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In-Vitro Conservation of Some Endangered Plant Species of India

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Procedures for in-vitro *multiplication and short-term conservation of 3 endangered medicinal plants, viz.,* Saussurea lappa. Picrorhiza kurroa *and* Podophyllum hexandrum *are reported. While first two species were multiplied via shoot multiplication, the third species was multiplied* via *both shoot multiplication and somatic embryogenesis. On MS + BAP (5×10^{-6} M) + GA_3 (3×10^{-6} M) shoots of* S. lappa *proliferated at a rate of 3.5 fold every 3 weeks and 90 per cent cultures of these shoots rooted in MS + NAA (5×10^{-7} M).* P. kurroa *multiplied 36-fold every 30 days on MS + BAP (1×10^{-6} M). Similarly, shoots of* P. hexandrum *muliplied 8-fold in 4 weeks on MS + BAP (2×10^{-6} M). The callus derived from zygotic embryos of* P. hexandrum *on MS + BAP (2×10^{-6} M) + IAA (5×10^{-7} M) differentiated globular embryos. Further development of these embryos occurred on MS + NAA and the somatic embros germinated on MS with or without growth regulators. The shoot cultures and embryogenic calli stored at 5°C in dark for 7 to 12 months without any intervening subculture survived with 70 to 100 per cent viability.*

Indiscriminate exploitation of natural resources, destruction of natural habitats and spread of harmful chemicals are some of the human activities responsible for the deterioration of natural ecosystem. It is estimated that about 1500 plant species are threatened to extinction in India (Nayar and Sastry, 1987). Of these, about 100 species have been declared as rare plants. The growing awareness of dreadful consequences of uncontrolled destruction of plant types has led to the establishment of national and international bodies to examine the seriousness of the problem and suggest measures to control it. At this stage of belated realization of the problem, a two-pronged approach is required. Firstly, the natural ecosystem and habitats should be protected and preserved by establishment of natural reserves in order to check further loss of species due to human activities. Secondly, the taxa whose number has fallen to such critical level that they are to be regarded as threatened or endangered under natural habitat should be preserved under safe, artificial conditions which would also allow their rapid multiplication for reintroduction into the wild. Plant tissue culture fulfills the requirements of the second approach.

In tissue cultures genotypes can be maintained as actively prolifering cells, somatic embryos or shoots through regular subcultures. The necessity of frequent subculture may be avoided by storing the cultures under growth-limiting condi-

tions, such as low temperature (5°-12°C) and the application of growth retardants through the culture medium (Westcott, 1981; Henshaw and O'Hara, 1983). Alternatively, cultured cells may be stored for long-term at cryogenic temperature (Kartha, 1984; Withers and Williams, 1986). It is now well established that plant cells can withstand —196°C, and plants have been regenerated from tips of pea and strawberry cryopreserved for over two years.

Under an All India Co-ordinated Project, sponsored by the Department of Environment, possibilities of *in-vitro* conservations of some endangered plant species were examined. The paper describes our observation on *in-vitro* multiplication of three endangered species of medicinal important, viz., *Podophyllum hexandrum*, *Picrorhiza kurroa* and *Saussurea lappa*. Attempts have also been made to preserve the cultures of these species at 5°C in dark for short-term.

MATERIALS AND METHODS

Plant material

Seeds of *Saussurea lappa* C.B. Clarke were obtained through the courtesy of the Lahul Kuth Growers Co-operative Marketing Society Ltd., Manali. Live plants with mature fruits of *Podophyllum hexandrum* Royle, and vegetative parts of *Picrorhiza kurroa* Royle ex Benth. were collected from Valley of Flowers, Ghangarea, Hemkund and Kedarnath regions (Altitude 3000 m-5000 m) in Uttar Pradesh, India.

Initiation of aseptic cultures

Seed and vegetative parts were sterilized with 0.2 per cent solution of mercuric chloride for 5-10 min. and were washed thoroughly in sterile distilled water 2-3 times. The seeds and *ca* 5 mm long leaf, root, rhizome segments and nodal and terminal cuttings were planted aseptically on the nutrient medium. In the case of *P. hexandrum*, in addition, decoated seeds and excised embryos (*ca* 3 mm long) were also cultured. In the case of *S. lappa* cotyledons, leaf, hypocotyl and root segments from 15 days-old aseptic seedlings were used to initiate cultures.

Shoot multiplication

Approximately, 5 mm long shoots with 1 or 2 young leaves from aseptic cultures were used to multiply the shoots *in-vitro*.

Somatic embryogenesis

Approximately 150 mg (fresh weight) of embryogenic calli was used as inoculum.

Culture medium

Murashige and Skoog's (1962) medium containing 3 per cent sucrose gelled with 0.8 per cent agar, hereafter referred to as MS, was used as basal medium. Depending upon the experiment, the MS was supplemented variously with benzylamino purine (BAP), 2-isopentenyl adenine (2ip), kinetin (Kn), 2,4-dichlorophenoxyacetic

acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) and gibberellic acid (GA_3). All the constituents were adjusted to pH 5.8. The molten medium was dispensed in 150 mm × 25 mm tubes (20 ml/tube), and plugged with non-absorbent cotton wrapped in a layer of cheese cloth and autoclaved at 121°C for 15 min.

Culture conditions

P. hexandrum cultures were normally maintained at 25 ± 2°C in 16 h photoperiod with 9.5 W/m² irradiance provided by cool, white, fluorescent tubes (Phillips TL 40 W/54). Cultures of *S. lappa* and *P. kurroa* were kept under continuous light. For cold storage, cultures were kept at 5°C in dark in a refrigerator for various periods.

RESULTS

Saussurea lappa C.B. Clarke

Commonly known as 'Kuth', *S. lappa* (Asteraceae), grows as a perennial herb in the Western Himalayas at an altitude of 2500-3000m. The dried roots of Kuth are used to cure cough, fever, asthma, rheumatism and skin diseases. The roots also yield an essential oil (costus oil) which is used for blending in high class perfumery. Owing to over exploitation of its natural populations for commerce and declining area under cultivation, *S. lappa* has become a threatened species in this country. The Kuth growers are shifting to potato cultivation solely for economic reasons.

Seed germination

On MS basal medium 30 per cent seeds germinated. Onset of germination was observed within 3 days of culture and a 15-day old seedling had two dark green and spathulate cotyledons, a long hypocotyl and a tap root. Up to two plumular leaves developed after eight weeks.

Regenerations

Cotyledons, hypocotyl, root and leaf segments from 15-day old seedlings were used to examine their potentiality for regeneration. While cotyledons and leaf explants differentiated shoot buds, the hypocotyl and root explants exhibited only callusing.

The cotyledons cultured on MS + BAP (5×10^{-6} M) + IAA (1×10^{-7} M) enlarged and callused at the cut ends. The callus was creamish, hard and nodular. When the callus and the parent explants were separately transferred to fresh medium of the same composition, both of them differentiated shoot buds, with calli showing higher regeneration (50 per cent) than the cotyledon pieces (25 per cent).

The leaf pieces cultured on MS + BAP (5×10^{-6} M) + IAA (1×10^{-7} M)

enlarged and formed nodular callus at cut end within one week. By the end of the 4th week, 70 per cent of these cultures differentiated adventitious shoot buds (Fig. 1a). The maximum number of buds per 5mm² explant was 8.

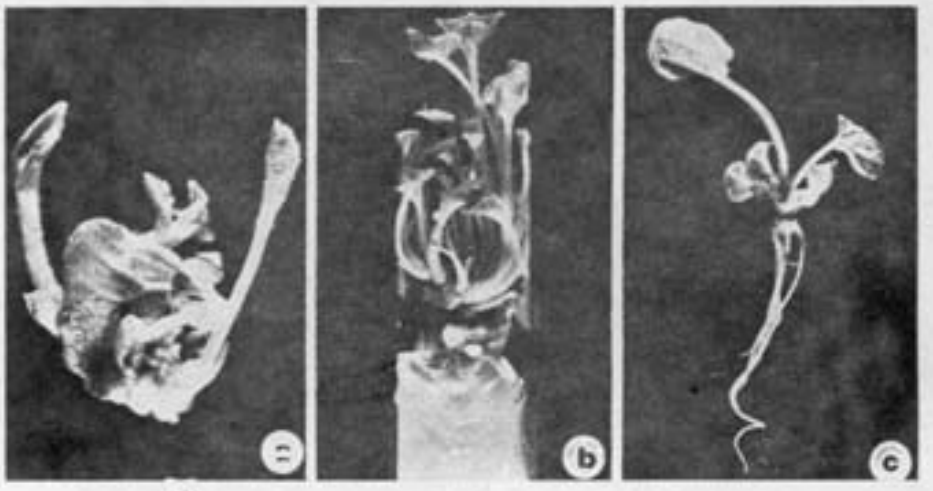

Fig. 1. *Saussurea lappa*

a : A 3-week old culture of leaf segment on MS + BAP (5×10^{-6} M) + IAA (10^{-5} M), showing differentiation of shoot buds from the cut end.

b : A cluster of shoots derived from a single adventitious shoot cultured on MS + BAP (5×10^{-6} M) + GA_3 (3×10^{-6} M) for 3 weeks.

c : An *in-vitro* multiplied shoot rooted on MS + NAA (5×10^{-7} M).

Shoot multiplication

The shoot buds differentiated from cotyledons and leaf pieces as terminal buds from aspectic seedlings were cultured on MS medium variously supplemented with growth regulators to achieve shoot multiplication. On MS + 3×10^{-6} M BAP, 3-4 healthy leaves developed and a hard nodular callus was formed at the basal cut end of the explant.

Shoot multiplication occurred in the combined presence of BAP (5×10^{-6} M) and GA_3 (3×10^{-6} M) (Fig. 1b). After one week of culture, a hard, slow growing callus developed at the basal cut end, which later differentiated shoot buds. On this medium, shoots have been multiplied for two years at a rate of 3.5 times every 3 weeks.

Rooting of shoots

Rooting of shoots occurred only in the presence of an auxin (Fig. 1c). NAA (5×10^{-7} M, 1×10^{-5}M, 1×10^{-6} M) induced rooting in 90 per cent cultures within 30 days. The roots were white, thick and hairy. The rooting was associat-

ed with callusing, the intensity of which was directly related to the concentration of the auxin. Thus, full plantlets have been established from *in-vitro* multiplied shoots.

Short term storage of shoot cultures

Seventy two *in-vitro* grown shoots were individually transferred to MS + BAP (5×10^{-6} M) + GA_3 (3×10^{-6} M) and shifted to cold storage after one week growth under culture room conditions (25°C, 16 h photoperiod). At monthly intervals, 5 cultures were withdrawn and placed under standard culture root conditions. The shoots cold stored up to 12 months remained fully viable. During cold storage, the original leaves dried and 1-2 new shoots with etiolated leaves had developed. The leaves turned green within a week after transfer to light. Individual shoots from these cultures exhibited a higher rate of multiplication (4.5 fold/4 week) under culture room condition than those not subjected to cold temperature.

Podophyllum hexandrum Royle

Commonly known as Himalayan May-apple, *P. hexandru* (Podophyllace) grow as a perennial rhizomatous herb in the inner ranges of Himalayas at an altitude of 2700 to 4200 m. The rhixome is a source of the resin (podophyllin) which contains the anticancerous principle, podophyllotoxin. While the resin is used for its purgative properties, the podophyllotoxin is used as a starting material for the production of certain semisynthetic anticancerous drugs. Resin from the Indian species yields higher amount of (40 per cent) podophyllotoxin, than the resin from the American species *P. peltatum* (10 per cent). Due to lack of organised cultivation and intense collection of its rhizome from the forests, *P. hexandrum* has become a threatened species.

Culture initiation

None of the vegetative parts responded in culture, except for young leaf segments showing little swelling on MS + 2,4-D (1×10^{-5} M) + BAP (1×10^{-6}). Similarly, the seeds and decoated seeds failed to show any change even after 8 weeks. The excised embryos, however, showed over 90 per cent germination within 7 days on MS basal medium. A 4-wk old seedling comprised two green cotyledons with free terminal lobes fused at the base forming a tube (CT) and a long tap root. The plumule situated at the base of the CT did not develop any leaf even after 3 subcultures. Even in nature the plant remains in seedling stage during the first year, and the first leaf emerges only during the second season (Badhwar and Sharma, 1963).

On MS + BAP (2×10^{-6} M) and MS + BAP (2×10^{-6} M) + IAA (5×10^{-7} M), the embryos showed expansion of cotyledons and slight elongation of the CT but root development was completely suppressed. Within 3 weeks the base of the CT became swollen and differentiated certain leafy structures on the surface

which later developed into normal shoots. In the presence of BAP and IAA, a nodular and compact callus also developed at the base of the CT. Shoots were subcultured for shoot multiplication and the calli were used to induce somatic embryogenesis.

Shoot multiplication

The adventitious shoot buds with one or two young leaves and a swollen base were excised from embryo cultures on MS + BAP and MS + BAP + IAA and transferred to MS supplemented with BAP alone at 3 different concentrations (2×10^{-6} M, 5×10^{-6} M, 1×10^{-5} M). In the first two weeks the petiole elongated, lamina expanded and the basal swelling enlarged. During the third week, white shoot buds emerged from the swollen base. Maximum proliferation of shoots (8 fold in 4 weeks) occurred in the presence of 2×10^{-6} M BAP (Fig. 2a).

Rooting of shoots

Satisfactory rooting of shoots is yet to be achieved. Only 25 per cent of the shoots developed 1 or 2 short, thick roots on MS (with 6 per cent sucrose) + 2×10^{-6} M BAP. Auxins, such as IAA, NAA and IBA were also tried for rooting but without any success.

Somatic embryogenesis

The calli from embryo cultures became friable and creamish, and in 70 per cent of the cultures abnormal looking leafy structures developed by the end of the fourth passage on MS + BAP + IAA. When transferred to MS + 2,4-D (1×10^{-5} M) + BAP (1×10^{-6} M), the calli turned slightly compact, light brown and differentiated a large number of globular embryos (Fig. 2b). On this medium proliferation of globular embryos continued but they failed to mature.

In order to induce further maturation, the globular embryos were transferred to MS supplemented with lower concentrations of 2,4-D or NAA ($1 \times 10^{-6} - 1 \times 10^{-5}$ M) and the cultures were maintained in dark. While the globular embryos proliferated on all the media, further maturation of embryo though heart, torpedo and dicotyledonous stages occurred only on media containing NAA (Fig. 2c). Maximum number of embryos (12/culture) were formed with 1×10^{-6} M NAA. Pluricotyledony, fused cotyledons and production of secondary embryos were common in these cultures.

Somatic embryos germinated on a range of media. On basal medium, a 4-wk old seedling possessed features similar to a zygotic seedling (Fig. 2d). Normal looking seedlings transferred to vermiculite : soil (1 : 1) mixture at 20°C in light remain and healthy up to 4 weeks, but none of the seedlings developed a plumular leaf.

Short-term Storage of Somatic Embryos

A preliminary study was undertaken on the storage of *Podophyllum* germplasm

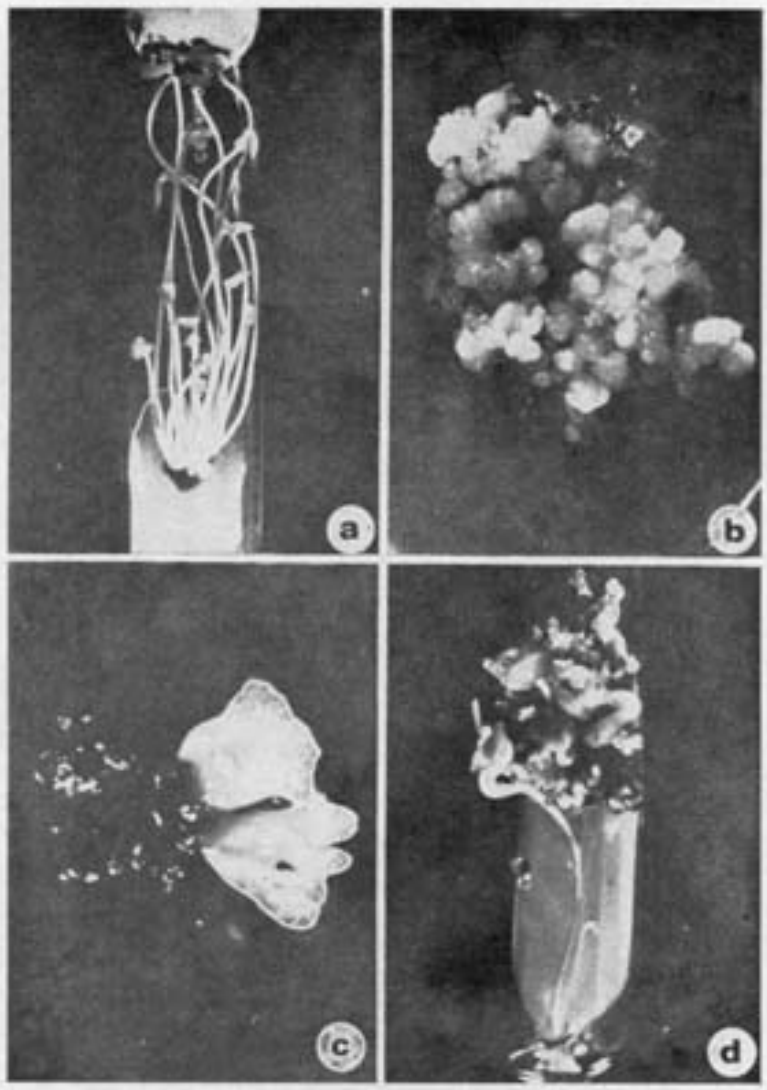

Fig. 2. *Podophyllum hexandrum*

a : A 4-week old shoot culture on MS + BAP (2×10^{-6} M).

b : Embryo callus cultured on MS + 2, 4—D (1×10^{-5} M) + BAP (1×10^{-6} M) for 4 weeks has developed numerous globular embryos.

c : Typical dicotyledonous somatic embryos attached to a piece of callus. Embryo maturation occurred on MS + NAA (2.5×10^{-6} M).

d : Somatic embryos germinated on MS basal seedling comprised two cotyledons whose base has fused to term a cotyledogary tube and a long tap root.

in the form of callus and somatic embryos at low temperature. Embryogenic calli cold stored for 7 months remained viable (95 per cent) and differentiated globullar embryos.

Picrorhiza kurroa Royal ex Benth.

Commonly known as Kutki, *P. kurroa,* (Scrophulariaceae) grows as a low hairy herb with a perennial woody rhizome, in the inner ranges of the Western Himalayas at an altitude of 3000-5000 m. The dried roots and rhizome of this plant are the source of a bitter tonic used in modern medicine as stomachic, purgative, and in dyspepsia and fever. Owing to over exploitation and the absence of systematic cultivation this species has become endangered. For the present study, the vegetative material was collected from Hemkund and Kedarnath regions of the Western Himalayas at an altitude of 4000 m.

Initiation of Aseptic Cultures

Surface sterilised terminal and nodal cuttings, measuring *ca* 2 cm, were cultured on MS, MS + BAP (5×10^{-6} M, 1×10^{-5} M, 5×10^{-5}) and MS + 2,4-D (1×10^{-6} M, 5×10^{-6} M). The sprouting of the cutting occurred only on BAP-containing media (Fig. 3A). On MS + BAP (5×10^{-6} M), the segments developed 2-3 leaves but the shoots failed to grow further. However, when these cuttings were transferred to MS with 3×10^{-6} BAP, the shoots resumed growth.

Shoot multiplication

The shoots developed in primary cultures on MS + 3×10^{-6} M BAP were excised and cultured on fresh media to test the effect of a range of concentrations of BAP, 2ip and kinetin on shoot proliferation. The cytokins were tried individually and in combination with IAA or GA_3. Shoot multiplication through forced axillary branching occurred in all the media. BAP at 1×10^{-6} M was found to be the best treatment (Fig. 3b). On an average, 12.6 shoots developed in 30 days on this medium. Higher levels of BAP were toxic. On MS + BAP (1×10^{-6} M) shoots have been multiplied for 14 months. At the end of each passage of 30 days, the long shoots were cut to prepared *ca* 2 cm long terminal and nodal cuttings (propagules) and individually transferred to fresh medium. Considering the number of propagules derived from each culture, a rate of 36-fold multiplication every 30 days was achieved.

Rooting of shoots

Attempts to root the shoots *in-vivo* were unsuccessful. However, 89 per cent of cultures on MS + 1×10^{-6} M NAA formed well developed roots (Fig. 3c), On this medium roots emerged within 20 days after culture and at the end of 30 days each shoot developed on an average 6 white stout roots. Increase in auxin level disturbed the health of the plantlet and in most cases induced callusing at the basal end of the shoot.

Short-term storage of shoot cultures

Individual shoots from MS + BAP (1×10^{-6} M) were transferred to fresh medium and after a week of acclimatisation under culture room conditions, they

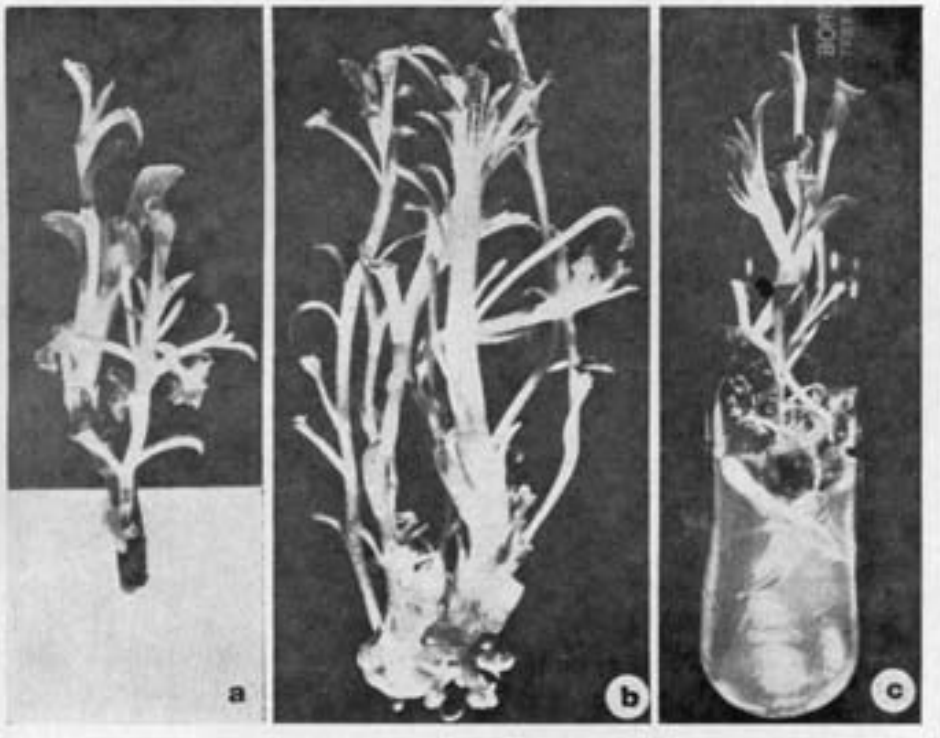

Fig. 3. *Picrorhiza kurroa*

a : Terminal shoot cutting cultured on MS + BAP (1×10^{-4} M) for 20 days, showing sprouting of terminal and an axillary bud.

b : A 15-day old proliferating shoot culture on MS + BAP (1×10^{-4} M).

c : An *in-vitro* formed shoot rooted on MS + NAA (1×19^{-4} M).

were transferred to cold storage conditions. The shoots received after 9 months of cold storage showed 70 per cent survival. A limited multiplication of shoots occurred at low temperature. When shifted to 25°C under 16 h light, viable cultures showed shoot multiplication comparable to the cultures maintained under culture room conditions throughout.

Shoot tip cultures

Shoot tips and globular embryos are the most suitable materials for long-term storage of plant materials at the super-low temperature (—196°C) of liquid nitrogen. However, cryopreservation of shoot tips for germplasm storage requires a protocol for high frequency plant regeneration from excised shoot tips. Therefore, an experiment was conducted to study the regeneration potential of shoot tips. Sub-millimeter shoot tips consisting of an apical dome and one or two leaf

primordia were excised from *in vitro* multiplied shoots, with the aid of a stereoscope, and cultured on MS, MS + 1×10^{-7} M IAA. Shoot generation occurred on all the three media, but MS + BAP (1×10^{-7} M) gave maximum regeneration (69 per cent).

DISCUSSION

In vitro multiplication of three important endangered medicinal plant species has been achieved. *Podophyllum hexandrum* has been multiplied through somatic embryogenesis and through shoot multiplication. *Saussurea lappa* and *Picrohiza kurroa* have been multiplied through shoot proliferation. Preliminary results with the storage of globular embryos of *Podophyllum* and shoot cultures of *Saussurea* and *Picrohiza* at low temperature are encouraging. Globular embryos and shoot tips will be subjected to cryopreservation for long term conservation of these species.

All the three species studied in the present work are new to tissue culture. Only one paper has been published on tissue culture of *P. kurroa* by Lal *et al.*, (1988). However, our observations are at variance with their results. This could be due to ecotypic differences in the plant materials used in the two studies. While we collected the plants from Hemkund-Kedarnath regions in Uttar Pradesh, Lal *et al.* (1988), obtained their material from Manikaran in Himachal Pradesh. Substantial ecotypic differences in *in vitro* response of *Trifolium repen* have been reported by Mahapatra and Gresshoff (1982).

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REFERENCES

Badhwar, R. L. and B. K. Sharma. 1963. A note on the germination of *Podophyllum* seeds. *Indian For.*, **89** : 445–447.

Henshaw, G. G. and J. F. O'hara. 1983. *In-vitro* approaches to the conservation and utilization of global plant genetic resources. *In* : S. H. Mantell, and H. Smith, (eds.), *Plant Biotechnology*, Cambridge University Press, U. K., pp. 219–238.

Kartha, K. K. 1984. Tissue culture techniques for virus elimination and germplasm preservation. *Plant Breed. Rev.*, **2** : 265–282.

Lal, N., P. S. Ahuja, A. K. Kukreja and B. Pandey. 1988. Clonal propagation of *Picrarhiza kurroa* Royle ex Benth. by shoot tip culture. *Plant Cell Rep.*, **7** : 202–205.

Mohapatra, S. S. and P. M. Gresshoff. 1982. Ecotypic variation of *in-vitro* plantlet formation in write clover (*Trifolium repens*). *Plant Cell Rep.*, **1** : 189–192.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassy with tobacco tissue culture. *Physiol. Plant.*, **15** : 473–497.

Nayar, M. P. and A. R. K. Sastry. 1987. Red Data Book of Indian Plants. Vol. 1 Botanical Survey of India, Calcutta.

Westcott, R. J. 1981. Tissue culture storage of potato germplasm. 2. Use of growth retardants. *Potato Research*, **24** : 343-352.

Withers, L. A. and J. T. Williams. 1986. *In-vitro* Conservation. Int. Board Plant Genet. Resour. Research Highlights, 1984-1985. IBPGR, FAO, Rome, pp. 21.