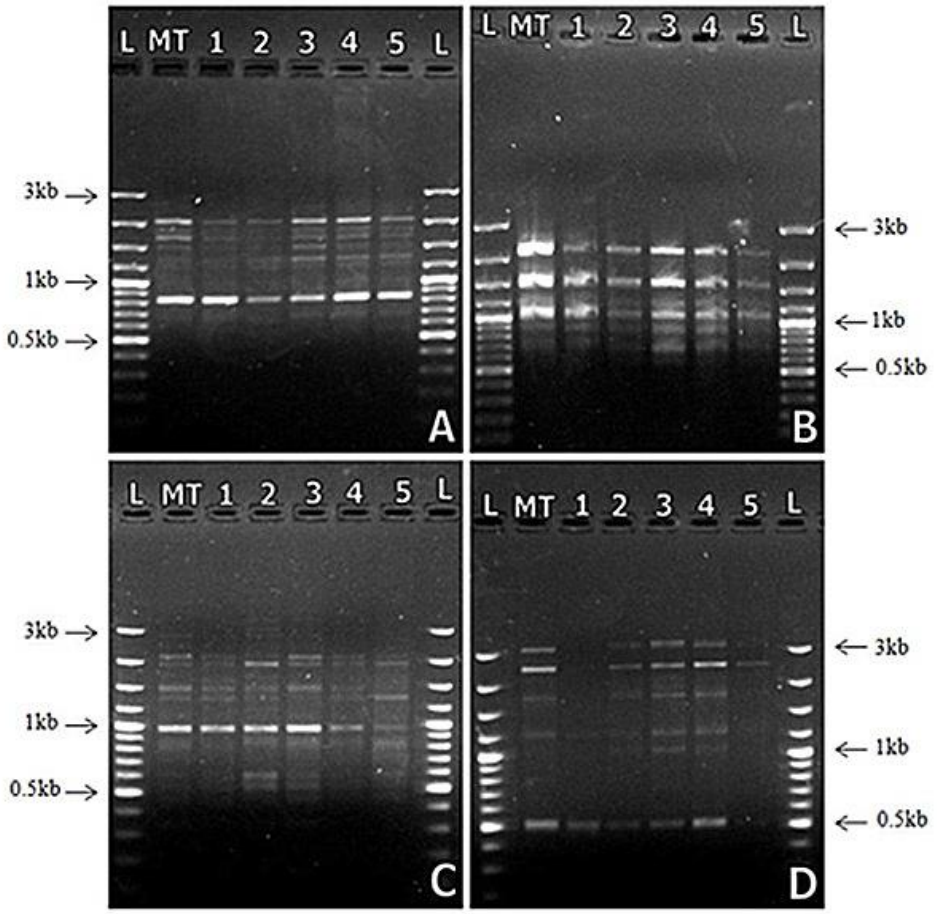

Fig. 3. DNA fingerprinting of mother tree (M) and tissue culture raised plants (1-5) of *H. binata*. **A.** RAPD analysis using primer OPL-16; **B.** Using primer OPI-06; **C.** Using primer OPL-02; **D.** Using primer OPI-18

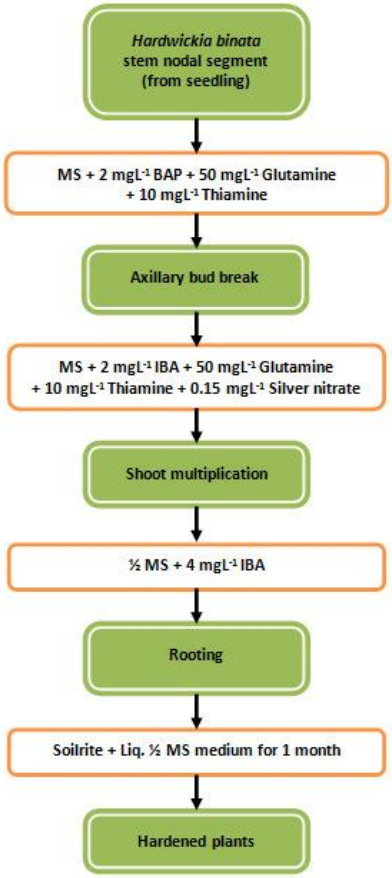

Fig. 4. Complete protocol for *in vitro* plant propagation of *H. binata*

References

ANURADHA M., KAVI KISHOR P. B. & PULLAIAH T. 2000. *In vitro* propagation of *Hardwickia binata* Roxb. *J. Indian bot. Soc.* **79**: 127-131.

CHAND S. & SINGH A. K. 2001. Direct somatic embryogenesis from zygotic embryos of a timber-yielding leguminous tree, *Hardwickia binata* Roxb. *Current Science.* **80**: 882-887.

DAS A. B., ROUT G. R. & DAS P. 1995. *In vitro* somatic embryogenesis from callus cultures of timber yielding tree *Hardwickia binata* Roxb. *Plant Cell Rep.* **15**: 147-149.

DAS P. 2011. *In vitro* somatic embryogenesis in some oil yielding tropical species. *Amer. J. Plant Sci.* **2**: 217-222.

GOMEZ S. M., RAMASUBRAMANIAN T. & MOHANKUMAR S. 2011. Potential RAPD markers for population studies in tree legumes. *Pak. J. Bot.* **43**: 1879-1883.

MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-497.

ZHAO Y. X., YOA D. Y. & HARRIS P. J. C. 1990. Nitrogen Fixing. *Tree Res. Rep.* **8**: 113-115.

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