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Effects of Additives in Shoot Multiplication and Genetic Validation in *Swertica chirayita* Revealed through RAPD Analysis

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Abstract

Enhanced *in vitro* caulogenesis has been tested in *Swertia chirayita* on MS supplemented with BAP, IAA, IBA, NAA and additives like adenine sulfate and d-glutamine with 2.5% sucrose. The best *in vitro* caulogenesis was observed in MS fortified with 4.43 μM BAP in combination with IAA (0.8 μM) that resulted increase in shoot multiplication rate. The multiplication and elongation of shoots were further enhanced by the addition of adenine sulfate (0.007%) that resulted in the further increase in multiplication fold. The addition of adenine sulfate reduced the use of other cytokinins with different auxins reported in the previous studies on *Swertia chirayita*. The study suggests adenine sulfate as a primary assimilable reduced nitrogen source for enhancing the shoot multiplication and elongation in *Swertia chirayita*. RAPD markers were employed to check the genetic variation among the clonal stock that resulted in 97% similarity.

Introduction

Swertia chirayita is a medicinal plant indigenous to temperate Himalayas found at the altitude of 1,200 - 3,000 m from Kashmir to Bhutan, and in the Khasi hills in Meghalaya (Kirtikar and Basu 1984, Duke 2002). It has an increasing pharmaceutical demand due to immense medicinal properties (Sharma et al. 2011), but the low viability and germination percentage of the seeds and the necessarily delicate field handling of the seedlings are some of the factors that

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discourage agro-technology development and, hence, commercial cultivation of *Swertia chirayita.* This has led to the wild extraction of plant from its natural habitat and has threatened its number in nature.

In situ propagation of the medicinal plants is limited by several factors (Jabeen et al. 2006). The potential of medicinal plants out of their natural habitat is poor. Propagation through seeds is limited with seed dormancy and depends upon rainfall, soil moisture, time of sowing etc. while vegetative propagation for these species is slow and time consuming to achieve large scale production of propagules. However, scaling up of any *in vitro* propagation protocol is severely hindered due to incidences of somaclonal variations. Hence, a stringent quality check in terms of genetic fidelity of progeny becomes essential. These variations arise in the cultured cells as a response to the stress imposed in tissue culture conditions.

Plant hormones have always done best with lower concentrations but the protocols used by the workers in past for *Swertia chirayita* have shown the effect of combinations of various cytokinins and auxins on the shoot multiplication and subsequent rooting but hardly any of them related it to the use of cytokinins and auxins along with the readily assessable additives containing adenins and amino acids that could possibly reduce the high concentration of the hormones and the negative effect of high concentration on the meristematic cell division including events of basal callusing and reduced *in vitro* rhizogenesis. Thus present investigation was taken up for assessing the effects of additives for enhanced rate of shoot multiplication (caulogenesis) in *Swertia chirayita* that has not been reported so far for this species. The clonally propagated shoots were analyzed by RAPD analysis for assessing the genetic purity.

Materials and Methods

The disease free young juvenile plants of *Swertia chirayita* grown *ex situ* were selected and collected from Hitech Nursury, Deovan, Chakrata (1400 meters), India, during the month of July. The flowering plants were dried and prepared herbarium was submitted to Botanical Survey of India, Northern Regional Centre, Dehradun (BSD) for identification up to species level and plants were given the Accession No. 113342 and identified as *Swertia chirayita*. Nodal segments measuring 5 - 8 mm in length were cut and sterilization was done as described by (Srivastava et al. 2010). The *in vitro* raised shoots were used as explant. Clonal authenticity of shoots was checked for each variable composition by random selection after each subculture and compared with the mother explant for variation through PCR-RAPD.

The basal media comprised of the mineral salts and organic nutrients of the MS containing 2.5% sucrose, solidified with 0.8% agar (Hi-Media, India) supplemented with different concentrations and combinations of plant growth hormones and additives. The pH of all media was adjusted to 5.8 ± 2 before autoclaving at 1.1 Kg/cm^2. All the glassware were used of Borosil, India and chemicals were of analytical grade (HiMedia, E. Merch and Banglore Genei). Cultures were incubated at 24 ± 2ºC under a photoperiod of 16h light/8 hrs dark with a light intensity of 45 μE/m^2/s. Subculturing was performed at an interval of four weeks. Each treatment was replicated 24 times and all experiments were repeated at least thrice. Genomic DNA was extracted from *in vitro* raised plants and mother plant using the protocol described by our research group (Sharma et al. 2010). *In vitro* grown shoots were randomly selected from multiplication and elongation experiments and the different hormonal (additives) interactions for assessing the genetic identity and fidelity of the clonal stock. The isolated DNA was quantified and all the samples were brought down to a uniform concentration of 20 ng/μl to be used as template DNA for PCR

A total of 30 decamer primers were used for PCR amplification. The PCR amplification was carried out in 25 l of reaction mixture in PCR tubes, in Master Cycler® Thermalcycler (Eppendorf). The optimized reaction mixture contained 2.5 M of each dNTP (Banglore Genei), 25 mM $MgCl_2$, 25 m primer, 20 ng DNA template and 1 unit of *Taq* DNA polymerase (Banglore Genei). The reaction was overlaid with mineral oil and amplification was performed. The amplification was performed by initial denaturation of template DNA at 94ºC for 1 min followed by 38 cycles of 1 min at 94ºC, 1 min at 35ºC, 2 min at 72ºC and final cycle having the final extension time of 8 min at 72ºC.

Results and Discussion

Explants cultured on MS supplemented with growth regulators alone or in combinations with different sets of concentrations, showed varied response that was highly significant with respect to the length and number of shoots buds obtained per explant. The effect of BAP (0.44 - 13.29 μM) in MS and MS (free of BAP) as control towards axillary bud proliferation was significantly influenced by the concentra-tion of growth regulator (BAP) in the medium. A number of 7 - 11 shoots were observed on MS supplemented with BAP (4.43 μM) after four weeks of culture (significant at 0.1% level). The number of shoots as well as the length of shoots decreased with the increase and decrease of the BAP concentration from 4.43 μM (Fig. 1A). The effect of BAP was found to be favorable with respect to the induction and number of shoots in initial phase of culture establishment. The results were found to be in agreement with the

previous reports that have showed a significant effect of BAP and other cytokinins for axillary bud proliferation (Joshi and Dhawan 2007). The shoot propagules obtained from the initial phase of culture establishment were subjected to study the effect of BAP alone and in combination with IAA (0.8 - 20 μM), IBA (0.49 - 12.3 μM) and NAA (0.54 - 13.4 μM) for determining the optimum rate of shoot multiplication and elongation. The combination of BAP with IAA, NAA and IBA showed a significant variation for shoot regeneration and number. Out of the three auxins the best number of shoots were observed in MS supplemented with BAP (4.43 μM) in combination with IAA (0.8 μM). This combination gave the higher multiplication fold as compared with BAP alone (27 shoots) and the length of the shoots was observed to be 2.70 cm. On an average 28.67 shoots were obtained with MS supplemented with BAP (4.43 μM) and 2.46 μM NAA with a length of 2.07 cm. Thirty one shoots were recorded for IBA (2.46 μM) but the length of the shoots was comparable to BAP + IAA i.e. 2.67 cm.

Effect of additives like adenine sulphate in concentration of 0.005 to 0.010% was tested in combination with BAP (4.43 μM) for best *in vitro* caulogenesis after determining the optimal cytokinins and auxins levels for shoot bud induction, multiplication and quality of shoots (Fig. 1C). Maximum number of healthy and profuse shoot emergence was observed in MS along with BAP (4.43 μM) supplemented with 0.007 % adenine sulfate (Fig. 1D). The medium supplemented with adenine sulfate showed a significant increase in the rate of shoot multiplication, showing highest numbers of shoots and highest shoot length as compared to control and other concentration tested and were significant at 0.1 % level. Effect of sucrose concentration was also studied to analyze the effect of adenine sulfate supplemented medium and 2.5% was found to be optimum for highest shoot multiplication (Data not shown). The concentration (2.5%) was most effective as compare to higher concentrations of sucrose as it produced highest number of shoots (42) and length (4.13 cm) (significant at 0.1% level) and less incidences of basal callusing. Among all the combinations, the best shoot multiplication was observed in BAP (4.43 μM) supplemented with 0.007% adenine sulphate, while the combination of NAA, IAA and IBA comparably resulted in the lowest multiplication. The superiority of BAP has been suggested over other cytokinins and auxins on medicinal plants (Dave et al. 2003, Martin 2003). There are several reasons for preferred use of BAP and slow degradation may be the one so that it can be autoclaved easily. The superiority of BAP has been established in this report while earlier reports on shoot multiplication and elongation on *Swertia chirayita* suggests the use of combinations of various cytokinins with BAP as Kn and 2iP along with auxin concentration and other components like CH and KNO_3 (Chaudhuri et al. 2007,

Wawrosch et al. 1999, Wang et al. 2009). The effects of higher concentration of cytokinins have been reported to reduce the number of shoots generated per explant (Wawrosch et al. 1999, Vincent et al. 1992). In the present study the use of higher concentrations of Kn and 2iP has been replaced with the supplementation of adenine sulphate at much lower concentration resulting in

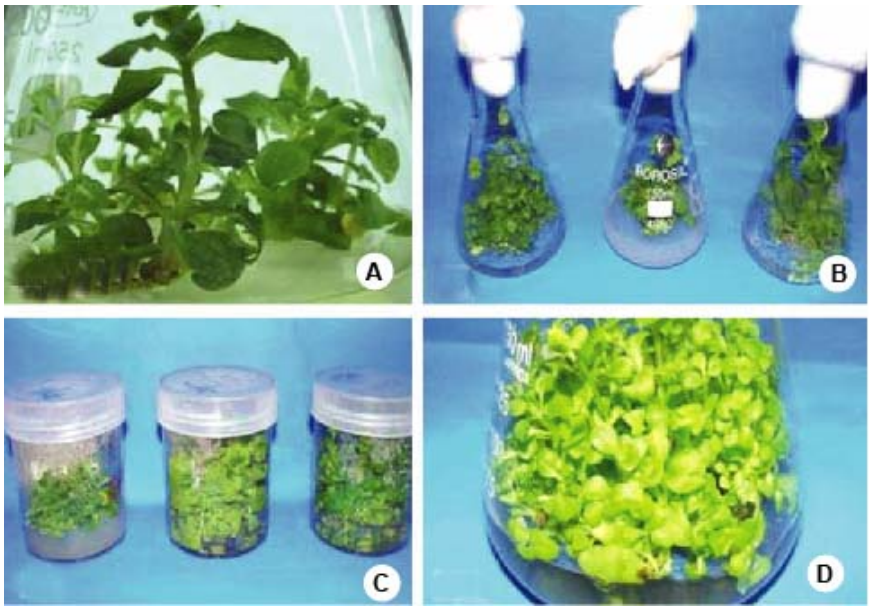

Fig. 1. *In vitro* shoot multiplication in *Swertia chirayita*. (A) Shoot multiplication in MS supplemented with BAP (4.43 μM). (B) Comparison of shoot multiplication in BAP with different auxins. (C) Comparison of MS basal with MS supplemented with BAP (4.43 μM) and adenine sulfate (0.001 - 0.010%). (D) Best shoot multiplication in MS basal with MS supplemented with BAP (4.43 μM) and adenine sulfate (0.007%).

higher ten-fold multiplication that has not been reported so far in *Swertia chirayita*. Also reduction of cytokinins and auxins supplementation results in the occurrence of reduced adventitious bud formation, basal callusing and hence reducing the chances of somaclonal variations. Despite the fact that nitrates and ammonium salts have been widely used in the conventional *in vitro* protocols as nitrogen source, many reports suggest the use of reduced nitrogen forms particularly adenins and amino acids as these additives can adjust the nitrogen utilization of *in vitro* grown culture by regulating primary nitrogen assimilation and can improve cell proliferation as well as regeneration in many species (Vasudevan et al. 2004, Shrivastava et al. 2008).

Present study was conducted to detect any event of genetic variation arisen among cultured plants due to the effect of the above mentioned cultural conditions. The components of PCR amplification were optimized for reproducible amplification of DNA using 30 random decamer primers. The primer sequences used for amplification are shown in Table 1. Three to five cloned plant propagules from multiplication tests of tissue culture protocol were selected for assessing the genetic fidelity. A total of 112 reproducible products were screened that showed no variation throughout the steps of micropropagation, justifying that the concentration of variable components imposed no variation or mutation in the *in vitro* raised shoots. The number of scorable bands with all primer ranged from 1 band to 7 bands in M-119 (Fig. 2A). The fragment size ranged from 0.3 to 1.5 kb with highest bp size in OPB-4 (Fig. 2B). The *in vitro*

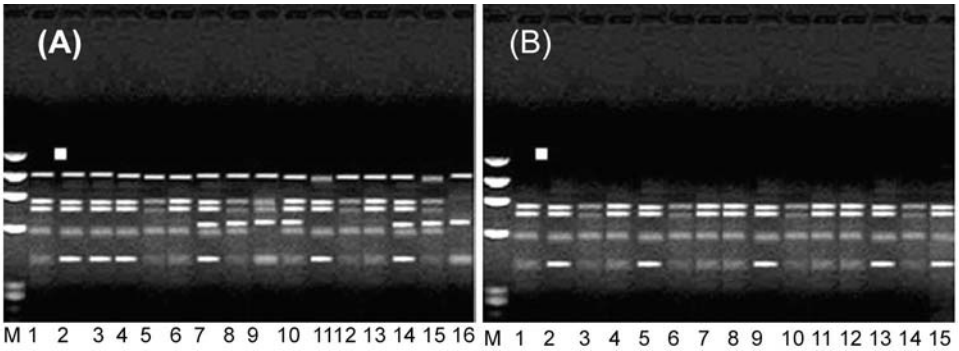

Fig. 2. RAPD fingerprinting pattern generated with primers M-119 (A) and OPB-4 (B) among the *in vitro* raised regenerants when compared with the donor plant. Lane (M) molecular weight marker φX174 *Hae* III digest lambda DNA, lane (1) donor plant, lane (2 - 16) micropropagated shoots.

raised shoot propagules were assessed for genetic analysis through cluster analysis along with the parental plants with *Swertia cordata* as an outlier and showed about 97% of similarity with the mother ex-plant of *Swertia chirayita*, and thus show high uniformity throughout the stock cultures with various treatments of media components during *in vitro* caulogenesis (Fig. 3).

The observed genetic uniformity might be ascribed to the employment of nodal segments and axillary buds as starting explant for micro-cloning as proposed for axillary and apical bud explants in many plant systems (Chaudhuri et al. 2007). Also the use of additives in present study lowers down the concentration of phytohormones and hence lowers the chances of somaclonal variations. Since *in vitro* multiplication or caulogenesis is one of the key parameters for the successful tissue culture protocol, genetic validation of the

Table 1. RAPD primers used and amplicons generated by 30 decamer primers in *Swertia chirayita*.

Sl. No.	Primer sequence (5′- 3′)	Total No. of amplicons	Mol. wt.t (kb)
1	M-29- CCGGCCTTA C	4	0.4 - 1.2
2	M-31- CCGGCCTTC C	3	0.3 - 1.2
3	M-33- CCGGCTGGA A	4	0.3 - 0.8
4	M-119- ATT GGG CGA T	7	0.3 - 1.0
5	M-66- GAG GGC GTG A	0	0.0
6	M-82- GCG CCC GAG G	3	0.5 - 0.9
7	M-83- GGG CTC GTG G	4	0.3 - 1.2
8	M-110- TAG CCC GCT T	4	0.3 - 1.0
9	M-116- TAC GAT GAC G	5	0.4 - 1.2
10	M-122- GTAGACGAG C	0	0.0
11	M-169- ACGACGTAG G	0	0.0
12	M-186- GTGCGTCGC T	1	0.7
13	M-188- GCTGGACAT C	3	0.4 - 0.7
14	M-191- CGATGGCTT T	1	0.4
15	M-131- GAA ACA GCG T	6	0.3 - 1.2
16	M-132- AGG GAT CTC C	5	0.3 - 1.2
17	M-147- GTG CGT CCT C	4	0.3 - 0.9
18	OPA-2- TGCCGAGCTG	4	0.3 - 1.0
19	OPA-8- GTGACGTAGG	4	0.4 - 1.2
20	OPA10- GTGATCGCAG	3	0.6 - 1.0
21	OPA 4- AATCGGGCTG	6	0.4 - 1.4
22	OPB 1- GTTTCGCTCC	4	0.3 - 0.7
23	OPB 2- TGATCCCTGG	4	0.3 - 1.1
24	OPB 3- TGATCCCTGG	3	0.4 - 0.7
25	OPB 4- GGACTGGAGT	6	0.4 - 1.5
26	OPB 5- TGCGCCCTTC	2	0.4 - 0.5
27	OPB 6- TGCTCTGCCC	4	0.3 - 0.9
28	OPB 7- GGTGACGCAG	7	0.5 - 1.4
29	OPB 8- GTCCACACGG	4	0.3 - 1.0
30	OPA 1- CAGGCCCTTC	5	0.4 - 1.2
Total number		112	

cloned stock further ensures the genetically identity and relatedness with the parental plants. As production of genetically true to type plants is a prerequisite for the conservation and propagation programme for the endangered plant

species. Thus present protocol provides the clear genetic status of the *in vitro* raised propagules where the fidelity of the plants was maintained throughout the stock.

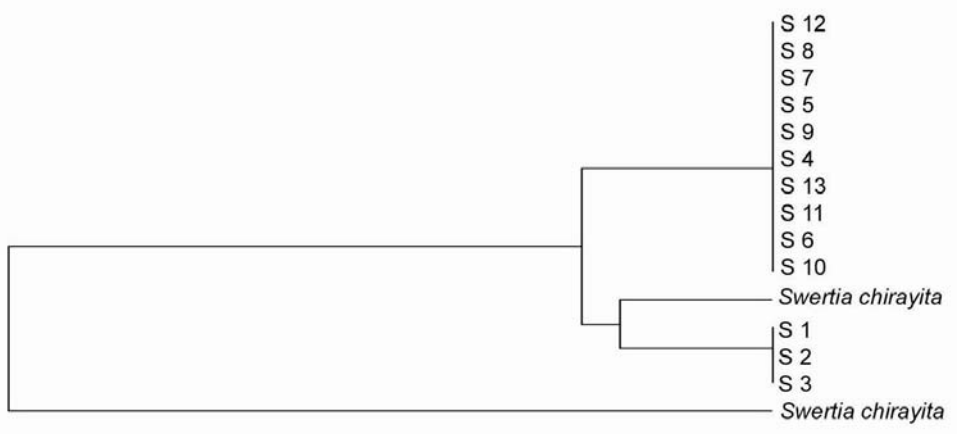

Fig. 3. Dendogram generated by UPGMA cluster analysis for similarity matrix obtained by Jaccard's genetic distance for 13 random samples obtained during *in vitro* caulogenesis.

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