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Efficient somatic embryogenesis and molecular marker based analysis as effective tools for conservation of red-listed plant *Commiphora wightii*

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Article info:
Received: 28 March 2014
Accepted: 9 June 2014

ABSTRACT

A refined and high efficiency protocol for *in vitro* regeneration of *Commiphora wightii*, a red-listed medicinal plant of medicinal importance, has been developed through optimized somatic embryogenesis pathway. Cultures from immature fruits were induced and proliferated on B5 medium supplemented with 2.26 µM 2,4-D. Embryogenic calli were obtained and then maintained for extended periods by alternately subculturing on modified MS medium supplemented with 1.11 µM BAP, 0.57 µM IBA and with 0.5% activated charcoal or without PGR every 3-4 weeks. Cyclic embryogenesis was obtained. Late torpedo and early cotyledonary stages somatic embryos were regularly harvested from PGR-free modified MS medium. It was found that percent moisture available in culture containers play a critical role in maturation and subsequent germination of somatic embryos of *C. wighti*. Maximum germination of more than 80% was achieved through media recycling.

Somatic embryo derived plants (emblings) were acclimatized. After 5 months, acclimatized plants were out-planted in experimental field. These morphologically normal plants have been surviving for over 3 years. Molecular polymorphism was clearly evident when these plants were tested using RAPD primers, making the plants suitable for use in its species restoration program.

Key words: somatic embryo, embling, oleo-gum-resin, field performance, genetic stability

Introduction

Commiphora wightii (Arn.) Bhandari, a member of family Burseraceae, is a red-listed medicinal plant of arid and semi-arid tracts of Northern Africa to Northwestern India. It is now on the verge of extinction and predominant reasons for its fast diminishing populations are over-exploitation, poor natural regeneration and inherent slow growth (Parmar & Kant, 2012; Kant et al. 2010a, 2010b). 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 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 et al., 1989; Paliwal, 2010). 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 species.

Pioneering effort was made in this direction by Kumar et al. (2003) resulting in development of multiplication protocol for *C. wightii* using indirect somatic embryogenesis pathway. While selecting the immature seeds as explants they could get

DOI: 10.69085/jbb20142169

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only 17% of fruits having immature embryo and to give only 0.03% embryogenic callus production leading to a meagre 25% germinated somatic embryos. This study has provided a clue that somatic embryogenesis can be induced in this species. Further, Prajapati (2008) has indicated that some improvement can be made in earlier findings (Kumar et al., 2003). Using a modified selection procedure of fruit collection, 40% fruits yielded immature embryos and more than 30% cultures produces embryogenic callus. However, the frequency and speed of embryogenic calli initiation, multiplication, maturation and germination of somatic embryo in the above described study was not so good as to consider the protocol fit for scaling up. Scope for improvement was felt possible in various phases of the process of somatic embryogenesis. Here, we report a refined and high frequency protocol for *in vitro* regeneration through indirect somatic embryogenesis of *C. wightii* from immature seed explants, including establishment of field trial and analysis of genetic stability of the regenerated plants.

Materials and Methods

Plant material

Generally, three types of fruits were observed on mature plants of *C. wightii* twice a year (summer: April-June and winter: October-December). These were immature (green), semi-mature (slight green) and mature (red, ripened fruits). Only immature fruits were collected from marked, visibly healthy plants of *C. wightii*, growing at the non-irrigated sites (Kumbatia enclosure, Kaylana, Jodhpur district and Charbhuja town, Rajsmand district) and at the irrigated sites (AFRI nursery, Jodhpur and 7 years old cuttings obtained from Mangliyawas Ajmer district growing in AFRI main campus, Jodhpur) in India.

Explant selection and preparation

Immature fruits were selected as a source of explant. These immature fruits were categorized according to size (small, medium and large) on the basis of weight, length and volume of settled and floating fruits by following the water submergence selection process optimized by Prajapati, (2008). Small size settled immature fruits served as the best source of green immature embryos in large numbers. These were used to establish the cultures (Figure 1a).

Surface sterilization

The settled small size fruits were washed under running tap water for 2 minutes to remove dirt and superfluous impurities. Fruits were then shaken in 100 ml RO water having 2 drops of tween-80 for 10 minutes, rinsed 3 times with sRO (sterilized reverse osmosis) water. To avoid bacterial and fungal contamination, fruits were treated for 15-20 minutes with a solution of 100 mg Bavestein and 50 mg streptomycin in 100 ml sRO water with gentle shaking and rinsed with sRO water once in a laminar flow clean air cabinet. Finally, immature fruits were treated with 5% NaOCl (v/v) solution (Rankem) for 5-7 minutes and rinsed with sRO water thrice. Surface sterilized immature fruits were then cut vertically, and the immature embryos were scooped out from locules. These were inoculated in autoclaved media for developing embryogenic cultures.

Autoclaving and growth room conditions

All media were adjusted at pH 5.8 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

Immature embryo explants obtained from small settled immature fruits were used for embryogenic culture initiation. B5 medium (Gamborg et al., 1968) supplemented with 2.26 µM 2,4-D was used for culture initiation (Prajapati, 2008). After 3-4 weeks, non-embryogenic calli were subcultured on medium with same composition for callus proliferation. Embryogenic callus, with different stages of embryos, were seen after subculturing callus on PGR-free B5 medium.

Multiplication and development of somatic embryos

Initiated embryogenic calli with different stages of embryos (proembryo, globular, early and late torpedo, early and late cotyledonary) were multiplied on modified MS (Murashige & Skoog, 1962) medium supplemented with PGRs (1.11 µM BAP and 0.57 µM IBA) and 0.5% activated charcoal for 3-4 weeks and thereafter subcultured on PGR-free modified MS medium supplemented with 0.5% activated charcoal.

Maturation of somatic embryos

Only late torpedo and early coteyledonary stages somatic embryos were used in maturation experiments. Effect of abscisic acid, osmotica (sorbitol, manitol and poly ethylene glycol), gelling agent (agar), sucrose concentrations, absence

DOI: 10.69085/jbb20142169

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of light, low temperature, M-shaped paper bridge and depleted medium were tested to achieve good maturation. Well matured, dehydrated and elongated white somatic embryos were harvested for germination.

Germination of somatic embryos

Matured somatic embryos were used for germination of somatic embryos. Abnormal germination of somatic embryos was also noticed in few instances. Effect of activated charcoal, gibberellic acid (GA_3), L-glutamine, L-arginine, gelling agent (agar), sucrose concentrations, absence of light, low temperature and M-shaped paper bridge were tested for finding out best germination response. Cyclic and adventitious embryogenesis was seen on junction of root and shoot of the regenerated plantlet (emblings).

Acclimatization and hardening of emblings

Routinely harvested mature embryos upon germination and formation of complete emblings were acclimatized. Emblings were transferred to glass jam jars filled quarter level with vermiculite and soaked with half-strength MS solution. After 4-5 weeks when plantlets showed new growth the plastic caps of the glass jars were unscrewed gradually over a period of 2-3 days to reduce relative humidity in the jar. The caps were removed completely from the jars on the third day. The emblings were then transferred to thermocol (closed-cell extruded polystyrene foam) cups containing vermiculite and farm yard manure (FYM) in ratio of 5:1 and placed in mist chamber. One fourth strength MS salts solution at one-week interval was used to water the emblings. After 3-4 weeks these were transferred to FYM:soil (1:2) mixture in plastic plantation bags (polythene-bags) of size 9x9x36 cm (2916 cm^3). These were then subjected to 90 second misting at 10 minutes interval (RH between 85 to 95%) in a mist chamber. The temperature of mist chamber was maintained between 28-30°C. After one month, emblings were transferred to green agro-net (proving 75% shade).

Field trial of in vitro raised plants

An open field trial of 50 plants was laid out in July, 2010 at AFRI Campus, Jodhpur in an area of 175.77sq.meter (1891.9 sq.ft.), at 2x2 meter spacing. Watering was done on a monthly basis initially during first year. Thereafter, no watering or fertilizer treatments were given to plants in fields. Established plants have been growing on rain fed conditions. Growth data (height, collar diameter, number of leaves, primary and secondary branches) are being collected at regular three month intervals for the last three years.

Genetic stability 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 et al. (2009) for *C. wightii* Arn. (Bhandari). RAPD analysis was performed based on the protocol of Samantaray et al. (2009, 2010) by using six highly polymorphic random and arbitrary 10-base primers (OPA 04, OPA 09, OPA 20, OPN 06, OPN 16 and OPN 20).

Statistical analysis

Experiments were set up in completely randomized design (CRD). Each treatment consisted of 15 replicates. Experiments were repeated thrice. 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 $P \leq 0.05$.

RAPD bands were scored visually as '1' for the presence and '0' for the absence of the band for each primer species combination for RAPD analysis. All the bands (Polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over/under estimation of the distance. Jaccard's coefficients of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficient generated by the Un-weight Pair Group method using arithmetic averages (UPGMA), (Sneath & Sokal, 1973) and Sequential Agglomerative Hierarchial Non-overlapping (SHAN) clustering. The statistical analysis was done using the software NTSYS-PC (Numerical Taxonomy System for Personal Computers, version 2.02i) (Rohlf, 1993).

Resolving power (Rp) of the RAPD primer was calculated according to Prevost & Wilkinson (1999). $Rp = \sum Ib_i$ where Ib_i (band informativeness) $= 1 - [2 \times (0.5 - P_i)]$, P_i is the proportion of samples containing the i^{th} band and Ib_i is the informativeness of the i^{th} band. The RAPD primer index was calculated from the polymorphic Index. The polymorphism information content (PIC) was calculated as $PIC = \sum_{i=0}^{n} P_i^2$ where, P_i is the band frequency of the i^{th} allele. Here, the PIC was considered to be $1-p^2-q^2$, where 'p' is the band frequency and 'q' is no band frequency (Ghislain et al., 1999). PIC value was then used to calculate the RAPD primer Index (RPI). RPI is the sum of the PIC of all the markers amplified by the same primer.

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Results and Discussion

Plant material

Visibly healthy mature plants of *C. wightii*, growing in the non-irrigated natural sites and in the irrigated sites were identified and selected for the present study. Environmental conditions and available resources on these sites seem to influence the growth of trees at each site. Some morphological differences in flowers, fruits, leaves and growth rate were visible between the two sites. Plants from both sites were therefore selected to ensure development of a protocol that is more universal.

Culture initiation

For improvement in embryogenecity response, only immature fruits were collected and categorized as small, medium and large sub-types. 'W*ater submergence selection process*' was used to separate the fruits as sinkers and floaters. Floaters were rejected as they were mostly found to have empty locules with small, improperly developed or sometimes no seed. Sinkers (small, medium and large) were cut and immature embryo were scooped out from locules and inoculated on B5 medium supplemented with 2.26 µM 2,4-D for 3-4 weeks. It was observed that the maximum numbers of immature embryos were obtained from small size immature settled sub-type in comparison of medium and large sub-types. These immature embryos gave the best callus induction response (95.6% callusing) followed by immature embryos obtained from medium and large sub-types (Table 1), (Figure 1b). This was a marked improvement in selection procedure of immature embryos compared to the earlier reports of Kumar et al. (2003) and Prajapati (2008). Therefore for callus initiation, only small settled immature fruits were used thereafter in all further experiments.

Table 1. *Effect of different size of immature fruits on callus initiation for somatic embryogenesis.*

Fruit size	Non-embryogenic calli (%)	Embryogenic calli (%)	Callus color and texture	Callus growth
Small	95.6	77.8	Dark brown, fragile	+++
Medium	77.8	57.8	Slight brown, smooth	++
Large	62.2	48.9	Slight brown, smooth	+

+ slow; ++ moderate; +++ fast; Number of explant-15; Replicates-3.

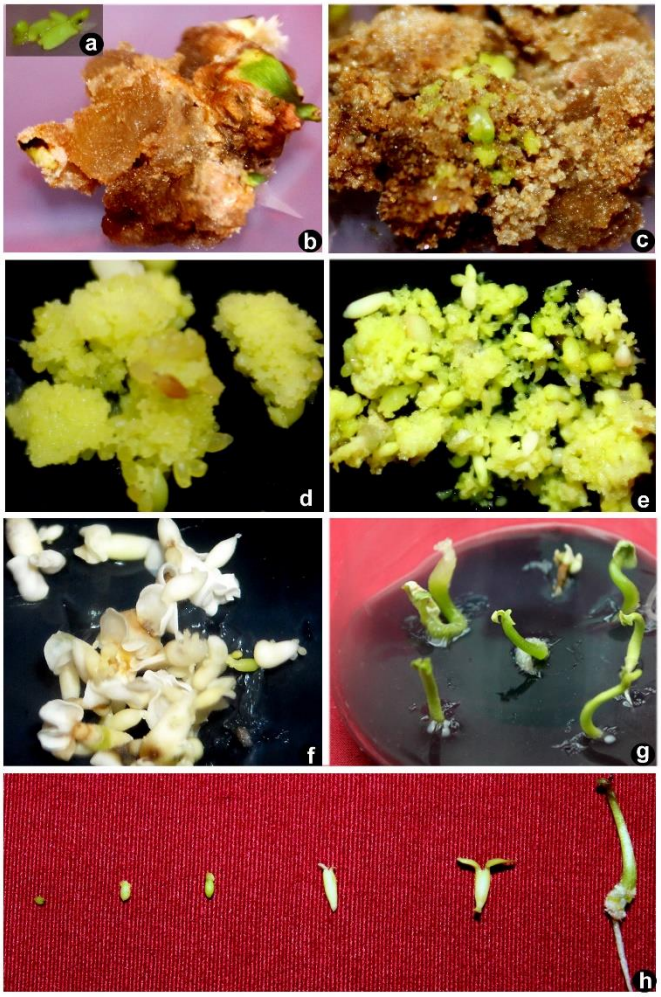

Figure 1. Somatic embryogenesis (a-h). ***a***- *Immature embryos obtained from small sized immature settled fruits;* ***b***- *After 3-4 weeks, callus obtained on B5 medium + 2,4-D (2.26 µM);* ***c***- *Conversion of non-embryogenic calli to embryogenic state on PGR-free B5 medium;* ***d***- *Multiplication on modified MS medium + BAP (1.11 µM) + IBA (0.56 µM) + 0.5% AC;* ***e***- *Multiplication on PGR-free modified MS medium + 0.5% AC;* ***f***- *Maturation on depleted medium (PGR-free modified MS medium + 0.5% AC);* ***g***- *Freshly prepared medium (PGR-free modified MS medium + 0.5% AC);* ***h***- *Different developmental stages of somatic embryos of C. wightii.*

Embryogenic callus initiation

The primary callus obtained on B5 medium supplemented with 2.26 µM 2,4-D from small sinker fruit derived immature embryos were found to be non-embryogenic. These non-embryogenic calli were subcultured on same initiation medium for 3-4 weeks for callus proliferation. The proliferated non-embryogenic calli was subcultured on PGR-

DOI: 10.69085/jbb20142169

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free B5 medium for 3-4 weeks resulting in conversion to embryogenic state. During the conversion period, small portions of dark brown non-embryogenic calli gradually converted to embryogenic calli and appeared green in color and friable in texture (Figure 1c). Sometimes anthocynin accumulation in calli was also observed in few cultures during multiplication and conversion procedure. Dass et al. (2008) has also reported the anthocyanin accumulation in callus culture of *C. wightii.* As a result callus turned slight pink or/reddish in colour but did not convert from non-embryogenic state to embryogenic. Kumar et al. (2003) and Prajapati (2008) have also reported B5 medium for embryogenic callus induction in comparison of others media.

Multiplication and development of somatic embryos

On B5 medium devoid of PGR, conversion of non-embryogenic calii to embryogenic calli was maximum. Embrogenic calli grouped and formed globular and further advanced stages of somatic embryos. Both types of calli (non-embryogenic and embryogenic) were further subcultured for proliferation and development towards further advanced stages (heart shaped, early and late torpedo, early and late cotyledonary) and finally germination to complete plants (Figure 1h).

Both types of calli (non-embryogenic and embryogenic) were subcultured on modified MS medium. In this medium, nitrogen content was reduced in form of ammonium ions [1.74 mM $(NH4)_2SO_4$] and nitrate ions (14.24 mM KNO_3), and also had reduced concentration of calcium (1.36 mM $CaCl_2$) and increased sulphur content for improvement in embryogenic response. A comparative study of contribution of elements/ions during medium was carried out using *'TC Lab'* software (ver. 1.0.0.1, Power Builder Enterprise series, USA). It was observed that concentration of elements/ions critically influenced the conversion of non-embryogenic calli to embryogenic state. It was observed that 75.9% increment in ammonium ions and 42.4% reduction in nitrate ions used in modified MS medium compared to B5 medium along with other nitrogen sources (33.4% reduction) led to initiation of embryogenic state of calli. Simultaneously, others elements/ions (B, Ca, Cl, H, Mg, Mn S, and Zn) were slightly increased while elements/ions (C, K, Na and O) were decreased in the modified MS medium that also seem to contribute to embryogenic calli initiation (Table 2), (Figure 1d, 1e). Nitrate and ammonium ions are important for plant differentiation (Halperin & Wetherell, 1965, Reinert et al., 1967) and for promoting embryo development (Joy et al., 1996). The total nitrogen content, the nitrate/ ammonium ratio and the inorganic/organic ratio, as well as the nature of the nitrogen source, have a considerable effect on the response of explants to somatic embryogenesis induction (Wetherell & Dougall, 1976; Grimes & Hodges, 1990; Mordhorst & Lorz, 1993).

Table 2. *Effect of percent element/ions concentrations level during somatic embryogenesis.*

Elements/ions	B5 (mM)	Modified MS (mM)	Modified MS in compare of B5 (mM)	
			+/-	%
N	**27.09**	**18.05**	**-9.04**	**-33.4**
NH_4	**2.03**	**3.57**	**+1.54**	**+75.9**
NO_3	**24.73**	**14.24**	**-10.49**	**-42.4**
B	0.05	0.1	+0.05	+100.0
C	4.78	4.43	-0.35	-7.3
Ca	**1.02**	**1.98**	**+0.96**	**+94.1**
Cl	**2.1**	**3.97**	**+1.87**	**+89.0**
H	**21.48**	**25.76**	**+4.28**	**+19.9**
K	**24.73**	**15.5**	**-9.23**	**-37.3**
Mg	1.01	1.5	+0.49	+48.5
Mn	0.06	0.1	+0.04	+66.7
Na	1.45	0.1	-1.35	-93.1
O	**93.31**	**66.21**	**-27.1**	**-29.0**
S	**2.22**	**3.42**	**+1.2**	**+54.1**
Zn	0.01	0.03	+0.02	+200.0

TC Lab' software (ver. 1.0.0.1, Power Builder Enterprise series, USA)

Trigiano et al. (1992) have also reported that reduction in nitrogen concentration in the form of ammonium ions influences somatic embryo quality and plant regeneration from suspension cultures of orchard grass. The effectiveness of various nitrogen sources in white spruce (*Picea glauca)* somatic embryogenesis has also been reported by Barrett et al. (1997). Fuentes-Cerda et al. (2001) optimized the nitrogen concentrations for embryogenesis between 3.75 and 15 mM nitrogen with a nitrate/ammonium molar ratio of 2:1 or 1:2 in *Coffea arabica.*

These embryogenic calli were maintained by alternately subculturing on modified MS medium supplemented with 1.11 µM BAP, 0.57 µM IBA and 0.5% activated charcoal or without PGR every 3-4 weeks. More than five years old embryogenic calli was maintained in our laboratory by this

DOI: 10.69085/jbb20142169

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alternate subculturing pattern. Precocious and abnormal germination was also observed during maintenance of embryogenic cultures. All well-developed advance stages like early and late torpedo stages, early and sometimes late cotyledonary stages were also observed. Abundance of proembryos and globular stages were frequently found in both medium (Table 3).

Table 3. *Long time maintenance of embryogenic calli on modified MS media.*

Media	A. Modified MS medium + IBA (0.1 mg/l) + BAP (0.25 mg/l) + activated charcoal (0.5%) + agar (0.8%) + sucrose (3%) pH 5.8 B. Modified MS media + activated charcoal (0.5%) + agar (0.8%) + sucrose (3%) pH 5.8
A	1. Brown, fragile and slightly green (proembryos) 2. Fast multiplication of globular and heart shape stages 3. Minimum conversion to advanced stages 4. Cyclic/adventitious embryogenesis after 3rd subculturing and 5. Also few with precocious germination
B	1. Globular embryos convert in heart, torpedo and cotyledonary stages 2. Late cotyledonary stages (white long mature sized of SEs) 3. Dehydrated and desiccated appearance of SEs 4. Regularly harvested for germination and 5. Maximum germination

We have found that activated charcoal (AC) plays an indispensable role in embryogenic cultures of *C. wightii*. The observation is supported by various reports (Paek & Hahn, 2000; Thomas, 2008; Carneros et al., 2009; Scherwinski-Pereira et al., 2010). Wang & Huang (1976) have reported the beneficial effects of activated charcoal on plant tissue and organ cultures. AC helps in SE induction, maturation or germination in various systems. Recently, Thomas (2008) has thoroughly reported the role of AC in tissue culture system. The effect of AC on *in vitro* nitrogen uptake had been evaluated in *Lagerstroemia indica*. Explants grown in medium with AC were capable of taking up both NO^{3-} and NH^{4+}, although NH^{4+} uptake was low and the pH of the medium was maintained between 5.5 and 6.0. In treatments which do not have activated charcoal, NH^{4+} uptake was preferential and the pH dropped to 3.1 (Eymar et al., 2000).

Similar to our findings in *C. wightii*, long term maintenance by establishing cyclic somatic embryogenesis has been reported in many species. Nair & Gupta (2006) reported secondary embryos formation from the radicular end of the primary somatic embryos which were originally derived from micropylar tissues of germinating seeds on growth regulator free SH medium in the absence of light, as also seen in our cultures.

Maturation of somatic embryos

Regularly, only late torpedo and early cotyledonary stages somatic embryos were harvested for maturation. Sometime precocious germinated somatic embryos were also seen and transferred to freshly prepared hormone free modified MS medium with 0.5% activated charcoal for elongation.

Outcomes of abscisic acid, osmotica (sorbitol, manitol and poly ethylene glycol), gelling agent (agar), carbon source (sucrose) concentrations, dark treatment, cold treatment, use of M-shaped paper bridge indicate that as such no one factor influenced the maturation of somatic embryos in *C. wightii* (Figure 2). It was found that after spending long time period on culture media (2 months or more), these somatic embryos of *C. wightii* underwent maturation. It was observed that percent moisture available in culture container played critical role in maturation of somatic embryos as well as germination of somatic embryogenesis of *C. wighti.*

By taking this critical factor into account, culture container containing modified MS medium supplemented PGR-free modified MS medium with 0.5% activated charcoal was used to examine the percent water content available after autoclaving and in culture room gradually for upto 9 weeks. Available water content was calculated every week for container containing medium. It was observed that during initial few days, culture container had higher percentage of water content and which later on gradually decreased. Between 1-2 weeks, available maximum water content and surrounding optimum temperature increased available moisture in culture container and significantly influenced germination of somatic embryos of *C. wightii*. At that point, maximum germination was achieved. After 5-6 weeks, available moisture in culture container became very low and significantly influenced maturation of somatic embryos of *C. wightii*. For that reason, 3-4 weeks older medium (already used medium for germination of somatic embryos) was used for maturation of somatic embryos. Recycling of media further helped in achieving higher rate of maturation and in

DOI: 10.69085/jbb20142169

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minimizing the reutilization of resources (Figure 1f).

The effects of drying rate and embryo moisture content on germination rate, root elongation, and plantlet regeneration were thoroughly examined by Bomal & Tremblay (1999). A fast drying rate to 4–5% embryo moisture content, obtained under 63% relative humidity (RH), was detrimental to germination and plantlet development. However, slower drying rates, obtained under 79–97% RH and generating 7–19% moisture content in the embryos, gave developmental responses. Synchronization of root emergence was improved only for embryos desiccated to approx 16% moisture content under 97% relative humidity (Bomal & Tremblay, 1999). Low moisture content as a means to achieve long term storage of dried somatic embryos has been reported for alfalfa (Senaratna et al., 1990), carrot (Lecouteux et al., 1992), and white spruce (Attree et al., 1995). For alfalfa, somatic embryos have been dried to 8–15% moisture content and stored for one year at room temperature without loss of vigor (Senaratna et al., 1990). Similarly, following sucrose pretreatment and desiccation leading to 25% moisture content, carrot somatic embryos were successfully stored at 4°C for 8 months (Lecouteux et al., 1992). Osmotic stress, through water depletion has been viewed as an important signal in directing proper embryo development in many plant species (Von-Aderkas & Bonga, 2000).

For this reason, 3-4 weeks old already used medium used was found to be the best for achieving synchronized and high frequency maturation of somatic embryos in our cultures of *C. wightii*.

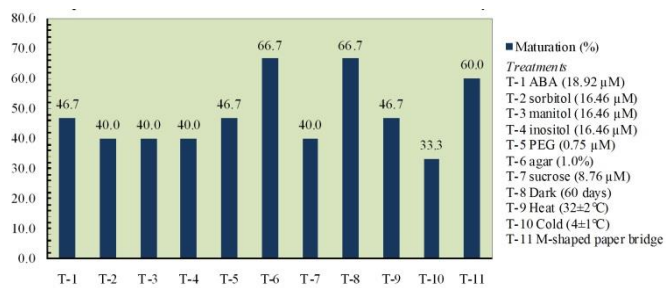

Figure 2. *Effect of different treatments maturation of somatic embryos.*

Germination of somatic embryos

Well matured, white, dehydrated, elongated somatic embryos were used for germination. These matured somatic embryos were harvested regularly from modified MS medium supplemented with 1.11 µM BAP, 0.57 µM IBA and with 0.5% activated charcoal and PGR-free modified MS medium supplemented with 0.5% activated charcoal. Different types of germination experiments involving use of activated charcoal, gibberellic acid (GA_3), L-glutamine, L-arginine, gelling agent (agar), carbon source (sucrose), dark treatment, cold treatment and M-shaped paper bridge were carried out with the aim of achieving improvement in germination frequency (Figure 3).

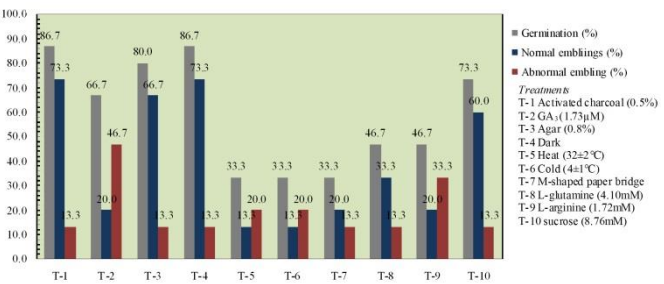

Figure 3. *Effect of different treatments on germination of somatic embryos.*

Available moisture content in culture container significantly enhanced germination of matured somatic embryos. Freshly prepared hormone-free modified MS medium with activated charcoal was used for germination. It was observed that during initial two weeks, maximum germination of more than 80% was achieved and later on germination frequency did not improve any further. After 2 weeks of germination, these were left for 1–2 weeks on same medium for elongation. After that period, emblings were safely removed and transferred for *in vitro* hardening and further acclimatization process. Emblings were easily taken out of the media having high water content. These depleted or already used medium was used again for maturation of somatic embryos. Recycling of media was done to save time and money without compromising percent maturation of somatic embryos, rather enhancing it (Figure 1g).

Acclimatization and hardening of somatic embryo derived plants (emblings)

Healthy and robust appearing emblings were transferred to sterilized culture bottle containing autoclaved vermiculite watered with half strength MS nutrient solution for *in vitro* hardening and for their further development and acclimatization. Hardening and acclimatization procedures for *C. wightii* were further improved and refined, which led to enhanced percent survival of plantlets.

Four to five weeks watering with half strength MS nutrient solution resulted in highest rate of survival (83%) during *in vitro* hardening of emblings. Significant increase in

DOI: 10.69085/jbb20142169

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growth rate of upto 5-7 cm height was achieved (Figure 4a, 4c). After completion of *in vitro* hardening, *ex vitro* hardening was carried out in mist-chamber, followed by green shade house and open nursery conditions. *Ex vitro* hardening of emblings was performed in plastic cups containing combination of vermiculite and FYM in ratio of 5:1. After 3-4 weeks, this combination gave best growth rate of upto 12 cm height of emblings with 100% survival rate.

After hardening in plastic cups, emblings were transferred in polythene-bags filled with mixture of FYM and soil in the ratio of 1:2 (v/v) and were kept in 75% agro-net shade for a month where they showed significant increase in growth. Finally, robust and healthy plants were transferred to field at the onset of rains (monsoon season) for field performance evaluation (Kant et al., 2010a; Parmar & Kant, 2012), (Figure 4b).

Field performance of in vitro raised Commiphora wightii plants

A comparative field trial of *C. wightii* tissue culture raised plants was laid out for growth performance evaluation. Field trial comprised of *in vitro* raised plants derived from axillary shoots proliferation pathway (8 plants) from mother plant Mangliawas, Ajmer district in India (A-series) as well as 42 plants from somatic embryogenesis (SE) pathway (among 27 SE plants from Charbhuja town, Rajsmand district (B- series) and 15 SE plants from Mangliawas mother plant, Ajmer district (C-series). *In vitro* raised *C. wightii* plants have been growing well in the field conditions for more than three years (from July-2010 to June-2013) with 100% survival. Flowering and fruit setting have been seen with multiple branches at basal portion of stem. Growth data of 27 plants derived from somatic embryogenesis based pathway of Charbhuja, Rajsmand (B-series) mother plant were collected from field. Mean growth data in terms of height (158.6±3.2cm.); collar diameter (2.29±0.0cm.); number of leaves (646±29.3); primary (38.21±1.7) and secondary (25.00±1.6) branches after two years and three month indicate robust plants with 100% survivability (Table 4).

Similarly, growth data of 15 plants derived from somatic embryogenesis based pathway of Mangliawas, Ajmer (C-series) mother plant were also collected from field. Mean growth data in terms of height (140.93±8.4cm.); collar diameter (2.35±0.1cm.); number of leaves (324.64±31.3); primary (38.00±1.9) and secondary (31.71±3.0) branches after two years and three month indicate robust plants with 100% survivability (Table 5), (Figure 4d).

Figure 4. Somatic embryogenesis (a-d). ***a****- In vitro hardening of somatic embryo derived plants (embling);* ***b****- Growth before plantation in field over 5 months in green-shade house;* ***c****- Well hardened in vitro raised embling;* ***d****- Growth of plants after more than 3 years with 100% survival rate.*

A second field trial of 110 SE derived plants was laid out in August, 2012. Somatic embryo derived *C. wightii* plants are growing well in the field condition from more than one year (from August-2012 to July-2013) with 100% survival.

Genetic stability test of somatic embryo derived Commiphora wightii plants

Banding pattern and amplification

***B-series*:** RAPD primers were tested for mother tree and 27 *in vitro* plants of *C. wightii* (B-series) for molecular characterization. Both polymorphic and monomorphic banding pattern were observed. Among them, maximum bands were observed as monomorphic. OPA 04 and OPN 16 reproduced maximum bands (196) with highest resolving power (Rp=13.143), while OPA 20 and OPN 06 reproduced minimum bands (140) with lowest resolving power (Rp=8.857 and Rp=9.214). Maximum RAPD primer index (RPI=0.202) was obtained in OPA 20 and minimum RAPD primer index (RPI=0.091) was obtained in OPA 09 and OPN 20 (Table 6), (Figure 5).

DOI: 10.69085/jbb20142169

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***C-series*:** RAPD primers were tested for mother tree and 15 *in vitro* plants of *C. wightii* (C-series) for molecular characterization. Maximum bends were showed monomorphic banding pattern. Maximum bands (120) were recorded in OPA 09 with resolving power (Rp=12.250) while minimum bands (64) were recorded in OPA 04 and OPN 06 with resolving power (Rp=7.000 and Rp=7.875). Maximum RAPD primer index (RPI=0.219) was obtained in OPA 03 and OPA 09, while minimum RAPD primer index (RPI=0.025) was obtained in OPN 20 (Table 7), (Figure 6).

Similarity index and RAPD tree constructions

Jaccard's similarity coefficient was followed for calculating similarity index. Similarity index value was compared for identifying genetic similarity with mother tree and among *in vitro* raised plants of *C. wighti.* The UPGMA dendrogram was obtained from the SHAN clustering analysis by using Jaccard's similarity coefficient of RAPD data.

Table 4. *Growth performance of somatic embryogenesis plants (Charbhuja, Rajsmand district) in experimental field over period of two years and three months.*

Years	Months	Mean height (cm) ± SE	Mean CD (cm) ± SE	Mean no. of primary branch ±SE	Mean no. of secondary branch ± SE	Mean no. of leave ± SE
2010	June-August	67.75±3.2^{e}	0.67±0.0^{f}	0.79±0.2^{f}	0.00±0.0^{d}	138.75±12.8^{cd}
	September-November	102.71±4.7^{d}	1.05±0.0^{e}	7.75±0.7^{e}	1.21±0.4^{d}	228.93±14.2^{c}
	December-February	112.00±4.5^{cd}	1.19±0.0^{d}	9.18±0.8^{e}	2.50±0.7^{d}	29.11±3.2^{e}
2011	March-May	113.41±4.5^{cd}	1.21±0.0^{d}	14.39±1.8^{d}	9.54±1.4^{c}	23.75±4.5^{e}
	June- August	115.21±4.3^{c}	1.22±0.0^{d}	18.50±1.4^{cd}	20.00±1.8^{b}	85.89±6.1^{de}
	September-November	118.61±4.2^{c}	1.24±0.0^{d}	18.96±1.5^{c}	21.82±1.8^{ab}	162.32±6.1^{cd}
	December February	144.21±4.1^{b}	1.93±0.1^{c}	31.50±2.2^{b}	25.39±1.7^{a}	341.61±63.0^{b}
2012	March- May	145.54±3.9^{b}	2.02±0.1^{c}	32.71±2.0^{b}	25.21±1.9^{a}	360.36±59.8^{b}
	June- August	147.64±3.5^{ab}	2.15±0.1^{b}	33.79±1.8^{b}	26.25±1.6^{a}	383.57±55.9^{b}
	September- November	158.50±3.2^{a}	2.29±0.0^{a}	38.21±1.7^{a}	25.00±1.6^{a}	646.43±29.3^{a}

DMRT; Means followed by different letters differ significantly at p≤ 0.05; CD-collar diameter; SE- standard error.

Table 5. *Growth performance of somatic embryogenesis plants (Mangliawas, Ajmer district) in experimental field-1over period of two years and three months.*

Years	Months	Mean height (cm) ± SE	Mean CD (cm) ± SE	Mean no. of primary branch ± SE	Mean no. of secondary branch ± SE	Mean no. of leave ± SE
2010	June-August	34.43±2.6^{d}	0.46±0.0^{e}	0.07±0.1^{e}	0.00±0.0^{c}	40.71±3.9^{de}
	September-November	62.29±5.2^{c}	0.80±0.0^{d}	2.00±0.7^{de}	0.29±0.3^{c}	104.29±8.6^{cd}
	December-February	78.43±7.3^{bc}	0.99±0.1^{c}	2.00±0.7^{de}	0.36±0.2^{c}	25.71±5.3^{e}
2011	March-May	79.29±7.4^{bc}	1.04±0.1^{c}	5.43±0.9^{d}	2.64±1.5^{c}	32.86±8.2^{de}
	June- August	83.07±7.2^{bc}	1.06±0.1^{c}	13.79±1.7^{c}	11.43±4.5^{b}	69.29±9.9^{de}
	September-November	88.14±7.1^{b}	1.14±0.1^{c}	15.50±1.7^{c}	11.93±4.6^{b}	152.86±8.5^{c}
	December February	134.86±8.4^{a}	2.09±0.1^{b}	32.14±2.4^{b}	18.93±2.4^{b}	464.29±41.4^{a}
2012	March- May	135.93±8.4^{a}	2.23±0.1^{ab}	33.79±2.5^{ab}	28.57±3.0^{a}	425.36±41.5^{a}
	June- August	135.93±8.4^{a}	2.23±0.1^{ab}	35.21±2.4^{ab}	30.93±2.9^{a}	259.64±29.6^{b}
	September- November	140.93±8.4^{a}	2.35±0.1^{a}	38.00±1.9^{a}	31.71±3.0^{a}	324.64±31.3^{b}

DMRT; Means followed by different letters differ significantly at p≤ 0.05; CD-collar diameter; SE-standard error.

DOI: 10.69085/jbb20142169

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Table 6. *Detail of RAPD analysis of 27 somatic embryogenesis in vitro plants of C. wightii (B-series, Charbhuja, Rajsmand district) using different six highly polymorphic primers.*

RAPD primer	Sequence of oligonucleotide	Approximate fragment size (bp)	Total bands	Polymorphic bands	Monomorphic bands	Resolving Power	RAPD primer index
OPA 04	5'-AATCGGGCTG-3'	2500-400	7	3	4	13.143	0.115
OPA 09	5'-GGGTAACGCC-3'	1400-500	6	2	4	11.429	0.091
OPA 20	5'-GTTGCGATCC-3'	2800-600	5	4	1	8.857	0.202
OPN 06	5'-GAGACGCACA-3'	2200-600	5	2	3	9.214	0.145
OPN 16	5'-AAGCGACCTG-3'	2300-700	7	2	5	13.214	0.106
OPN 20	5'-GGTGCTCCGT-3'	2800-600	6	2	4	11.429	0.091

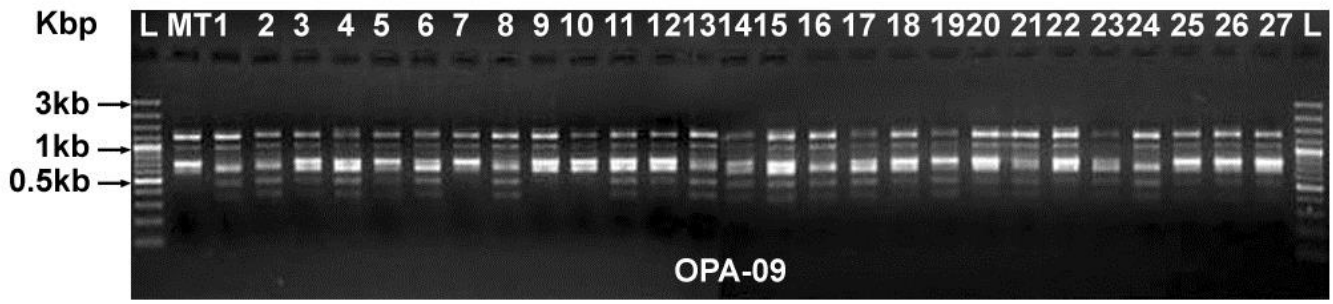

Figure 5. *Genetic fidelity test of somatic embryo derived tissue cultured plants (27 emblings) of C. wightii was done using six highly polymorphic RAPD primers of Operon series (showing representative banding pattern obtained from OPA-09). L-100bp ladder, MT- Mother Tree (Charbhuja, Rajsmand district, India).*

***B-series*:** Total 942 bands were amplified from 6 highly polymorphic RAPD primers form 27 somatic embryogenesis derived *in vitro* plants with mother tree (Charbhuja, Rajsmand district). Similarity index varied between 0.771 to 0.941 in 27 somatic embryo derived plants compared with mother tree. Maximum similarity index (SI=0.941) was observed for B9 plant with mother tree while minimum similarity index (SI=0.771) was observed for B19 plant. Range of variation was very high which clearly showed the genetic dissimilarity with mother tree (Figure 7). The dendrogram showed multiple branches. The genetic distance between somatic embryos derived *in vitro* plants and mother tree varied from 0.84 to 1.00. Plant B9 was found to be genetically the closest to the mother plant.

Table 7. *Detail of RAPD analysis of 15 somatic embryogenesis in vitro plants of C. wightii (C-series, Mangliawas, Ajmer district) using different six highly polymorphic primers.*

RAPD primer	Sequence of oligonucleotide	Approximate fragment size (bp)	Total bands	Polymorphic bands	Monomorphic bands	Resolving Power	RAPD primer index
OPA 04	5'-AATCGGGCTG-3'	2200-500	4	3	1	7.000	0.219
OPA 09	5'-GGGTAACGCC-3'	1600-300	7	4	3	12.250	0.219
OPA 20	5'-GTTGCGATCC-3'	2000-700	5	3	2	9.250	0.139
OPN 06	5'-GAGACGCACA-3'	2200-700	4	1	3	7.875	0.031
OPN 16	5'-AAGCGACCTG-3'	2300-700	5	3	2	9.000	0.180
OPN 20	5'-GGTGCTCCGT-3'	2700-600	5	1	4	9.875	0.025

DOI: 10.69085/jbb20142169

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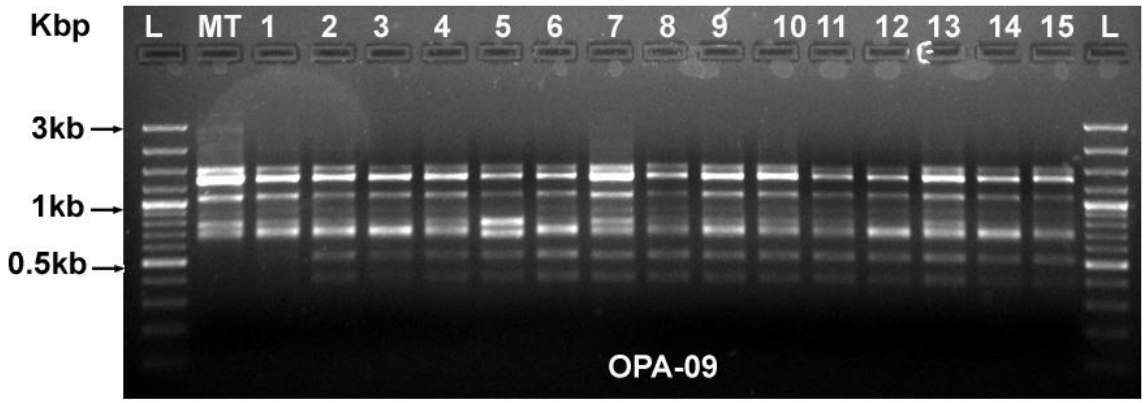

Figure 6. *Genetic fidelity test of somatic embryo derived tissue cultured plants (15 emblings) of C. wightii was done using six highly polymorphic RAPD primers of Operon series (showing representative banding pattern obtained from OPA-09). L-100bp ladder, MT- Mother Tree (Mangliawas, Ajmer district, India).*

***C-series*:** Total 442 bands were amplified from 6 highly polymorphic RAPD primers form 15 somatic embryogenesis derived *in vitro* plants with mother tree (Mangliawas, Ajmer district). Similarity index varied between 0.700 to 0.923 in 15 somatic embryogenesis derived plants compared with mother tree. Maximum similarity index (SI=0.923) was observed between C1 and the mother tree while minimum similarity index (SI=0.700) was observed between C2, C6 and C14 plants. Range of variation was very high which clearly showed the genetic dissimilarity with mother tree (Figure 6). The dendrogram showed multiple branches. Dendrogram showed that the genetic distance between somatic embryos derived *in vitro* plants and mother tree varied from 0.79 to 1.00. Plant C1 was found to be genetically the closest to the mother plant.

RAPD markers have been used to evaluate the genetic fidelity of plants regenerated from somatic embryos of *Vitis vinifera* and RAPD profiles revealed that regenerated plants were monomorphic and similar to those of the field grown donor plants (Yang et al., 2008) an at some time, RAPD markers have also been able to detect somaclonal variation among micropropagated plants of *Picea glauca* (Isabel et al., 1996), peach (Hashmi et al., 1997), sugarcane (Taylor et al., 1995). Rani et al. (2000) has reported the presence of genetic variability in the commercially well-established somatic embryogenesis-derived plants of this important *Coffea arabica.*

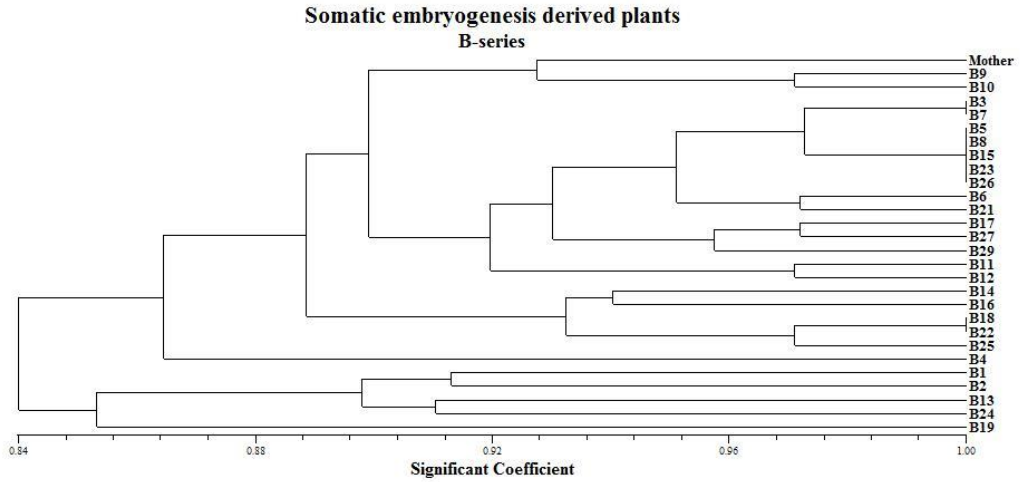

Figure 7. *Dendrogram showing genetic similarity between and within mother plant and 27 somatic embryo derived in vitro raised plants of C. wightii (B-series, Charbhuja, Rajsmand district, India) by cluster analysis of RAPD markers.*

DOI: 10.69085/jbb20142169

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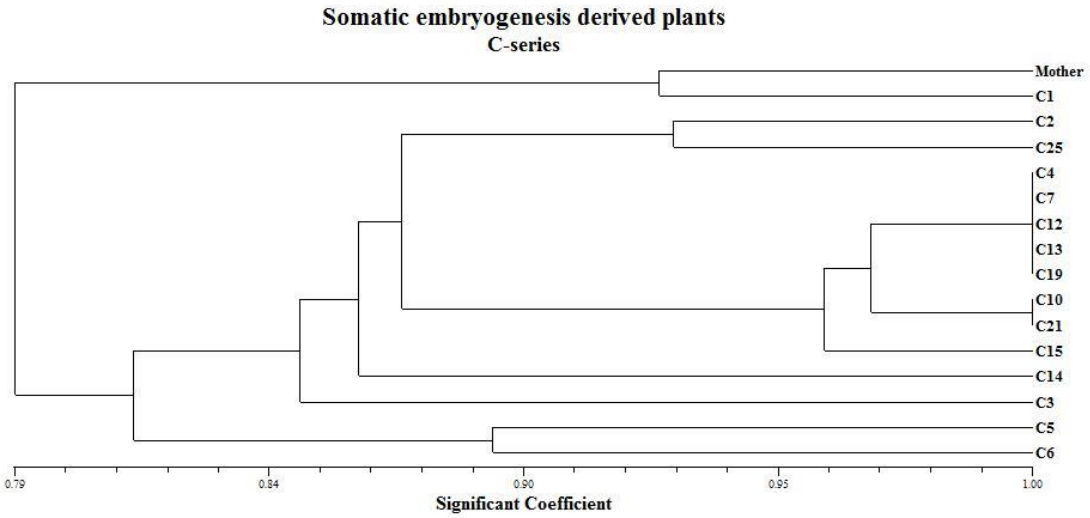

Figure 8. *Dendrogram genetic similarity between and within mother and 15 somatic embryo derived in vitro plants of C. wightii (C-series, Mangliawas, Ajmer district, India) by cluster analysis of RAPD markers.*

Conclusion

Commiphora wightii is an important, red-listed yet a much sought after species due to its valuable oleo-gum-resin having tremendous medicinal importance. *In vitro* raised plants of *C. wightii* developed through indirect somatic embryogenesis were successfully regenerated at a good efficiency under this investigation. Effectiveness of complete *in vitro* propagation protocol and adaptability of the hardened plants under field conditions over a period of more than three years has been proved successfully. Evaluation of growth performance and genetic similarity test of these *in vitro* raised plants has been successfully done. This is the first report on the long term field performance of tissue culture *C. wightii* plants and their genetic similarity test of somatic embryo derived plants on this species. This protocol would provide an effective strategy for the conservation of this over-exploited medicinal plant. Hardening procedure which were not well established earlier have been fine-tuned with hundred percent survival of the plants under field trial. Variation range was found to be high in somatic embryo derived plants which clearly show the genetic dissimilarity with mother plant. With a narrowing genetic base of this threatened species, using somatic embryo derived plants with wide variations for plantation purpose will aid in fast recovery of the specie in its natural habitat thereby ensuring species restoration.

Acknowledgement

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.

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