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Sustainable Production of a Therapeutically Important Tree (*Holarrhena antidysenterica*) of India

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ABSTRACT

Holarrhena antidysenterica (Linn.) Wall is an important medicinal plant of the Indian System of Medicine, the Ayurveda. The stem bark of the tree is used for antidiarrhoeal activity, owing to the presence of the bioactive compound conessine, a steroidal alkaloid. Due to the difference in environmental conditions or in genotype, the plant shows much variation in its therapeutic efficacy, hence there is a need to have more plants with an optimum and uniform therapeutic efficiency. In this study, we established a protocol for clonal multiplication of this important medicinal tree. The superior mother plant was selected on the basis of a higher concentration of the bioactive constituent, conessine, by phytochemical analysis. The maximum multiplication rate of axillary buds was observed in 8 μM 6-benzyladenine on Murashige and Skoog (MS) medium. Multiplied shoots of 7-9 cm were transferred to rooting medium (MS medium with 0.5 μM indole-3-acetic acid). The rooted plantlets were then transferred for primary and secondary hardening and finally transferred to the field. A 70-80% field survival was observed in regenerated plants. The established protocol offers a sustainable method for consistent production of this elite, medicinally-important tree.

Keywords: axillary bud multiplication, conessine, Kada Chhal, Kurchi, plantlets
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **KIN**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, α-naphthaleneacetic acid; **PGR**, plant growth regulator

INTRODUCTION

Holarrhena antidysenterica (Family: Apocynaceae) is an important Indian medicinal tree locally known as Kada Chhal or Kurchi. The medicinal properties of the plants were known as early as 1000 B.C. Many traditional systems of medicine all over the world include the use of stem bark as a principal remedy of various types of dysentery (Gopal and Chauhan 1996). The bark is also reported to have astringent, anthelmentic, stomachic, febrifugal and tonic properties (Anonymous 2001). Water decoction of the bark showed significant antiulcer activity in albino rats at a dose of 400 mg/kg (De *et al*. 1997). Steroidal alkaloids (~4%) were reported as the major chemical constituents of the plant (Bhutani *et al*. 1984) and are responsible for the medicinal properties of the plant. A total of 48 alkaloids were reported to be present in the plant while conessine (0.4%) was reported to be one of the major bioactive compounds of the plant (Siddiqui 1936).

Phytochemical studies on the plant reveal a high degree of variation in the chemical constituents, especially in the concentrations of the bioactive constituents (Srivastava *et al*. 2004) which in turn affects its medicinal properties. These variations could be genotypic or geographic. Hence, there is a need to have a superior quality, genetically uniform plantation of this important tree of Ayurvedic Medicine. Conventional vegetative propagation methods, e.g. airlayering, grafting or cutting, are not sufficiently rapid and efficient, and thus *in vitro* techniques like micropropagation via multiple axillary branching offers a rapid means for multiplication of elite and rare germplasm and an alternative method for *ex situ* conservation (Anand *et al*. 1998; Boggetti *et al*. 1999).

The present study describes an efficient protocol for axillary bud multiplication and plantlet regeneration of *H. antidysenterica* from a superior mother plant selected on the basis of its phytochemical evaluation.

MATERIALS AND METHODS

Selection of mother plants

The mother plant was selected on the basis of total alkaloid as well as conessine content in the stem bark of the plant collected from various geographical locations of India.

Culture media and incubation conditions

Murashige and Skoog (1962), or MS medium with 20 g l^{-1} sucrose and 8 g l^{-1} agar (S.D.Fine-Chem Ltd., Mumbai, India) at pH 5.7 was used as the basal nutrient medium. Cultures were incubated at 24±2°C with 80-90% humidity under cool white fluorescent lights (Philips, India with total light intensity of 1500 lux) for a 16:8 h photoperiod. The cultures were transferred at four weeks interval onto fresh medium. Changes occurring in the explant during the incubation period were recorded on a daily and weekly basis. All experiments were repeated three times with 10 replicates in each case.

Axillary bud multiplication and plantlet formation

Green, healthy axillary buds (2^{nd} to 6^{th} from apex) from the selected superior mother plant were collected to initiate cultures. The buds were washed thoroughly and cleaned with water and polysorbate 80, thereafter they were surface sterilized in mercuric chloride (0.1% w/v). The sterilized explants were then cut into the desired size (internode tissue was cut 0.3 cm above and 1.0 cm below the node having opposite axillary buds) and inoculated onto MS medium supplemented with various concentrations of plant growth regulators (PGRs) like 6-benzyladenine (BA), kinetin (KIN) and α-naphthaleneacetic acid (NAA), individually or in combination. For histological analyses, a thin section of the ex-

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plant was cut from the site of axillary bud multiplication and stained with saffranin and methylene blue.

For elongation, microshoot clusters (5-6 shoots per cluster) 1-2 cm long were excised, and transferred to fresh medium optimized for shoot multiplication, as the same medium was also found suitable for shoot elongation. After elongation, the shoots were excised from the base and transferred individually to full (MS salts with original concentration) and half (MS salts with half concentration) strength liquid and solidified MS medium supplemented with and without auxins and various concentrations (1, 2, 3%) of sucrose for rooting. Various concentrations (0.2-2.0 μM) and combinations of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were also tested for induction and elongation of roots. The obtained plantlets (elongated shoots with roots) were washed with sterile distilled water to remove adhering culture medium and transferred to pots containing a hardening mixture of sterile sand, soil, vermiculite, perlite and cocopeat in different combinations and ratios [cocopeat: soil (3:1), cocopeat: sand: soil (1:1:1), vermiculite: soil (1:1), vermicultite: soil (3:1), perlite: sand: soil (1:1:1)]. For secondary hardening, the primary hardened shoots were transferred to a combination of soil and sand (3:1). The shoots were kept in the greenhouse at 28±2°C and 60-70% relative humidity. The hardened shoots were then transferred to net house and subsequently to the field.

RESULTS AND DISCUSSION

Selection of mother plant

The phytochemical analysis of the stem bark samples collected from various states of the country showed that the maximum amount of total alkaloid was present in the bark sample collected from Gujarat State of India. The conessine content was also found to be highest in the bark sample collected from Gujarat amongst all the samples analyzed (Srivastava *et al*. 2004). Hence, the explant material for tissue culture studies was used from plants growing in Gujarat region.

Axillary bud multiplication and plantlet formation

The induction of multiple shoots through axillary branching is now recognized as a useful technique for propagation. To reduce the risk of somaclonal variability during multiplication, preformed apical and axillary meristems, as sources of new plants are usually preferred over indirect organogenesis (Boggetti *et al*. 1999). Various concentrations (2-12 μM) of BA and KIN were attempted individually for the multiplication of axillary buds (**Table 1**). Activation of quiescent meristems and sprouting of axillary buds was observed within one week at all concentrations of BA as well as of KIN but the extent of response varied with concentration (**Fig. 1**). Multiplication of axillary buds (1→2) was observed after two weeks of incubation at all concentrations of BA except for 2 μM. The highest multiplication rate (1→5) was observed in medium supplemen-

Table 1 Effect of growth regulator on bud sprouting and multiplication (n=20).

Growth regulators			Number of	Number of	Callus
BA (μM)	KIN (μM)	NAA (μM)	axillary buds sprouted	axillary buds multiplied	Phase
2	-	-	9 ± 1.158	0	-
4	-	-	10 ± 1.528	6 ± 0.577	-
6	-	-	14 ± 2.082	11 ± 1.155	-
8	-	-	19 ± 1.155	18 ± 1.000	-
10	-	-	12 ± 1.155	11 ± 1.155	-
12	-	-	12 ± 1.000	8 ± 0.577	-
-	2	-	6 ± 0.577	0	-
-	4	-	7 ± 1.528	0	-
-	6	-	9 ± 1.000	0	-
-	8	-	13 ± 0.578	0	-
-	10	-	8 ± 1.732	0	-
-	12	-	4 ± 1.528	0	-
8	2	-	10 ± 0.577	6 ± 0.239	-
8	4	-	9 ± 0.738	2 ± 0.098	-
8	2	0.05	5 ± 0.577	2 ± 0.098	++
8	2	0.1	12 ± 1.000	8 ± 0.130	+++
8	2	0.2	5 ± 0.623	0	+++
8	-	0.05	8 ± 0.795	3 ± 0.110	+
8	-	0.1	15 ± 1.234	10 ± 0.378	+++
8	-	0.2	10 ± 1.000	2 ± 0.073	+++

+ : Extent of callus formation

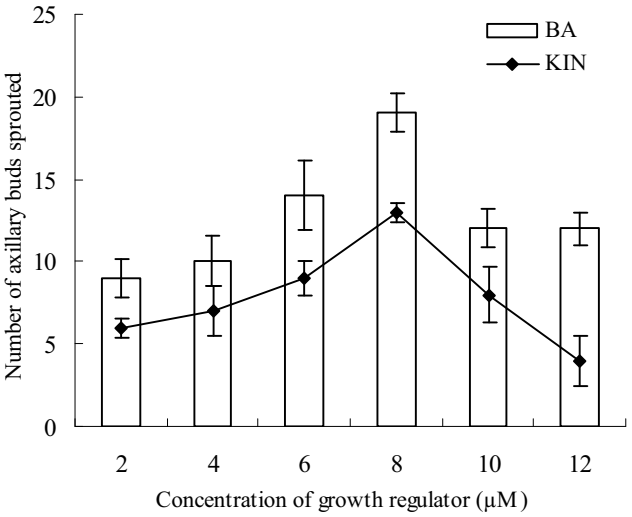

Fig. 1 Axillary bud sprouting in different concentrations of BA or KIN. Values are mean of 10 replicates (each replicate with two axillary buds) in each treatment. Bars indicate SD.

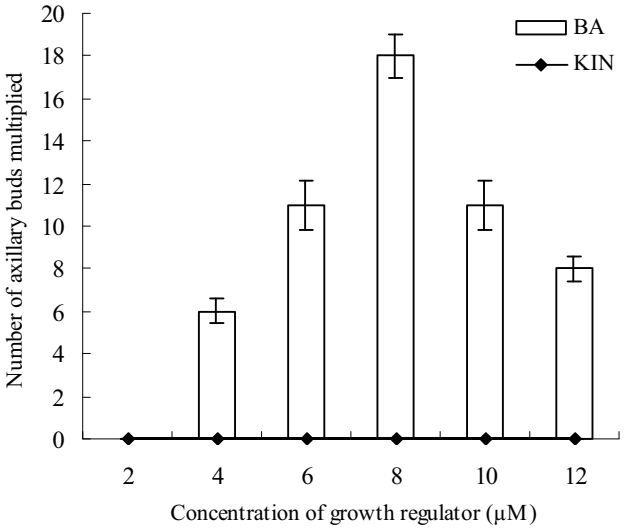

Fig. 2 Axillary bud multiplication in different concentrations of BA and KIN. Values are mean of 10 replicates (each replicate with two axillary buds) in each treatment. Bars indicate SD.

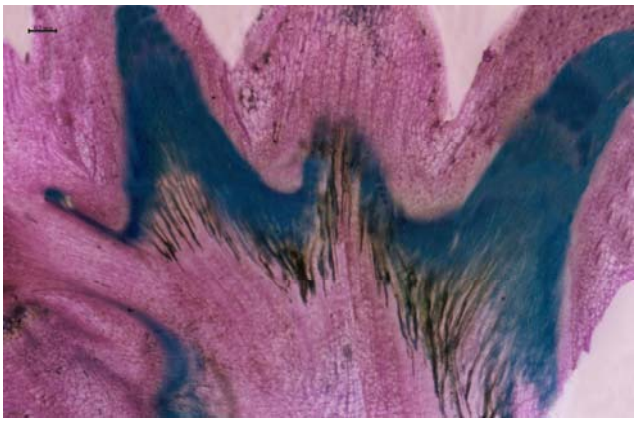

Fig. 3 Histology of axillary bud multiplication in medium supplemented with 8 μM BA. Scale bar = 200 μm.

ted with 2% sucrose and 8 µM BA (**Figs. 2, 3**). A linear increase was observed in the percentage response of bud sprouting, shoot multiplication, and the number and length of microshoots with an increase in BA concentration reaching a maximum at 8 µM. The microshoots were developed and multiplied from pre-existing quiescent axillary meristems from both sides of nodal explants without any visible deformities or callus formation. Similar results were observed in a number of medicinal plants such as *Cleistanthus collinus* (Quraishi *et al*. 1996), *Sapindus mukorossi* (Philomina and Rao 2000), *Terminalia arjuna* (Pandey and Jaiswal 2002), *Balanites aegyptiaca* (Ndoye *et al*. 2003) and *Clerodendrum inerme* (Kothari *et al*. 2006), where BA alone at concentration ranging from 0.4 to 22 µM resulted in the maximum rate of multiplication and gave better results than KIN. *Rauvolfia tetraphylla* and *R. micrantha* plants are also reported to respond in similar manner in response to BA (Patil and Jayanthi 1997). Earlier studies on *in vitro* clonal propagation of *H. antidysenterica* also revealed maximum multiplication of the nodal explants in BA alone but the number of shoots multiplied per explant was less even at high concentration of BA (Agrawal *et al.* 2005) as compared to our results. The addition of auxin at low concentrations is reported to favor the multiplication rate of axillary buds in many reports. Anand *et al*. (1998) reported an increased rate of multiplication of axillary buds when NAA was added with BA to *Uraria picta* cultures. In our experiment, we did not find favourable results with the addition of auxin, NAA (0.05-2 µM; **Table 1**). Low concentrations of NAA did increase the rate of multiplication of axillary buds but intervening callus phase was also observed at the base, which is an undesirable phenomenon in clonal multiplication. BA proved to be the best amongst all the PGRs tested for axillary bud multiplication in *H. antidisenterica*.

Elongation of the multiplied microshoots was observed better in medium optimized for shoot multiplication (MS medium with 8 µM BA and 2% sucrose). However, other growth hormones were also tried in order to reduce the time for elongation. MS medium supplemented with IBA and IAA individually at the concentration range of 0.05-0.2 µM were also found to be suitable for elongation of the shoots but a significant difference was not observed in the time taken for elongation of microshoots hence the shoots were allowed to elongate on shoot multiplication medium. After microshoots attained a length of 7-9 cm (**Fig. 4**), they were separated and placed on various rooting medium such as full or half concentration of MS salt with and without auxins and various concentrations (0, 0.5, 1.0 and 2.0%) of sucrose for root induction. No root induction was observed on half strength MS medium and medium supplemented with lower concentrations of sucrose. Full strength MS medium with 1 and 2% sucrose in the absence of PGRs resulted in root formation but the rate of response and quality of roots was very poor. Root induction was also observed in MS medium supplemented with IAA and IBA individually and also in combination [IAA (0.2-2.0 µM), IBA (0.5 µM) and IAA + IBA (0.05 + 0.05, 0.1 + 0.1 µM)]. The response of shoots towards root induction was better in the presence of IAA than IBA. The highest number of plantlet formation (root induction in shoots) was seen in the presence of 0.5 µM IAA. The roots formed were thin and soft. Both liquid medium and medium containing a gelling agent were tried to support rooting. The addition of gelling agent improved the thickness and texture of the roots in all cases but there was no significant increase in rooting response except in MS medium (full strength) supplemented with 0.5 µM IAA (**Fig. 5**). Induction of roots was observed in all cases after 3 weeks. After 2 weeks of root induction (total after 5 weeks), the plantlets were transferred for primary hardening to different combinations and ratios of hardening mixture (**Fig. 6**). After 2 months of primary hardening period, the shoots were transferred for secondary hardening to mixture of soil and sand (3:1). The shoots were allowed to grow for 4 to 5 weeks, after which they were transferred to soil and also to a net house. After maintaining the shoots in the net house for 5 to 6 weeks, they were finally transferred to the field. Survival rate of the regenerated plant in the field was observed to be 70-80%. The time-lines for each of the stages of micropropagation shows that the total time period required from inoculation of explant to field transfer of plantlet is 25-34 weeks (**Fig. 7**).

Fig. 4 (left) Elongated multiplied shoots in medium supplemented with 8 µM BA. Fig. 5 (right) Rooting of elongated shoots in medium supplemented with 0.5 µM IAA.

Fig. 6 Primary hardening of plantlet in mixture of perlite: sand: soil (1: 1: 1).

CONCLUDING REMARKS

The present work demonstrates a simple procedure for the rapid clonal multiplication of *Holarrhena antidysenterica* through axillary buds. This approach offers a means for producing identical propagules from phytochemically superior mature trees selected for desired therapeutic properties.

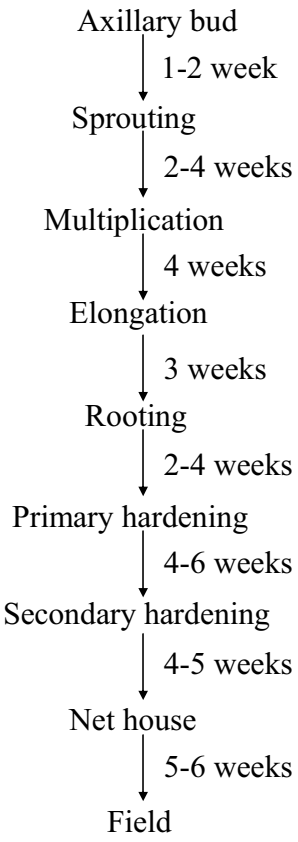

Fig. 7 Flowchart of timelines for each of the stages of micropropagation.

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