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Conference Paper · January 2012

DOI: 10.13140/RG.2.1.4108.1689

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(Re-accredited A+ by NAAC)

Title: Impact of Plant Tissue Culture On Advances in Plant Biology

ISBN No.: 978-93-80066-83-7

Published by Gujarat Sahitya Prakash, P.B. 70, Anand – 388001 *and* Fr. (Dr.) Vincent Braganza, Convener PTCA 2012, C/o Loyola Centre for Research and Development, St. Xavier's College Campus, Navrangpura, Ahmedabad – 380009 INDIA.

Printed at **Rachana Corporation**, FF-7, Devshruti Complex, Mithakhali, Ahmedabad - 380 006. M : 98250 16474 E : shashikant24@gmail.com

Supported by:
The Department of Biotechnology (DBT), Ministry of Science and Technology, The Energy and Resources Institute (TERI), IHC Complex, Lodhi Road, New Delhi - 110 003.

Micropropagation of Hibiscus radiatus- A Medicinal Plant

Mafatlal M. Kher and M. Nataraj*

Lab-302/B, B. R. D. School of Biosciences, Sardar Patel Maidan,
Vadtal Road Post box no. 39 Sardar Patel University, Vallabh
Vidyanagar-388120 (India).
*Corresponding author:
Email: nattusamy@yahoo.com Tel: +91-2692-234412 extension-303

Abstract

A micropropagation method for *Hibiscus radiatus*- a medicinal herb of Malvaceae has been developed. This study provides an efficient micropropagation protocol for multiplication of *H. radiatus* from nodal explants. Surface sterilized nodal segments were cultured on Murashige and Skoog medium supplemented with various concentrations (0.00, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/l) of BAP and KN, and gelled with 0.8% w/v agar. After four weeks these were subcultured on fresh medium of the same composition. MS medium supplemented 1.50mg/l of BAP showed maximum of 3.50 ± 0.52 shoots per node, where as MS medium supplemented with 2.0 mg/l KN produced 2.91 ± 0.54 shoots per node. Shoots were rooted in ½ MS medium supplemented with 0.50 mg/l NAA.

Keywords: KN, BA, NAA, Hibiscus radiatus, axillary shoot proliferation.

Abbreviations:BA: 6- benzyl adenine, D/W: Distilled water, IBA: Indol-3 butyric acid, KN: Kinetin, MS: Murashige and Skoog (1962) medium, NAA: Naphthalene acetic acid, PGRs: Plant growth regulators

Introduction

Hibiscus radiatus, is native to southern and southeast Asia. This plant is very similar to *H. cannabinus* except that its leaves lack glands found in the latter species. It has yellow flowers that have a purple center. Leaves are dentate, with upper leaves lobed into three or five parts. It is frequently used as a vegetable and medicine (Lawton 2004).

This plant is reputed as cure for fever and as a blood purifier all plant parts are cut and boiled, and the liquid is used (Anonymous, 1914). The stem is an important source of good quality of fiber (Maity, 1997). Sexually propagated plants (through generation of seeds) demonstrate a high amount of heterogeneity since their seed progenies are not true to type whereas asexual reproduction (by multiplication of vegetative parts) gives rise to genetically identical copies of parent plant. (Malueg *et al.* 1994).

To date, genetic improvement of *H. radiatus* has been mainly achieved by conventional plant breeding methods (Manzel and Wilson, 1961, 1966), but more recent techniques in plant genetic engineering have advanced and opened a new avenue for plant improvement. The availability of an effective *in vitro* regeneration system is pre requisite for the genetic transformation of most plants (Thiruvengadam *et al.*, 2010), and plant regeneration protocol from explant is essential for the application of genetic engineering in *H. radiatus*.

In the present research, work was carried out to develop a protocol for micropropagation of *H. radiatus* using node as a source of explant. *In vitro* methods have been reported for some related species of *H. radiatus* such as *H. rosa sinensis*,

Christensen *et al.,* 2008; Bhalla *et al.,* 2009) *H. cannabinus* (Srivatanakul *et al.,* 2000; Herath *et al.,* 2004; Chen *et al.,* 2010) , *H. sabdariffa* (Sié *et al.,* 2010), *H. acetosella* and *H. moscheutos* (Sakhanokho and Kelley, 2009), *Kosteletzkya pentacarpos* (Piovan *et al.,* 2010), *Gossypium hickii* (Yang *et al.,* 2010). The objective of this study was, therefore, to establish an efficient reproducible protocol for high frequency regeneration from nodal explants. We believe that our findings may facilitate *in vitro* propagation of this important plant species and may be also applicable to other related plant species of Malvaceae.

Materials and Methods

Collection of explant and surface sterilization

Third or fourth nodes were taken as explants from healthy *H. radiatus* plant from BRD School of Biosciences Campus, Sardar Patel University during December, 2010 to March, 2011. The collected explants were washed thoroughly in running tap water for 30 min. Then explants were washed with 3% Neutral Rankleen detergent (RFCL-India) for 5-10 min, followed by 30 minutes washing in running tap water. The explants were sterilized with 0.1% mercuric chloride (w/v) solution for 2-3 minutes. They were rinsed properly with sterile D/W, 3-4 times inside the laminar air flow to remove all traces of mercuric chloride. All the experimental manipulations were carried out under strict aseptic conditions in laminar air flow bench fitted with bactericidal U.V. tubes (25 W). The floor of the chamber was thoroughly wiped with spirit. The surface of all the vessels and other accessories such as sprit lamps, lighter, were cleaned with spirit. Forceps, scalpels, needles, and spatula were autoclaved before inoculation. All the accessories and media were kept in the chamber and then sterilized with U.V. rays continuously for 30 minutes. Forceps and scalpels were flame sterilized in the chamber. Sterilized explants were excised from both ends, using fine sterile forceps and scalpel and inoculated into respective media in culture tubes. The culture tubes were closed with non absorbent cotton plugs wrapped with muslin cloth to prevent microbial contamination.

Culture media and conditions

The Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) plant tissue culture grade agar (SRL, India) was used throughout the experiments. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving at 121°C and 104 kPa pressure for 20 min. All the cultures were maintained at 25 ± 2°C under 16 hours photoperiod with a photosynthetic photon flux density (PPFD) of 40-50 µmol m^{-2} s^{-1} provided by cool white florescent tubes (40 W, Philips, India) and with 50–60% relative humidity.

Bud breaking and multiple shoot induction: To study the effect of PGRs on bud breaking, nodal explants were cultured on MS medium with different concentrations of BA (0.00, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/l) or KN (0.00, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/l). For shoot multiplication, explants along with regenerated shoots were transferred to MS medium supplemented with1.5 mg/l of BA. The cultures were further multiplied by newly formed shoots and were transferred to optimized shoot

multiplication medium (MS medium containing 1.50 mg/l BA) up to five passages to study the effect of successive transfer of the mother cultures on shoot multiplication. Subculturing of initial cultures was done after 4 weeks of each passage.

Rooting of regenerated shoots

For the root induction under *in vitro* condition, shoots (2–5 cm in length) were excised from mother explants and transferred to half-strength MS medium supplemented with different concentrations of NAA or IBA alone (0, 0.5, 1, 1.5, 2 and 2.5 mg/l).

Statistical analysis

All the experiments were conducted with twelve replicates per treatment and repeated three times. Each replicate represents one explant per culture tube. The data were analyzed statistically through one-way ANOVA using GraphPad PRISM ver.3.00 (GraphPad Software, USA). The significance of difference among means was carried out by Dunnet test and the results are expressed as mean ± SD of three repeated experiments.

Results and Discussion

As the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators, the proportion of these regulators in the media can be manipulated to induce the regeneration of each meristem into viable shoots (Varghese *et al.,* 1992, 1993). In this study, growth regulator type, concentrations were assessed to determine optimal culture conditions to regenerate shoots of *H. radiatus in vitro*.

Effect of cytokinin on multiple shoot induction

The response of nodal explants cultured on MS medium supplemented with various concentrations of BAP and KN is presented in (Table 1). Nodal explants showed minimal response in terms of: percentage response (PR), number of shoot (NS) and shoot length (SL), in PGR free medium, where as medium supplemented with cytokinin showed bud breaking within 2-3 weeks of inoculation. Among the two cytokinins tried, BA proved to be more effective than KN, in terms of PR, NS and SL (Table-1). The maximum numbers of shoots were obtained in MS medium supplemented with 1.50 mg/l BA, 100 percent of the cultures responded and average 3.50 ± 0.52 numbers of shoots were formed with shoot length of 2.53 ± 0.31 centimetres within four weeks (figure 1A). As the concentration of BA increases the number of shoots increases significantly as compared to control ($p < 0.01$), contrastingly medium supplemented with KN showed nonsignificant increase then control ($p > 0.01$), but shoot length was increased significantly ($p < 0.01$) in all the concentrations of both BAP and Kinetin. In medium supplemented with 2.00 mg/l of kinetin, best response was seen among all the kinetin concentrations, where 91.67% cultures were having average number of shoot 2.91 ± 0.54 with shoot length of around 2.34 ± 0.59 centimetres. Therefore, BAP is the best and suitable cytokinin over Kinetin for *in vitro* propagation of *H. radiatus* using nodal explant.

Though both cytokinins were capable of inducing shoot formation, BA was found to be significantly more effective than KN in these studies. Similar observations have been reported in *Hibiscus cannabinus* (Herath *et al.*, 2004), *Hibiscus acetosella* (Sakhanokho *et al.*, 2008), *H. rosa-sinensis* (Bhalla *et. al.*, 2009), *Gossypium bickii* (Yang *et al.*, 2009).

The nodal pieces of shoots grown on MS medium supplemented with 1.50 mg/l BA were subcultured every four weeks on the same medium (Figure 1-B). The shoot numbers and shoot length increased significantly during second and third subcultures (Figure-2 and 3) then it decreases. The increase in shoot number may be due to suppression of apical dominance during subculture that induced basal meristematic cells to form new shoots (Tripathi and Kumari, 2010). Similar effect of subculturing on shoot multiplication was reported by (Siddique and Anis, 2009) in *Balanites aegyptica.*

Table 1 Effect of cytokinins (BAP or KN) on bud breaking and shoot growth from nodal explants of *H. radiates*

KN (mg/l)	BAP (mg/l)	Response (%)	Number of shoots per explant (mean ± SD	Average shoot length (centimetre) (mean ± SD)
0.00	0.00	25.00	1.33 ± 0.58	0.60 ± 0.10
0.50	-	66.67	1.75 ± 0.46*	1.30±0.30***
1.00	-	75.00	2.00 ±0.50**	1.73 ± 0.25***
1.50	-	91.67	2.36±0.67***	2.05 ± 0.46***
2.00	-	91.67	2.91±0.54***	2.34 ± 0.59***
2.50	-	81.33	2.20±0.42***	1.93 ± 0.43***
-	0.50	66.67	1.88 ± 0.35	1.39 ± 0.25***
-	1.00	75.00	2.33±0.50***	1.84 ± 0.25***
-	1.50	100.0	3.50±0.52***	2.53 ± 0.31***
-	2.00	91.67	2.82±0.40***	2.39 ± 0.20***
-	2.50	81.33	2.30±0.48***	1.84 ± 0.21***

Note: *, ** and *** indicates level of significant at *p> 0.05 i.e. non significant, **p ≤ 0.05, *** p ≤ 0.01 significant.

Table 2 Effect of auxins in half strength MS medium supplemented with (IBA or NAA) on *in-vitro* root induction and growth from isolated in vitro shoots of *H. radiatus*

IBA (mg/l)	NAA (mg/l)	Response (%)	Average number of roots (mean ± SD)	Average root length (centimetre) (mean ± SD)
0.0	0.0	41.67	2.80 ± 0.84	3.22 ± 0.52
0.5	-	83.33	4.00±0.94***	3.47 ± 0.63*
1.0	-	75.00	3.00 ± 0.71*	2.87 ± 0.47*
1.5	-	50.00	2.50 ± 0.55*	2.53±0.44***
-	0.5	100.0	6.50±0.90***	3.58 ± 0.64*
-	1.0	91.67	5.18±0.75***	2.92 ± 0.54*
-	1.5	91.67	4.27±0.65***	2.58 ± 0.40**

Note: *, ** and *** indicates level of significant at *p ≥ 0.05 i.e. non significant, **p ≤ 0.05, *** p ≤ 0.01 significant.

Rooting of regenerated shoot

For any micropropagation protocol, successful rooting of microshoots is a pre-requisite to facilitate their establishment in soil. Regenerated shoots of about 2-3 cm.

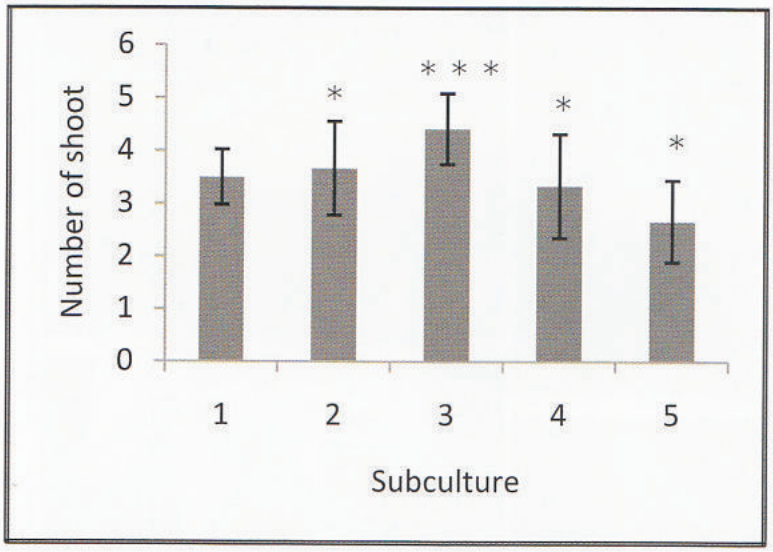
long were excised and transferred to half strength MS medium supplemented with different concentrations of auxins to investigate the effect of auxins on root induction (Table-2).

Reduced strength of basal medium is commonly used for rooting of adventitious shoot (Sivanesan and Jeong, 2007) and so was employed in this study. Roots started to emerge from the cut end of the shoots within 12 days of transfer to rooting medium. The MS medium devoid of plant growth regulators is also capable of root formation *in vitro* (figure 1-D). Around 41.67 % of cultures responded to form 2.80 ± 0.84 roots per shoot with average length 3.22 ± 0.52 centimeters. Data was recorded after 45 days (Table 2).

Figure 1. Micropropagation of H. radiatus **from nodal explants of field grown plant.**

A) Bud breaking from nodal explants on MS medium supplemented with 1.50 mg/l of BAP. B) Shoot multiplication on MS medium supplemented with 1.50 mg/l of BAP after third subculture. C) *In vitro* rooted shoots on half strength MS medium supplemented with mg/l IBA, arrow indicates callusing along with root. D). Formation of microshoots (MSH) at the base along with root on half strength basal medium. E) In vitro rooted shoots on half strength MS medium supplemented with 0.50 mg/l NAA.

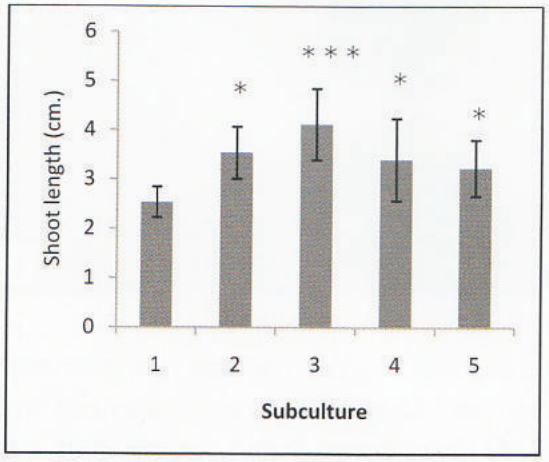
Figure 2 : Effect of successive transfer of mother cultures on shoot multiplication of *H. radiatus*



Note: *, ** and *** indicates level of significant at *p ≥ 0.05 i.e. non significant, **p ≤ 0.05, *** p ≤ 0.01 significant.

One interesting result found in basal medium without auxin, is the formation of microshoots along with root in this medium (Figure 1- D). This may be because of the accumulation of some amount of cytokinin which helps in shoot formation from preexisting meristem or cells are committed to form shoots due to extensive subculture in cytokinin rich medium. The best response with 100 % root induction and an average 6.50 ± 0.90 roots per shoot after 45 days of culture was achieved on a ½ strength MS basal medium supplemented with 0.50 mg/l NAA (figure 1-E). Concentration of 0.50 mg/ l IBA also yielded a significant number of roots with callusing at the cut end (Figure 1-C).

Figure 3 **:** Effect of successive transfer of mother culture on shoot length of *H. radiatus*



Note: *, ** and *** indicates level of significant at *p ≥ 0.05 i.e. non significant, **p ≤ 0.05, *** p ≤ 0.01 significant.

Conclusion

The present study describes for the first time protocol for a rapid and efficient method for direct regeneration of *H. radiatus*. Considering the medicinal importance of this plant, the micropropagation protocol developed and described here offers a potential system for rapid multiplication and germplasm conservation.

Acknowledgement

We are thankful to Dr. A.S. Reddy, plant taxonomist from BRD School of Biosciences, Sardar Patel University for identification of the plant and Mr. Sandip L. Patel, Research Scholar at BRD School of Biosciences for the collection of plant materials and Mr. Vinay R. Patel, Research scholar at BRD School of Biosciences, for photography.

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