J. Plant Develop.
19(2012): 29-40

EFFICIENT MICROPROPAGATION AND EVALUATION OF GENETIC FIDELITY OF *IN VITRO* RAISED PLANTS OF *COMMIPHORA WIGHTII* ARN. (BHANDARI) – A MEDICINALLY IMPORTANT RED-LISTED SPECIES OF ARID REGIONS

PARMAR ASHOK KUMAR[1], KANT TARUN [1]

Abstract: A refined and an efficient protocol for *in vitro* clonal propagation of *Commiphora wightii*, a red-listed desert plant of medicinal importance, has been developed from nodal segment of mature plant. Nodal segments from new branches having 6-7 nodes were excised after discarding the initial 5-6 cm terminal portion and were surface sterilized with 2.5% NaOCl (sodium hypochlorite), (v/v). MS medium [MURASHIGE & SKOOG, 1962] with different concentrations of BAP (6-benzylaminopurine) was used alone and in combinations with IAA (indole-3-acetic acid), NAA (α-naphthalene acetic acid), Kn (kinetin) and other additives for shoot induction. Best bud break response (84.5%) was obtained on MS medium supplemented with 8.88 µM BAP, 0.57 µM IAA and additives (50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid and 25 mg l^{-1} adenine sulphate) within 2 weeks of inoculation. The micro-shoots were subcultured and maintained for further elongation on the same medium for 4 weeks. Best shoot multiplication was obtained on same medium as used for shoot initiation. Best rooting was obtained when the shoots were initially given a 24 h pulse treatment in liquid MS medium supplemented with 4.92 µM IBA (indole-3-butyric acid) and 5.71 µM IAA under dark condition, followed by transfer to semi-solid half-strength hormone-free MS medium supplemented with 2% (w/v) sucrose and 0.5% (w/v) activated charcoal. High (86.7%) percent rooting was achieved after 4-5 weeks with 3-4 multiple adventitious roots of 5-6 cm length. These *in vitro* raised well rooted plantlets were acclimatized in a two step manner. During *in vitro* hardening step, the survival was 61.5% and during *ex vitro* hardening step it was 100%. Hardened plants (10-12 cm in height) were transferred to polythene-bags filled with mixture of soil and FYM in the ratio of 2:1 (v/v) and were kept in 75% agro-net shade for one month, where they gained height up to 60 cm. Five month old hardened plants were planted in open field condition for evaluation of these tissue cultured raised plants. There is cent percent survival of these field grown plants over period of two years with no visible morphological abnormalities. Genetic fidelity test was carried out for these *in vitro* raised plants by using RAPD primers (OPA and OPN). Uniform banding pattern was observed in all plants without any polymorphism.

Key words: tissue culture, axillary shoot, rooting, hardening, oleo-gum-resin

Introduction

Commiphora wightii (Arn.) Bhandari (family Burseraceae) is well known by its vernacular names guggal (Hindi), guggul (English), guggulu (Sanskrit), etc. *C. wightii* is an endangered medicinal plant of arid and semi arid regions of Rajasthan, Gujarat, Maharashtra, Karnataka and Madhya Pradesh in India and also found Pakistan to gulf countries. It is now on the verge of extinction and predominant reasons for its fast diminishing population are over-exploitation, poor natural germination rate and slow growth. It has been listed in IUCN Red Data Book under Data Deficient Category [IUCN, 2012]. The species yield a valuable oleo-gum-resin from its shoot. The resin has

[1] Forest Biotechnology & Molecular Biology Laboratory, Forest Genetics and Tree Breeding Division, Arid Forest Research Institute, New Pali Road, Jodhpur 342005 – India; e-mail: tarunkant@icfre.org

tremendous value as cholesterol reducing agent and hence a favourite of Ayurvedic medicine industry [SATYAVATI, 1990]. In India, there is a gap between demand and supply of guggul gum and it is being imported from Pakistan and gulf countries moreover, the imported guggul gum is highly adulterated. The plant dies due to indiscriminate tapping for oleo-gum-resin [BHATT & al. 1989]. Natural regeneration is very poor and artificial propagation has limitations through seed as well as through stem cuttings [KANT & al. 2010a, 2010b]. Biotechnological tools have potential to overcome the problems of traditional methods of propagation for large scale production of high quality planting material, remove the gap between demand and supply in pharmaceutical market and also maintain the gene bank of this marvelous plant. *In vitro* propagation thorough axillary bud proliferation has tremendous scope of large scale production without losing the genetic identity of material.

Pioneer effort was made in this direction by BARVE & MEHTA (1993) through mature nodal segments of elite tree of *C. wightii*. Thereafter PRAJAPTI (2008) reported 40% bud break with only one shoot sprouted from each explant. These results depict that *C. wightii* tree is a recalcitrant and very slow growing species. An efficient protocol for *in vitro* clonal propagation of *C. wightii* is still a need for commercialization. Here we report a refined and efficient protocol for *in vitro* clonal propagation of *C. wightii* from nodal segments of mature plants, developed at Arid Forest Research Institute, Jodhpur with aim of selection of appropriate nodal segment explants, improvement in percent bud break response and multiplication rate, acclimatization and hardening of plantlets and growth performance in field condition. Morphological and Molecular characterization were done by collecting growth data from regular interval and by DNA finger printing of plants respectively.

Materials and methods

Plant material. Comiphora wightii nodal segments were collected from marked, visibly healthy trees growing in the natural population cluster in Kaylana region, Jodhpur district and Magliyavas, Ajmer district from where cuttings were brought, rooted and used as source of explants.

Explant selection and preparation. A young juvenile branch of mature *C. wightii* tree was categorized in three parts: immature, semi-mature and mature. Nodal segments from new branches having 6-7 nodes (semi-mature) were excised after discarding the initial 5-6 cm terminal portion (immature) of mature plant and used as an explant having 1-2 nodes.

Surface sterilization. Branches (15-20 cm) were washed under running tap water for 2 minutes to remove dirt and superfluous impurities. Explants were then shaken in 100 ml. RO water (produced from Millipore RiOS5) having 2 drops of tween-80 for 10 minutes, rinsed 3 times with sRO water (sterilized water from Reverse Osmosis). The cleaned explants were then treated for 10 minutes with a solution of 200 mg bavestein and 50 mg streptomycin in 100 ml sRO water with gentle shaking at 50 rpm and rinsed with sRO water once in a laminar flow clean air cabinet. Finally nodal segments were treated with 2.5% NaOCl (v/v) solution (5% available chlorine, Rankem) for 7-10 minutes and rinsed with sRO water thrice.

Autoclaving and growth room conditions. All media were adjusted at pH 5.8 and followed by autoclaving at 121 °C and 20 psi (137,900 pa) pressure for 15 minutes. All the cultures were aseptically inoculated and manipulated under a sterile laminar flow hood and incubated in tissue culture racks in an aseptic culture room having a temperature of 26 ± 2 °C, 16 h/d photoperiod and 1600 lux intensity light (via white cool florescent tubes).

Culture initiation. Sterilized nodal segments were vertically inoculated on autoclaved MS [MURASHIGE & SKOOG, 1962] medium supplemented with different concentrations of BAP (4.44, 8.88, 13.32, 17.75, 22.19 µM) and in combination with NAA (2.69 µM). Second treatment comprised of MS medium supplemented with different concentrations of BAP (4.44, 8.88, 13.32, 22.19 µM) and IAA (0.57 µM) with additives (50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} adenine sulphate) along with a positive control, earlier reported by BARVE & MEHTA (1993) on BAP (17.75 µM) and Kn (18.59 µM) with additives (glutamine 100 mg l^{-1}, thiamine HCL 10 mg l^{-1}, activated charcoal 0.3%). Further, most responsive treatments in terms of bud break response were tried on different media types MS, B5 [GAMBORG, 1968] and WPM [LLOYD & MCCOWN, 1968] media for suitability of the most responsive nutrient medium for improvement in terms of bud break response, length and numbers of shoots.

Multiplication & elongation of micro-shoots. After 3-4 weeks of inoculation, newly sprouted axillary buds were subcultured for multiplication on existing medium and on MS media supplemented with low concentrations of cytokinins (BAP and Kn) and combination with auxins (IAA and NAA) and with additives.

Rooting of micro-shoots. Micro-shoots were initially given a 24 h treatment in liquid MS and White's medium [WHITE, 1954] supplemented with 4.92 µM IBA and 5.71 µM IAA under dark condition, followed by transfer to semi-solid half-strength hormone-free MS and White's medium supplemented with 2% sucrose and 0.5% activated charcoal.

Acclimatization and hardening of plantlets. Well-rooted plantlets were transferred to glass jam jars filled-up to quarter level with vermiculite and soaked with half strength MS salt solution. After 4-5 weeks when plantlets showed new growth the plastic cap of the glass jar was unscrewed gradually over a period of 2-3 days to reduce relative humidity in the jar, then finally the caps were removed completely from the jars on the third day. The plantlets were then transferred to thermocol (Styrifoam) cups containing FYM : vermiculite :: 1:5 soaked with one fourth strength MS salt solution at one-week interval. These plantlets were placed in mist chamber. After two weeks these were then transferred to FYM : soil :: 1:2 mixture in plastic plantation bags (polythene-bags) of size 9x9x36 cm (2916 cm^3). In mist chamber, 90 second misting at ten minutes interval was given to maintain RH between 85 to 95%. The temperature of mist chamber was maintained between 28-30 °C. After one month of transfer to poly-bags plantlets were transferred under green-75% agro-net shade.

Field trial of in vitro raised plants. A field trial of *C. wightii* tissue culture raised plants was established in July, 2010 at AFRI Campus, Jodhpur in field area of 175.77 sq.meter (1891.9 sq.ft.), and an elevation at 725ft. above sea level and an altitude of N26˚13.865’ and latitude of E073˚01825’ in pit (size 1.5 x 1.5ft.) having distance of 2 x 2 meter. Watering was done on a monthly basis initially for a year. Later no watering was done and established plants have been growing on rain fed conditions. The trial comprised of 8 plants originated from axillary shoot proliferation based pathway (reported here) as

well as 42 plants somatic embryogenesis based pathway (results not reported here) and 10 from seedlings for comparative growth performance evaluation. Growth data (height, collar diameter, number of leaves, primary and secondary branches) are being collected at regular three month intervals.

Genetic fidelity test. Fresh semi mature leaves were collected from field trial of tissue culture raised *C. wightii* plants and from mother plant for DNA extraction and purification. DNA was isolated by following the protocol developed by SAMANTARAY & al. (2009) for *C. wightii* Arn. (Bhandari). RAPD analysis was performed based on the protocol of SAMANTARAY & al. (2009, 2010) by using six highly polymorphic randomly and arbitrarily 10-base primers (OPA 04, OPA 09, OPA 20, OPN 06, OPN 16 and OPN 20) synthesized from Xcelris genomics Labs Ltd., Ahmedabad (Gujrat) [WILLIAMS & al. 1990].

Statistical analysis. Experiments were set up in completely randomized design (CRD). Each treatment consisted of 15 replicates. Experiments were repeated thrice. The data were collected every 3-4 weeks and all requisite parameters were taken for data analysis. The data were analyzed statistically using SPSS ver 8.0 (SPSS, Chicago, IL). Significance of differences among means was compared by using analysis of variance and Duncan Multiple Range Test (DMRT) at $P \leq 0.05$.

Results and discussions

Plant materials. Abundance of undifferentiated cells in immature and semi-mature portion of young juvenile branch helps in establishing the culture quickly as compare to well differentiated mature portion of branch. This concept was kept in mind for selection of appropriate nodal segment explants which resulted in high percentage of bud breaks response. BARVE & MEHTA (1993) also suggested that mature nodal explants were collected after 8 days of excision of apical buds which resulted in high percent bud break response.

Explants sterilization. A study was carried out on responsiveness of different explants (nodal segment, leaf, internodes and semi mature fruits) to sterilization procedures for *C. wightii* by using NaOCl. It was concluded that semi-mature nodal segments treated with 2.5% NaOCl solution (5% available chlorine) for 7-10 minutes sterilize the explants up to 80-90%, which is comparable to the efficiency of $HgCl_2$ (mercuric chloride), Hence 2.5% NaOCl solution was used as an effective surfactant.

Culture initiation. MS medium supplemented with different concentration of BAP and combination with NAA were tried out for establishing *in vitro* culture, all concentrations resulted in axillary bud break, but the maximum of 46.7% bud break response was observed on MS medium supplemented with BAP (4.44 µM), NAA (2.69 µM), followed by 40.0% on MS medium supplemented with BAP (8.88 µM), NAA (2.69 µM). New shoots sprouted from each nodal area was not more than two which represent the effect of different concentrations of BAP and along with NAA were not significant. On the other hand, shoot length difference in two treatments was significant but not more than 0.5 cm in length which depict that the *C. wightii* is a recalcitrant and very slow growing species and these hormonal combination are not effective in further improvement of bud break response (Tab. 1).

Tab. 1. Effect of BAP and combination with NAA on bud break response

PGRs concentrations (μM)	Response (%)	Mean no. of shoots with ±SE	Mean shoot length (cm) with ±SE
©	6.7	1.00±00	0.20^{b}±0.0
4.44 BAP	20.0	1.33±0.3	0.20^{b}±0.0
8.88 BAP	13.3	1.00±0.0	0.50^{a}±0.0
13.32 BAP	20.0	1.67±0.3	0.40^{a}±0.1
17.75 BAP	6.7	1.33±0.3	0.40^{a}±0.1
22.19 BAP	6.7	1.00±00	0.50^{a}±0.0
4.44 BAP + 2.69 NAA	46.7	1.80±0.2	0.50^{a}±0.0
8.88 BAP + 2.69 NAA	40.0	1.50±0.3	0.48^{a}±0.0
13.32 BAP + 2.69 NAA	26.7	1.25±0.3	0.50^{a}±0.0
17.75 BAP + 2.69 NAA	26.7	1.33±0.3	0.17^{b}±0.2
22.19 BAP + 2.69 NAA	33.3	1.67±0.3	0.50^{a}±0.0

DMRT, Mean followed by different letters differ significantly at $p \leq 0.05$; © = control; SE, standard error.

Second treatment involved MS medium supplemented with different concentration of BAP and IAA with additives (50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} adenine sulphate) along with a positive control media which was reported by BARVE & MEHTA (1993) viz. MS medium supplemented with BAP (17.75 μM) and Kn (18.59 μM) with additives (100 mg l^{-1} glutamine, 10 mg l^{-1} thiamine HCL, 0.3% activated charcoal), (Tab. 2). After the end of 2nd week of inoculation, it was observed that a maximum 84.5% bud break response on BAP (8.88 μM) and IAA (0.57 μM) with additives (50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} adenine sulphate) was obtained. There was no further increase in the next two weeks (Tab. 2). After 3-4 weeks, these PGRs combination was found most responsive in terms of bud break with maximum shoot sprout (2.23±0.1 with 1.64±0.2 cm length of shoots). This response was better than the results earlier reported by BARVE & MEHTA (1993) and also used as positive control in the experiment. This positive treatment resulted in 75% bud break response, shoots sprouted up to 2.18±0.1with 1.59±0.2 cm length of shoot. These results clearly showed the effect of additives on bud break response and improve in the shoot number and shoot length (Fig. 1*a*, 1*b*). The positive effects of different additives on bud break response and shoot multiplication were reported earlier by BARVE & MEHTA (1993), SHEKHAWAT & al. (1993), NEELAM & CHANDEL (1992), KOMALAVALLI & RAO (1997). However our results are better than all earlier reports.

Tab. 2. Effect of BAP, IAA and additives on bud break response

PGRs concentrations (mg l^{-1})	Response (%)	Mean no. of shoots with ±SE	Mean shoot length (cm) with ±SE
©	75.0	2.18^{a}±0.1	1.59^{a}±0.2
BAP 1.0+IAA 0.1+ additives^{A}	54.8	1.67^{bc}±0.2	0.62^{b}±0.1
BAP 2.0+IAA 0.1+ additives^{A}	84.5	2.23^{a}±0.1	1.64^{a}±0.2
BAP 3.0+IAA 0.1+ additives^{A}	47.6	1.78^{bc}±0.2	0.94^{b}±0.2
BAP 5.0+IAA 0.1+ additives^{A}	28.6	1.33^{c}±0.3	0.77^{b}±0.1

DMRT, Means followed by different letters differ significantly at $p \leq 0.05$; ©- BAP 4.0+ Kn 4.0+ additives^{B} (BARVE & MEHTA, 1993); **A-** 50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} adenine sulphate; **B-** 100 mg l^{-1} glutamine, 10 mg l^{-1} thiamine HCL, 0.3% activated charcoal; SE, standard error.

Further experiment was carried out on different media (MS, B5 and WPM) supplemented with best responsive plant growth regulators (PGRs) and with additives in terms of bud break response among the aforesaid experiments for establishing the best nutrient medium (Tab. 3).

Tab. 3. Effect of different media on bud break response on best responsive PGRs combination

Media		Response %	Mean no. of shoots with ±SE	Mean shoot length (cm) with ±SE	Mean no. of leaves with ±SE
MS	A	84.6	1.64^{b}±0.2	0.65±0.2	4.50±0.7
	B	73.1	1.78^{ab}±0.3	0.72±0.2	4.00±1.0
	C	38.5	1.17^{b}±0.1	0.56±0.1	4.25±0.6
B5	A	53.8	1.50^{b}±0.2	0.35±0.1	5.00±1.1
	B	34.6	1.60^{b}±0.4	0.54±0.1	6.40±1.4
	C	19.2	1.11^{b}±0.1	0.31±0.0	4.33±0.5
WPM	A	65.4	1.40^{b}±0.2	0.28±0.0	4.40±1.0
	B	53.8	2.40^{a}±0.2	0.44±0.1	4.20±0.4
	C	23.1	1.40^{b}±0.2	0.34±0.1	5.60±0.9

DMRT, Means followed by different letters differ significantly at p≤ 0.05; **A**- BAP (8.88 µM)+IAA (0.57 µM)+additives: 50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} adenine sulphate; **B**- BAP (17.75 µM)+Kn (18.59 µM)+additives: 100 mg l^{-1} glutamine, 10 mg l^{-1} thiamine HCL, 0.3% activated charcoal; **C**- BAP (8.88 µM)+NAA (2.69 µM); SE, standard error.

Multiplication & elongation of micro-shoots. After 3-4 weeks of inoculation, newly sprouted axillary buds were sub-cultured for multiplication on existing medium and on various MS media supplemented with lower concentrations of cytokinin (BAP and KIN) and with combination of auxins (IAA and NAA) and additives (Tab. 4). After 3-4 weeks, no micro-shoots multiplied and leaves turned yellow. Defoliation was clearly seen in all cultures.

Tab. 4. Effect of different cytokinin, auxins and additives on multiplication of newly sprouted microshoot

S. no.	Treatment (µM)	Mean no. of shoots with ±SE	Mean shoot length (cm) with ±SE
1.	4.44 BAP	1.20^{a}±0.1	0.44^{c}±0.0
2.	4.44 BAP+0.54 NAA	1.53^{ab}±0.1	0.36^{abc}±0.0
3.	4.44 BAP+0.57 IAA	1.60^{b}±0.1	0.46^{c}±0.1
4.	4.44 BAP+4.65 Kn+0.54 NAA	2.20^{a}±0.1	0.24^{a}±0.0
5.	4.44 BAP+4.65 Kn+0.57 IAA	1.87^{bc}±0.1	0.38^{abc}±0.0
6.	4.44 BAP+4.65 Kn+activated charcoal	1.80^{b}±0.1	0.39^{bc}±0.1
7.	4.44 BAP+4.65 Kn+additives(Th. HCl+ glu +ads + AC)	1.93^{bc}±0.2	0.30^{ab}±0.0
8.	4.44 BAP+4.65 Kn+ additives(Th. HCl+ glu+ ads+ AC)	1.87^{bc}±0.1	0.28^{ab}±0.0
9.	4.44 BAP+4.65 Kn+ additives (Th. HCl+ aa +ca+ AC)	1.73^{b}±0.1	0.28^{ab}±0.0
10.	4.44 BAP+0.54 NAA+ additives (Th. HCl+ glu+ ads)	1.67^{b}±0.1	0.26^{ab}±0.0

DMRT, Means followed by different letters differ significantly at p≤ 0.05; Th. HCl- Thiamine HCL (10 mg l^{-1}), glu-glutamine (100 mg l^{-1}), ads- adenine sulphate(25 mg l^{-1}), AC- activated charcoal (0.3%), aa- ascorbic acid (50 mg l^{-1}), ca- citric acid (25 mg l^{-1}); SE, standard error.

The micro-shoots were subcultured and maintained for further elongation on the same medium for 4 weeks (Fig. 1c). Maximum three shoots were observed per explant. Only well elongated micro-shoots were found suitable for rooting. PREECE (1995) suggested that the accurate concentration of nutrients in the medium removes the stress in explants and help to improve the *in vitro* performance, which can not be achieved solely by the use of plant growth regulators. Further refinement is still needed for multiplication of micro-shoot.

Rooting of micro-shoots. Micro-shoots of 2-3 cm length were initially given a 24 h treatment in liquid MS and White's medium supplemented with 4.92 µM IBA and 5.71 µM IAA under dark condition, followed by transfer to semi-solid half-strength hormone-free MS and White's medium supplemented with 2% sucrose and 0.5% activated charcoal. High (86.7%) percent rooting was achieved after 4-5 weeks with 2.85 ± 0.5 multiple adventitious roots of 6.46 ± 0.4 cm length (Fig. 1d) on half-strength hormone-free MS medium with 0.5% activated charcoal, while 46.7% rooting was observed on White's medium with 0.5% AC (Tab. 5). Earlier, BARVE & MEHTA (1993) also reported these hormonal combinations were found best for rooting of micro-shoots of *C. wightii*. The marked promoting effect of auxins (IBA and IAA) and darkness on adventitious root formation in micropropagated shoots of different species were clearly reported by FOURET & al. (1986), GEROGE (1993), MONTEUUIS & BON (2000) and influence of agar and activated charcoal on *in vitro* morphogenesis in plants were reported by YASSEEN (2001).

Tab. 5. Effect of different media on *in vitro* hardening of microshoot.

S. No.	Media	Rooting %	Mean root length (cm) with ±SE	Mean root/ shoot with ±SE
1.	MS medium	86.7	6.46±0.4	2.85±0.5
2.	White's medium	46.7	6.29±0.4	2.29±0.2

SE, standard error

Acclimatization and hardening of plantlets. Chemoautotrophic well-rooted plantlets were gradually hardened in different phases; *in vitro* hardening, *ex vitro* hardening in plastic cups at mist-chamber and in polythene-bags in green-shade house to make them ready for field transfer as complete photoautotrophic plants. The well-rooted plants (4-5 cm) were gently removed from the vessels, washed initially to remove adhered traces of the depleted medium and then washed for 5-10 min in autoclaved distilled water [THIRUVENGADAM & al. 2006]. Within 4-5 weeks, these plants gained 3-5 cm height and showed 62% survival rate during *in vitro* hardening in culture room (Fig. 1e). During *ex vitro* hardening plants in mist-chamber and green-shade house remained showed 100% survival and attained 10-12 cm height (Fig. 1f). After five months, plants attained average height of 60 cm. Finally robust and healthy plants were transferred to field at the onset of rains (monsoon) season for field performance evaluation (Fig. 2a, 2b).

Field trial of in vitro raised plants. A comparative field trial of *C. wightii* tissue culture raised plants was laid out for growth performance evaluation and comprised of *in vitro* raised plants derived from mature axillary shoots (nodal segments), somatic embryogenesis pathway and seed derived plants. Plants are growing well in the field condition from last two years with 100% survival (Fig. 2c), (Tab. 6). Mean growth data in

terms of height (123.6±5.7 cm); collar diameter (2.1±0.1 cm); number of leaves (406.3±44.8); primary (24.4±2.4) and secondary (11.8±2.2) branches after two years and three month indicate robust plants with good growth rates compared to seedling derived plants.

Tab. 6. Growth performance of axillary shoot derived plant in experimental field over period of two years and three months.

Years	Months	Mean height (cm.) with ±SE	Mean CD (cm.) with ±SE	Mean no of primary branch with ±SE	Mean no of secondary branch with ±SE	Mean no of leave with ±SE
2010	June-August	86.9^{b}±7.5	0.8^{e}±0.1	1.6^{f}±0.4	0.0^{e}±0.0	167.3^{b}±40.1
	September-November	99.8^{ab}±9.5	1.1^{d}±0.1	6.4^{e}±0.9	1.0^{e}±0.6	172.5^{b}±11.9
	December-February	103.6^{ab}±9.6	1.2^{d}±0.1	5.5^{e}±1.4	2.0^{e}±0.8	24.4^{d}±8.4
2011	March-May	104.4^{ab}±10.3	1.2^{d}±0.1	8.8^{de}±0.7	$6.8\pm^{bc}2.1$	14.4^{d}±7.9
	June- August	105.5^{ab}±9.7	1.2^{d}±0.1	10.1^{d}±0.9	13.1^{ab}±4.9	53.1^{d}±10.2
	September-November	108.1^{ab}±9.4	1.3^{d}±0.1	11.4^{cd}±0.9	15.8^{a}±4.3	118.1^{bc}±11.6
	December February	109.0^{ab}±9.0	1.4^{cd}±0.1	13.9^{bc}±1.2	17.1^{a}±3.6	43.1^{d}±3.7
2012	March- May	110.3^{ab}±8.5	1.6^{bc}±0.1	16.9^{b}±0.9	17.1^{a}±2.2	77.5^{cd}±5.9
	June- August	112.0^{ab}±8.5	1.9^{ab}±0.1	21.3^{a}±1.3	18.3^{a}±2.5	125.0^{bc}±4.6
	September-November	123.6^{a}±5.7	2.1^{a}±0.1	24.4^{a}±2.4	11.8^{ab}±2.2	406.3^{a}±44.8

DMRT, Means followed by different letters differ significantly at $p \leq 0.05$; CD, collar diameter; SE, standard error.

Fig. 1. Axillary Shoot Proliferation (a-f): a, b - Axillary bud sprouted from mature nodal segment; c - Multiplication and elongation of micro-shoot; d - Rooting in micro-shoots; e - *In vitro* hardening of plantlets; f - *Ex vitro* hardening of plantlets in plastic cups.

Fig. 2. Axillary Shoot Proliferation (a - c): a, b - Plantlets in mist-chamber and in green-shade house; c - *C. wightii* tissue culture plants over a period of two years.

*Genetic fidelity test***.** DNA was extracted from semi-mature leaves of tissue culture *C. wightii* field growing plants after one year plantation and from mother plant growing at its natural sites by following the protocol developed by SAMANTARAY & al. (2010). RAPD analysis was performed based on earlier screened highly polymorphic RAPD primers for *C. wightii* by SAMANTARAY & al. (2009, 2010); WILLIAMS & al. (1990). These six highly polymorphic randomly and arbitrarily 10-base primers (OPA 04, OPA 09, OPA 20, OPN 06, OPN 16 and OPN 20) were used for genetic fidelity test. All banding profiles from micropropagated plants through axillary shoot proliferation were monomorphic and similar to those of the mother plant (Fig. 3). The genetic stability of *in vitro*-propagated plants has been confirmed in many numbers of species like *turmeric* [SALVI & al. 2001], *Pinus thunbergii* [GOTO & al. 1998], *Gerbera jamesonii* [BHATIA & al. 2011], *Chlorophytum arundinaceum* [LATTOO & al. 2006].

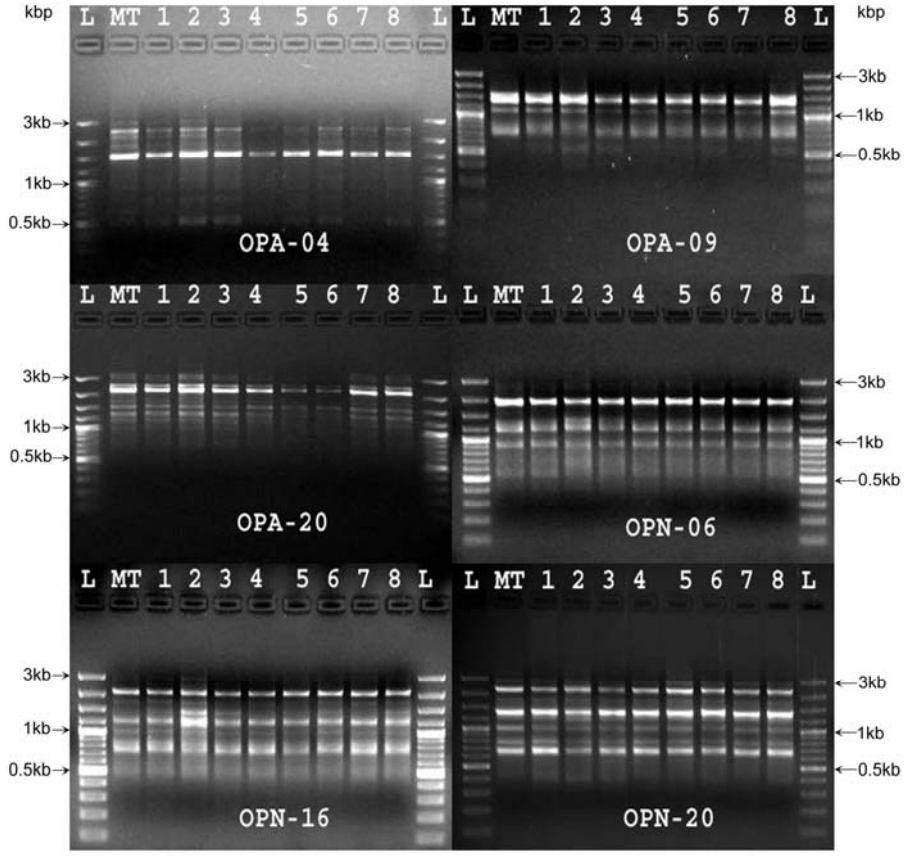

Fig. 3. Genetic fidelity test of axillary shoot proliferation derived tissue culture plants of *C. wightii* by using six highly polymorphic RAPD primers of Operon series (OPA-04, OPA-09, OPA-20, OPN-06, OPN-16 and OPN-20). L-100bp ladder, MT- Mother Tree (Mangliawas, Ajmer district, growing near AFRI Lab.)

Conclusions

Commiphora wightii is an important, endangered and sought after species due to its valuable oleo-gum-resin having tremendous medicinal importance. *In vitro* raised plants of *C. wightii* through axillary shoot proliferation was successfully regenerated at a good efficiency in this investigation by developing a complete protocol and their adaptability in field over a period of two years as proved. Evaluation of growth performance and genetic fidelity test of these *in vitro* raised plants has been successfully done. This is the first report on the field performance of tissue culture *C. wightii* plants and their genetic fidelity test. This protocol would provide an effective strategy for the conservation of this over-exploited medicinal plant. Multiplication rate of micro-shoots is still low. Bud break response has been improved compared to earlier reports. Hardening procedure which was not well established earlier has been fine tuned with the cent percent field performance of plants more than two years in open field.

Acknowledgements

This research work has been financially supported by Indian Council Forest Research & Education, Dehradun and National Medicinal Plants Board, New Delhi for which the authors are grateful. AKP also thank Council of Scientific & Industrial Research for fellowship grant.

References

1. BARVE D. M. & MEHTA A. R. 1993. Clonal propagation of Mature Elite trees of *Commiphora wightii*. *Plant Cell Tiss. Org. Cult.* **35**(3): 237-244.
2. BHATIA R., SINGH K. P., SHARMA T. R. & JHANG T. 2011. Evaluation of the genetic fidelity of *in vitro*-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers. *Plant Cell Tiss. Org. Cult.* **104**: 131-135.
3. BHATT J. R., NAIR M. N. B. & MOHAN RAM H. Y. 1989. Enhancement of oleo-gum-resin production in *Commiphora wightii* by improved tapping technique. *Current Science.* **58**: 349-357.
4. FOURET Y., ARNAUD Y., MALDINEY R., SOTTA B. & MIGINIAC E. 1986. Relation entre rhizogenèse et teneur en auxine et acide abscissique chez trois clones de *Sequoia sempervirens* (Endl) issus d'arbres d'âge différent. C R Acad Sc Paris t 303, série III, **4**: 135-138.
5. GAMBORG O. L., MILLER A. & OJIMA K. 1968. Nutrient requirements of suspension cultures of soyabean root cells. *Exp. Cell Res.* **50**: 151-158.
6. GEORGE E. F. 1993. Plant Propagation by Tissue Culture. Exegetics Ltd, Basingstoke, UK.
7. GOTO S., THAKUR R. C. & ISHII K. 1998. Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. *Plant Cell Rep*. **18**: 193-197.
8. IUCN. 2012. "IUCN Red List of Threatened species" Version 2012.2. http://www.iucnredlist.org. Cited at 04 December 2012.
9. KANT T., PRAJAPATI S. & PARMAR A. K. 2010b. Efficient micropropagation from cotyledonary node cultures of *Commiphora wightii* (Arn.) Bhandari, An endangered medicinally important desert plant. *J. Plant Develop.* **17**: 37-48.
10. KANT T., TOMAR U. K., PRAJAPATI S. & PARMAR A. K. 2010a. *In vitro* propagation as a viable conservation strategy for *Commiphora wightii*. *Conservation evidence.* **7**: 94-99.
11. KOMALAVALLI N. & RAO M. V. 1997 *In vitro* micropropagation of *Gymnema elegans* W& A, a rare medicinal plant. *Indian J. Exp. Biol.* **35**: 1088-1092.
12. LATTOO S. K., BAMOTRA S., SAPRU DHAR R., KHAN S. & DHAR A. K. 2006. Rapid plant regeneration and analysis of genetic fidelity of derived plants of *Chlorophytum arundinaceum* Baker – an endangered medicinal herb. *Plant Cell Rep*. **25**: 499-506.
13. LLOYD G. & MCCOWN B. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Intern Plant Pop Soc Proc.* (PCTOC). **30**: 421-427.
14. MONTEUUIS O. & BON M. 2000. Influence of auxins and darkness on *in vitro* rooting of micropropagated shoots from mature and juvenile *Acacia mangium. Plant Cell Tiss. Org. Cult.* **63**: 173-177.
15. MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum.* **15**: 473-497.
16. NEELAM S. & CHANDEL K. P. S. 1992. Effects of ascorbic acid on axillary shoot induction in *Tylophora indica* (Burm. F.) Merr. *Plant Cell Tiss. Org. Cult.* **29**: 109-113.
17. PRAJAPATI S. 2008. Tissue Culture Studies in an Important Medicinal Plant of the Arids and Semi-arids *Commiphora wightii* (Arn.) Bhandari. Ph.D Thesis, Forest Research Institute University, Dehradun, Uttarakhand.
18. PREECE J. E. 1995. Can nutrient salts partially substitute for plant growth regulators? *Plant Tissue Cult. Biotechnol.* **1**: 26-37.
19. SALVI N. D., GEORGE L. & EAPEN S. 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tiss. Org. Cult.* **66**: 113-119.
20. SAMANTARAY S., GEETHA K. A., HIDAYAT K. P. & MAITI S. 2010. Identification of RAPD markers linked to sex determination in guggal [*Commiphora wightii* (Arnott.)] Bhandari. *Plant Biotechno. Rep.* **4**: 95-99.

21. SAMANTARAY S., HIDAYAT K. P. & MAITI S. 2009. An isolation protocol of genomic DNA from *Commiphora wightii* (Arnott.) Bhandari: An endangered medicinal plant. *International Journal of Integrative Biology*. **6**(3): 127-131.
22. SATYAVATI G. V. 1990. Use of plant drugs in Indian traditional systems of medicine and their relevance to primary health care. In: Wagner H. & Farnsworth N. R. eds. Economic and medicinal plant research, London: Academic Press. **4**: 39-56.
23. SHEKHAWAT N. S., RATHORE T. S., SINGH R. P., DEORA N. S. & RAO S. R. 1993. Factors affecting *in vitro* clonal propagation of *Prosopis cineria*. *Plant Growth Reg.* **12**: 273-280.
24. THIRUVENGADAM M., REKHA K. T. & JAYABALAN N. 2006. An efficient *in vitro* propagation of *Momordica dioica* Roxb. Ex. Willd. *Philip Agric Sci.* **89**: 165-171.
25. WHITE P. R. 1954. The Cultivation of Animal and Plant Cells. 1st edition. Ronald Press. New York.
26. WILLIAMS J. G. K., KUBELIK A. R., LIVAK K. J., RFALSKI J. A. & TINGEY S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535.
27. YASSEEN Y. M. 2001. Influence of agar and activated charcoal on uptake of gibberellin and plant. *In Vitro Cell Dev. Biol.-Plant*. **37**: 204-205.