Indian J. Pl. Genet. Resources 8(1): 107-112, 1995; Special Issue (2)

IN VITRO CONSERVATION OF THREATENED PLANTS OF MEDICINAL IMPORTANCE

Neelam Sharma, K.P.S. Chandel and Paul K. Anderson

National Plant Tissue Culture Repository,
National Bureau of Plant Genetic Resources,
Pusa Campus, New Delhi 110 012

Procedure for *in vitro* multiplication and *in vitro* conservation of six threatened/endangered medicinal plants are reported. Various combinations of growth regulators were tested to select optimal medium for initiation and further shoot multiplication. Slow growth experiments were performed and the shelf-life of shoot cultures on multiplication and/or modified medium could be extended for 11-20 months depending on the species.

Key words : *In-vitro* conservation, *Colens forskohlii, Gentiana kurroo, Picrorhiza kurroa, Rauvolfia serpentina, Sausseona lappa, Tylphora indica*

More than 1100 plant species are used in Indian ayurvedic medicine system for the formulations of different drugs. Owing to large scale and indiscriminate collection of wild materials from natural habitat for commercial purposes coupled with insufficient attempts to allow their replenishment or their cultivation, quite a few species of known medicinal importance have become threatened, vulnerable, or even extinct. Thus, there is need to conserve these species through *in vitro* conservation on priority basis. The communication discusses the achievements in the direction of *in vitro* conservation of *Coleus forskohlii, Gentiana kurroo, Picrorhiza kurroa, Rauvolfia serpentina, Saussurea lappa* and *Tylophora indica.*

MATERIALS AND METHODS

Initiation of aseptic cultures

Plants of *Coleus forskohlii, Gentiana kurroo, Picrorhiza kurroa, Rauvolfia serpentina* and *Tylophora indica* maintained *ex situ* served as source of explants. Seeds of *Saussurea lappa* were germinated aseptically and these seedlings were used as experimental material. Seeds and small twigs were washed thoroughly, surface sterilized with 0.2 or 0.1 per cent solution of mercuric chloride ($HgCl_2$) and were washed thoroughly in sterile distilled water 2-3 times. Single nodal stem segments (1.0-1.5cm) and seeds were implanted vertically onto the nutrient medium. In the case of *S. lappa*, terminal buds (shoot tip of 15 days old aseptic seedlings) were used to initiate cultures.

Shoot multiplication and rooting

The shoots regenerated from stem cuttings were subcultured after 3-4 weeks. At each subculture the individual shoots were separated and cut into approximately 1.0cm segments and transferred to multiplication medium. For rooting, individual shoots with 2-4 nodes were implanted onto rooting medium.

Culture medium

Murashige and Skoog's (1962) basal medium with 3 per cent sucrose, 100 mg/l myoinositol and 0.8 per cent agar were used as basal medium (BM). Depending on the experiment, BM was supplemented with benzylamino purine (BAP), kinetin (KN), indole-3-acetic acid (IAA), indole-3- butyric acid (IBA), napthaleneacetic acid (NAA), gibberellic acid (GA_3), and mannitol. All the the ingredients were added to molten agar and pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min in culture tubes closed with either cotton plugs or polypropylene caps.

Culture conditions

For multiplication, cultures were maintained at 25° ± 2°C and 16 photoperiod. For low temperature storage, the cultures were kept at culture room conditions for one week to eliminate contamination. The cultures were then transferred to incubators maintained at 5°C, 10°C and 15°C with or without light.

RESULTS AND DISCUSSION

In vitro multiplication

Attempts were made to standardise a procedure of shoot multiplication of the species. Details of surface sterilization and the best responding media for each species are given in Table 1. Results of the experiments are summarised below (see also Table 2) :

Coleus forskohlii

Surface sterilized terminal nodal segments measuring (1.0 - 1.5 cm) were cultured on MS, MS + KN (0.5 - 2.0 mg/l) in combination with IAA (0.5-2.0 mg/l) or MS + BAP (1.0 or 2.0 mg/l) in combination with IAA (1.0 mg/l) as reported earlier (Sharma *et al.*, 1991).

Table 1. *In vitro* multiplication of endangered plant species of medicinal value under study

Plant species	Surface % $HgCl_2$	Sterilization Time (min)	Multiplication medium	Rooting medium
Coleus forskohlii	0.1	10	MS+KN 2.0 mg/l + IAA 1.0 mg/l	MS+IAA 1.0 mg/l
Gentiana kurroo	0.1	10	MS+ BAP 2.0 mg/l + NAA 0.2 mg/l	MS + IBA 0.1--1.0 mg/l
Picrorhiza kurroa	0.1	10	MS+BAP 0.225 mg/l	MS+NAA 0.5 mg/l
Rauvolfia serpentina	0.2	10	MS+BAP 1.0 mg/l + NAA 0.1 mg/l	MS + NAA 1.5 mg/l
Saussurea lappa	0.2	10*	MS+BAP 1.0 mg/l + GA_3 1.0 mg/l	MS+NAA 0.5 mg/l
Tylophora indica	0.1	7	MS+BAP 5.0 mg/l + NAA 0.5 mg/l + ascorbic acid 100 mg/l	B5 + IBA 1.0 mg/l

* For seeds

Table 2. *In vitro* conservation responses of threatened plants of medicinal importance under study

Species	Shoot multiplication	Conservation
Coleus forskohlii	12 shoots/4 weeks	18M at 25°C
Gentiana kurroo	5 shoots/4 weeks	11M at 4°C
Picrorhiza kurroa	30 shoots/6 weeks	16M at 10°C
Rauvolfia serpentina	20 shoots/4 weeks	15 M at 15°C 20M at 25°C
Saussurea lappa	4 shoots/ 3 weeks	15M at 4°C
Tylophora indica	5 shoots/5 weeks	12m at 25°C

Sprouting of single shoot was observed on almost all medium but multiple shoot formation was observed in all combination of KN and IAA tested. Optimal response in terms of shoot number and growth of shoots was attained in MS + KN (2.0 mg/l) + IAA (1.0 mg/l): Single explant cultured on this medium produced an average of 12 shoots every six weeks.

For rooting, individual shoots with 3-4 nodes were placed on MS medium supplemented with 0.5 -2.0 mg/l of an auxin (IAA NAA or IBA). Optimal rooting occurred on MS + IAA (1.0 mg/1). The plantlets thus obtained were successfully transferred and established in soil.

Gentiana kurroo

Shoot tips and nodal segments surface sterilized for 10 minutes with 0.1 per cent mercuric chloride were used (Sharma *et al.*, 1993). Different concentrations of BAP (0.2 -2.0 mg/l) and KN (0.2-2.0 mg/l) either alone or in combinations with NAA (0.1-1.0 mg/l) or IAA (0.1-1.0 mg/l) were tested. 50 per cent axillary buds sprouted only in medium with BAP (2.0 mg/l) and NAA (0.5 mg/l). The shoots developed in primary cultures were subcultured to fresh medium to test the effect of different concentrations of BAP in combination with NAA. BAP (2.0 mg/l) and NAA (0.2 mg/l) was found to be the best in terms of number of shoots and shoot growth. An average of 3.5 shoots of 4.2 cm length with 3-4 nodes were produced on this medium providing 15 propagules for the next cycle of shoot multiplication. Of the various media tested best rooting was achieved on basal medium containing 6 per cent sucrose.

Picrorhiza kurroa

Cultures were initiated and multiplied from terminal and nodal cuttings (1.0 - 1.5 cm) using the procedure of Upadhyaya *et al.* (1990). The shoots were proliferated on standard medium supplemented with 0.2 mg/l BAP. These *in vitro* multiplied shoots could be induced to form roots on MS + NAA (0.5-1.0 mg/l).

Rauvolfia serpentina

Terminal and nodal cuttings of field grown plants of *R. serpentina* were used to initiate cultures (Sharma and Chandel, 1992a). The shoots proliferated and optimal response in terms of number of shoots and growth was attained on MS + BAP (1.0 mg/l) + NAA (0.1 mg/l). These shoots were subcultured every 4-6 weeks to generate sufficient material for conservation experiments. Rooting of shoots occurred only in presence of an auxin. NAA (1.5 mg/l) induced rooting in 90 per cent of culture. Rooting was sometimes associated with callusing.

Saussurea lappa

20-30 per cent surface sterilized seeds (0.2% $HgCl_2$ for 10 minutes) germinated on MS basal medium. Shoot tips of 15 days old seedlings served as explant for shoot multiplication. BAP (0.5-2.0 mg/l) in combination with NAA (0.05-1.0 mg/l) or GA_3 (0.1-1.0 mg/l) was tested to achieve shoot multiplication. On an average 4.0 shoots per explant were produced every three weeks.

For rooting, individual shoots (3 weeks old) were implanted onto rooting media consisting of MS supplemented with 0.05, 0.1 0.5, 1.0 mg/l of IAA,

NAA or IBA. Best rooting occurred on MS+NAA (0.05 mg/l). Increasing the auxin concentration induced callusing. Shoot multiplication of *Saussurea* through hypocotyl and leaves was earlier reported by Arora and Bhojwani (1989).

Tylophora indica

Following the procedure of Sharma and Chandel (1992b), cultures were initiated and multiplied on MS medium supplemented with BAP (5.0 mg/l) + NAA (0.5 mg/l)+ ascorbic acid. Addition of ascorbic acid was essential to induce axillary bud break. Rooting of *in vitro* shoots was readily achieved on MS + IAA (1.0 mg/l). These *in vitro* produced plantlets were successfully transferred to pots and maintained under field conditions.

***In vitro* conservation**

Conservation experiments involved employing minimal media, osmoticum or incubation at low temperature. The results obtained so far showed that culture could be maintained for 8-16 months depending on the species and storage conditions (Table 2).

Shoot cultures of these remain viable for 3-5 months (*Saussurea, Rauvolfia*) in culture vessel covered with cotton plugs. In comparison, those covered with polypropylene caps remained healthy for a longer period eg., 12 months in *Coleus* and *Tylophora,* thus, polypropylene caps alone were found to be better than cotton plugs for enhancing subculture cycle. (Sharma and Chandel, 1992). This may be due to avoidance of excessive dessication coupled with nutrient depletion as compared to cultures covered by cotton plugs.

Low temperature incubation appears to be highly promising as *in vitro* cultures of *Rauvolfia, Saussurea* and *Tylophora* were conserved for 15, 15 and 12 months at 15°C, 4°C and 10°C respectively. In case of *Saussurea* and *Picrorhiza* also low temperature under dark condition was more effective.

Shoot cultures of these species exhibited relatively slow growth with the inclusion of mannitol in the medium. This treatment resulted in maintainance of cultures well over 9 months at 25°C while 15 months under low temperature in *Rauvolfia, Saussurea* and *Picrorhiza.* Experiments are currently under way to study the effect of mannitol and other osmoticum under low temperature in other species also.

In vitro techniques are being increasingly used for multiplication and conservation of germplasm of medicinal importance threatened with extinction (Arora and Bhojwani, 1989; Bhojwani *et al.*, 1989; Chandel *et al.*, 1988; Constable, 1990; Sharma and Chandel, 1992a, b). The present study described results of *in vitro* conservation experiments on six species of medicinal importance.

CONCLUSIONS

Protocol for *in vitro* multiplication of *Rauvolfia serpentina, Tylophora indica, Saussurea lappa, Picrorhiza kurroa, Coleus forskohlii* and *Gentiana kurroo* were standardized. Shoot cultures could be successfully conserved for 11-20 months without subculture. Addition of mannitol and high sucrose/agar reduced growth. Its effect on increasing shelf-life of cultures is presently being further investigated. Encapsulation of explants for cryopreservation and propagation is a potential alternative being investigated under *in vitro* conservation programme at NFPTCR.

ACKNOWLEDGEMENTS

We thank Director, National Bureau of Plant Genetic Resources, New Delhi for the necessary facilities. Financial support provided by the Department of Biotechnology, Ministry of Science & Technology, New Delhi is gratefully acknowledged.

REFERENCES

Arora, R. and S.S. Bhojwani. 1989. *In vitro* propagation and low temperature storage of *Sassurea lappa* C.B. Clarke, -an endangered medicinal plant. *Plant Cell Rep.* **8**: 44-47.

Bhojwani, S.S., N. Arumugam, R. Arora and R.P. Upadhyaya. 1989. *In vitro* conservation of some endangered plant species of India. *Indian J. Pl. Genet. Resources* **2**: 103-113.

Chandel, K.P.S., R. Pandey, R. Choudhary, S.M. Balachandran and N. Sharma. 1988. Biotechnology — its role in conservation of plant genetic resources. *In* : Paroda, R.S., Arora, R.K. and K.P.S. Chandel (Eds.). Plant Genetic Resources - Indian Perspectives (p 499-516), NBPGR Publ., India.

Constable, F. 1990. Medicinal plant biotechnology. *Planta Med.* **56**: 421-425.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiology. plant.* **15**: 473-497.

Sharma, N., K.P.S. Chandel and V.K. Srivastava. 1991. *In vitro* propagation of *Coleus forskohlii* Briq. - A threatened medicinal plant. *Plant Cell Rep.* **10** : 67-70.

Sharma, N. and K.P.S. Chandel. 1992a. Low temperature storage of *Rauvolfia serpentina* Benth. ex Kurz.: An endangered, endemic medicinal plant. *Plant Cell Rep* **11** : 200-203.

Sharma, N. and K.P.S. Chandel. 1992b Effects of ascorbic acid on axillary shoot induction on *Tylophora indica* (Burm.f.) Merrill. *Plant Cell Tiss. Org. Cult.* **29** : 109-113.

Sharma, N., K.P.S. Chandel and A. Paul. 1993. *In vitro* propagation of *Gentiana kurroo* - an indigenous threatened plant of medicinal importance. *Plant Cell Tiss. Org. Cult.* **34**: 307-309.

Upadhyaya, R., Arumugam N. and S.S. Bhojwani. 1989. *In vitro* propagation of *Picrorhiza kurroa* Royle. ex Benth. — an endangered species of medicinal importance. *Phytomorphology* **39**: 235-242.