In vitro propagation as a viable conservation strategy for *Commiphora wightii*, an endangered medicinally important desert tree, India

Tarun Kant*, Uttar K. Tomar, Sushma Prajapati & Ashok K. Parmar
Biotechnology Laboratory, Forest Genetics and Tree Breeding Division, Arid Forest Research Institute, New Pali Road, Jodhpur 342005 India

*Corresponding author e-mail: tarunkant@icfre.org

SUMMARY

Commiphora wightii is an endangered tree of arid and semi-arid tracts of northern Africa to northwest India. It is an important medicinal plant well-known for its oleo-gum-resin with cholesterol reducing properties. However, it has been over-exploited such that it is on the verge of extinction in the Indian part of its range. The present study reports the use of tissue culture as a viable alternative to propagation via stem cuttings as well as seedlings for conservation of this valuable plant. The work presented describes the development of two tissue culture based pathways for plant production, their acclimatization and successful field transfer. Plants derived from *in vitro* propagation have been growing well under field conditions for over three years (April 2007 to August 2010). Flowering and fruiting has taken place, as would be expected in similarly sized wild plants, and plants have exhibited a good rate of growth.

The initiative has proven cost-effective in terms of producing plants from culture initiation stage to a hardened plant of size suitable for transplanting into the field; the cost of a single plant produced through a somatic embryogenesis pathway was about Indian Rupees (INR) 19 (equivalent to Pound Sterling (GBP) 0.26), while that produced through a cotyledonary node based protocol was INR 27 (GBP 0.37). The study clearly indicates the applicability and benefits of using tissue culture technology to assist in conservation of *C. wightii*.

BACKGROUND

Once a prominent species of the arid tracts of Rajasthan and Gujarat states (northwest India), *Commiphora wightii* is now on the verge of extinction over much of its Indian range and is listed as endangered (IUCN 2010). The predominant reasons for its fast diminishing populations are over-exploitation (tapping of woody shoots for its oleo-gum-resin), poor natural germination rate and slow growth rate. This resin is considered by some to have tremendous value as cholesterol reducing agent and hence a favorite of the Ayurvedic medicine industry. This has resulted in widespread indiscriminate tapping for the resin. The magnitude of the conservation problem facing *C. wightii* through this exploitation is greatly exacerbated by the fact that a plant after being tapped through deep cutting, usually dies within two to six months of a single tapping episode (Bhatt *et al* 1989, Paliwal 2010). It is not yet clear as to why plants die after tapping. Various theories exist, but none are scientifically proven. Several researchers are currently working to ascertain the reason for this, and only if this becomes known can strategies of sustainable gum tapping perhaps be implemented.

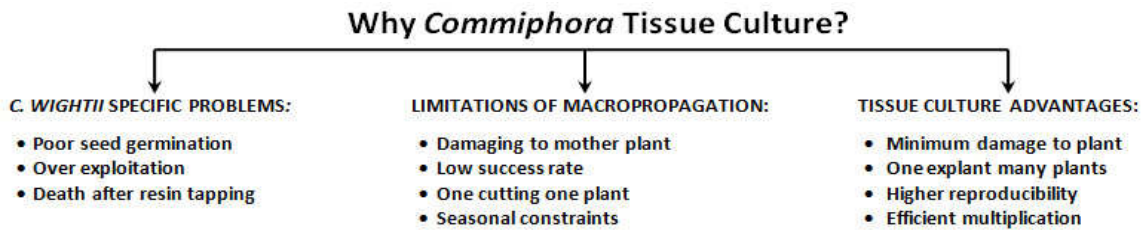

Figure 1. Summary of problems leading to decline of wild populations of *Commiphora wightii*, limitations of macro-propagation via stem cuttings and potential advantages of tissue culture based propagation.

The need of the hour however, is production of planting stock to enable species restoration efforts through transplanting initiatives. The current situation is that, the state forest departments (SFDs) of Rajasthan and Gujarat are not able to produce sufficient plants through propagation by seeds and stem cuttings to meet their own demands for plantation activities. In this situation, tissue culture technique can supplement such demands due to the high potential of mass multiplication at faster rate, though at slightly higher costs. These costs may be considerably reduced if protocols are further refined to make them more efficient and reliable. The benefits of tissue culture based mass multiplication verses propagation via cuttings are summarised in Figure 1.

Here, details of two micropropagation protocols (one somatic embryogenesis based and the other cotyledonary node based) developed at the Arid Forest Research Institute (AFRI), Jodhpur (Rajasthan) are presented. Plants obtained from these micrcroprogation techniques have been out-planted in an experimental field site to evaluate their performance and to establish the suitability of this system for *ex situ* conservation efforts for the species. In the longer-term, it is hoped that the plants produced through these protocols can be used in *C. wightii* species recovery programmes. Aims include both the bolstering of dwindling wild populations and potential establishment of cultivation activities to support incomes of local village communities traditionally involved in resin collection.

ACTION

Mother plants were selected from four sites in Rajasthan for the propagation experiments (conducted in 2002 to 2007, and currently ongoing): Kaylana area and JNV University (both in Jodhpur district); Mangliawas (Ajmer district); and Charbhuja (Rajasmand district).

Cotyledonary node based micropropagation protocol: For the development of the micropropagation protocol from cotyledonary nodes, mature fruits (40 fruits per treatment) were collected from the mother plants. The fruits were subjected to flotation in water to segregate 'floaters' from 'sinkers' (approximately 50% each). Only sinkers were used as floaters were usually found to be empty fruits lacking viable seeds. These selected fruits were de-pulped to remove the exocarp and mesocarp. They were then surface sterilized by washing in running tap water for 2 min (to remove dirt) and shaken in 100 ml RO water (Millipore RiOS5) with two drops of Tween-80® (polysorbate 80; Ranbaxy Fine Chemicals Ltd.) for 10 min, rinsing three times with autoclaved RO water. The cleaned fruits were then treated for 10 min with a solution of Bavestein and Streptomycin and were finally treated with NaOCl solution (providing 5% available chlorine) for 5 min and rinsed with autoclaved RO water thrice. The endocarp was then broken open with a sharp sterile scalpel and seeds were scooped out and inoculated on germination medium. The seeds were germinated and cotyledonary nodes were used as explants. The cotyledonary nodes were inoculated on MS medium with various hormonal combinations to achieve bud break and subsequent steps leading to development of complete plantlets.

Somatic embryogenesis micropropagation protocol: For the development of somatic embryogenesis based protocol, immature fruits (rather than mature ones) were collected from the mature selected mother plants; somatic embryogenesis was found to be higher through use of immature fruits compared to mature fruits where somatic embryogenesis percentage was very poor. The sinkers were selected and surface

sterilized as described above and the seeds extracted and inoculated on various types of media [B5 (Gamborg, 1968) and MS (Murashige and Skoog, 1962)] with and without hormones (2,4-D, IBA and BAP) to derive somatic embryo based plantlets. All experimental treatments consisted of 25 explants, each replicated three times.

All media were sterilized 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 25± 2°C, 16 hour photoperiod and 1600 lux intensity light (via cool florescent lamps and incandescent light bulbs).

Acclimatization was carried out in mist chamber (90 sec misting at 10 min intervals to maintain a relative humidity of between 85 to 95%) at a temperature of 28 to 30°C. Somatic embryo derived complete plants and rooted micro-shoots derived from cotyledonary nodes were transferred to vermiculite and wetted with Hoagland's solution (Hoagland, 1950) for primary hardening for 4-5 weeks and then finally transferred to plastic cups containing vermiculite, placed in mist chamber. Plantlets were transferred to a soil: farm yard manure (FYM) mix (ratio 1:1) in polythene plantation bags (9 x 9 x 36 cm). After one month of transfer to polythene bags, plantlets were transferred under green 50% agronet shade for complete acclimatization and finally transplanted to the experimental field site in April 2007.

CONSEQUENCES

Development of plants from cotyledonary node micropropagation: Multiple microshoots were obtained on MS medium supplemented with NAA and BAP. Elongation of microshoot was significantly observed on IBA and BAP supplemented MS medium. Efficient rooting was obtained by transfer to White's (1954) medium without hormones and high concentration of activated charcoal (Fig. 2 F-H). Complete procedure from primary culture initiation to acclimatization of the plants takes 24 weeks.

Development of plants from somatic embryo micropropagation: Immature seeds when cultured on B5 medium supplemented with the auxin 2,4-D; callus formation was achieved within four weeks after which the calli were sub-cultured on hormone free B5 medium for somatic embryo induction. Subsequently, these were transferred to MS medium supplemented with BAP and IBA for formation of early and late torpedo stage somatic embryos. Trials of various stress treatments optimized the somatic embryo maturation and the mature somatic embryos germinated on hormone free MS medium (Fig. 2 I, J). Complete procedure from primary culture initiation to acclimatization of the plants through this pathway takes 32 weeks.

Acclimatization of *in vitro* raised plants and establishment of field trial: Acclimatization is a very important step for success of a tissue culture based regeneration protocol. Without proper hardening, the success rate of a tissue cultured plant surviving subsequent to transplanting into (often harsh) field conditions will be minimal. A suitable hardening protocol was developed and standardized (Fig. 2 K, L).

A total of 35 plants were obtained after acclimatization out of which 15 were used in field trail, while 20 were handed over to private volunteers for raising. There is presently no data available on survival and growth of these 20 plants. The remaining 15 acclimatized plants (10 derived from cotyledonary node micropropagation and five from somatic embryos) were transferred to a 'natural' field site (situated in AFRI's experimental field near Jhalamand in Jodhpur). The site is protected by a boundary wall (to prevent incursion of livestock and other large herbivores) and also houses other plant species trial plots. Initially, to enhance establishment of the plants, weekly watering in the first month followed by fortnightly watering for 3 months was undertaken. After that no watering has been done. Six-monthly weeding is undertaken as a regular cultural practice. Plants have been growing well under field condition for the last 3 years (Fig. 3). Flowering and fruiting has taken place, at a slightly earlier age as compared to seed raised plants of the same age and size.

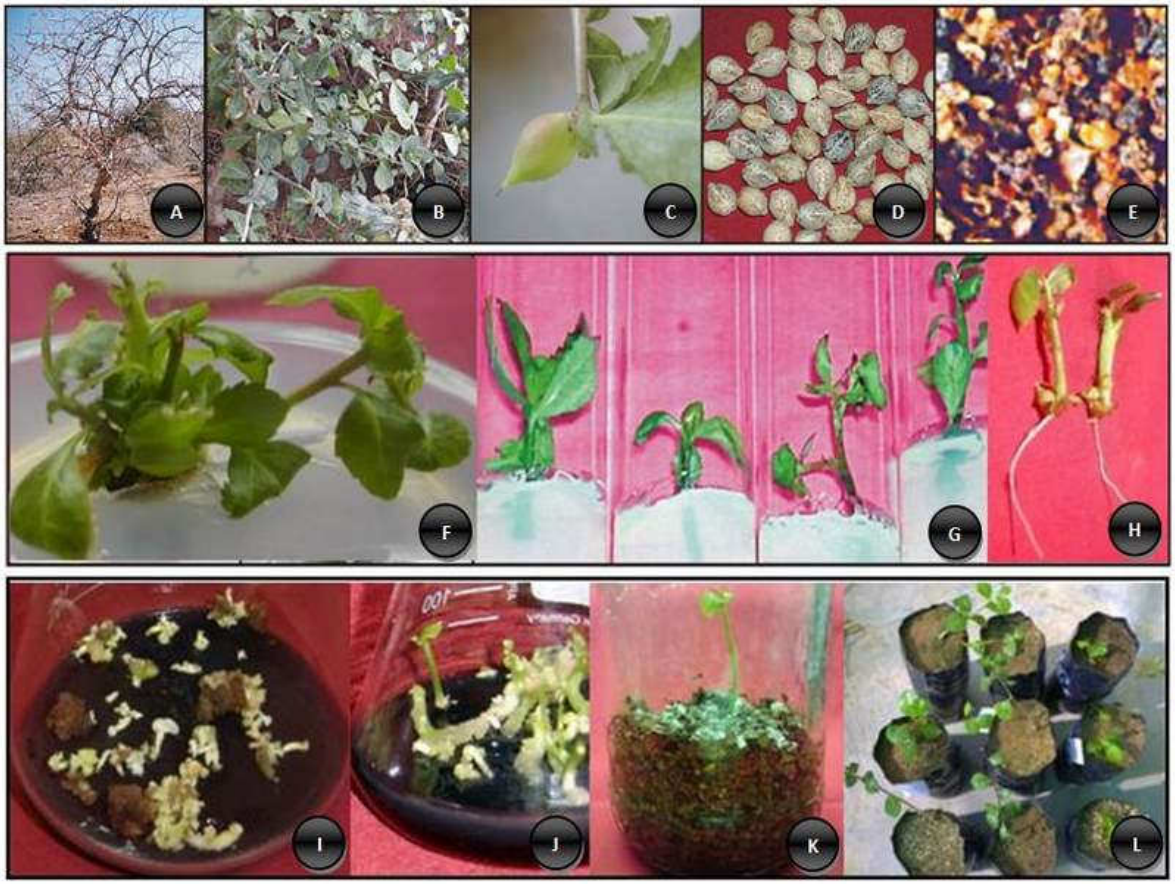

Figure 2. *In vitro* propagation of *Commiphora wightii* (A-L): A) mature mother plant; B) close-up of stems bearing leaves; C) fruit; D) de-pulped fruits; E) crystalized oleo-gum-resin as used by the pharmaceutical industry (not part of the propagation methods); F) multiple shoot production from cotyledonary node explants; G) elongation of micro-shoots; H) *in vitro* rooted shoots; I) somatic embryos; J) germinating somatic embryos; K) acclimatization; L) acclimatized plants ready for field transfer.

Figure 3. A) tissue culture raised *Commiphora wightii* plants after one year of establishment in field conditions at Jodhpur (Rajasthan); B) a tissue culture raised plant growing under field conditions after three years of establishment (Note: this photograph was taken in June 2010; during hot summer months the plant remains largely leafless).

Growth data was collected indicating that these plants showed good incremental growth rate (Fig. 4) comparable to wild plants. After the success of this initial field trial, recently a larger field trial has been laid out at AFRI campus that comprises of 42 plants derived from somatic embryos (data not shown). This has been a result of further refinement of our initial *in vitro* propagation protocol described. The field trial is in its initial stage and data on survival percentage and growth increment is yet to be collected. The initial observations indicate cent percent survival and good establishment.

Cost: Per plant cost from our protocols has been calculated using the equation formulated by Tomar *et al* (2007). This formula takes into account all the stages from initiation to acclimatization up to the plantable size. The cost of single plant produced through somatic embryogenesis pathway, is equivalent to Indian Rupees (INR) 19, while that produced through cotyledonary node based protocol is INR 27. This is substantially lower compared to that reported by Soni (2010) at INR 80 per plant.

Conclusion and discussion: The present study demonstrates that tissue culture protocols (through cotyledonary node cultures and somatic embryogenesis) for *in vitro* mass multiplication of *C. wightii* are effective (and provide a viable alternative to propagation via stem cuttings and seeds) for quality plant production. The plants so obtained can directly assist in various conservation efforts, including restoration of wild populations. Through a tissue culture based system, continuous supply of quality planting stock can be ensured at a reasonable cost. At a per plant cost ranging from INR 19 to 27 for somatic embryogenesis and cotyledonary node based pathways respectively are economical. This costing also incorporates the critical hardening (acclimatization) step which is the most important factor for ensuring field survival of tissue culture raised plants and often ignored in most such studies. The present study also demonstrates long term survival and production of fertile tissue cultured plants that are performing very well under natural field conditions.

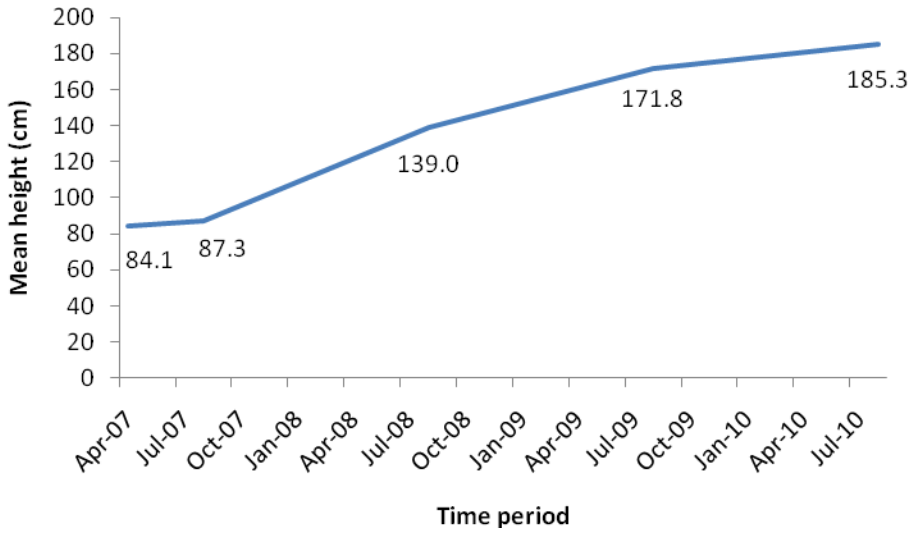

Figure 4. Height increment growth of tissue culture raised *Commiphora wightii* plants (n=15) growing under field conditions at Jodhpur (Rajasthan) over a period of 40 months.

ACKNOWLEDGEMENTS

The authors thank ICFRE, Dehradun and NMPB, New Delhi for financial support. AKP thanks CSIR for fellowship grant.

REFERENCES

Bhandari M.M. (1990) *Flora of Indian Desert*.MPS Repros, Jodhpur, India

Bhatt J.R., Nair M.N.B. & Mohan Ram H.Y. (1989). Enhancement of oleo-gumresin production in *Commiphora wightii* by improved tapping technique. *Current Science*, **58**, 349-357.

Gamborg O.L., Miller A. & Ojima K. (1968) Nutrient requirements of suspension cultures of soyabean root cells. *Experimental Cell Research*, **50**, 151-158.

Hoagland D.R. & Arnon D.I. (1950) *The water culture method for growing plants without soil*. California Agricultural Experimental Station Circular, Berkely, California, USA.

IUCN (2010) IUCN Red List of Threatened species, Version 2010.2 http://www.iucnredlist.org Accessed: 13 July 2010.

Kumar S. & Shankar V. (1982) Medicinal plants of Indian desert: *Commiphora wightii* (Arnott) Bhandari. *Journal of Arid Environments*, **5**, 1-11.

Murashige T. & Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473-497

Paliwal A. (2010) Guggal faces sticky end. *Down to Earth* **19**, 24-25.

Soni V. (2010) Efficacy of *in vitro* tissue culture versus stem cuttings for propagation of *Commiphora wightii* in Rajasthan, India. *Conservation Evidence*, **7**, 91-93.

Tomar U.K., Negi U., Sinha A.K. & Dantu P.K. (2007) An overview of the economic factors influencing micropropagation. *My Forest*, **43**, 523-532.

White P.R. (1954) *The cultivation of animal and plant cells*. The Ronald Press Company, New York, USA.