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MICROPROPAGATION OF *URARIA LAGOPUS*, A MEDICINAL PLANT, THROUGH AXILLARY BUD CULTURE AND CALLUS REGENERATION

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ABSTRACT

Micropropagation of Uraria lagopus, a leguminous herb, was achieved through axillary bud culture and nodal callus culture. Bud break was best when nodes were cultured on Murashige and Skoog (1962) (MS) medium supplemented with 2.5 µM a-naphthalene acetic acid and 2.5 µM N6-benzyladenine. Optimum shoot multiplication was obser ved in aden inesulphateat2.0µM concentration. Competent callus was initiated around the nodal ring of the explanton thebasalmedium supplemented with cytokinins and auxin ({x-naphthalene acetic acid and N6-benzyladenine), which regenerated into new profusely growing shoots on transferring to 2.5µM N6-benzyladenine. Shoots elongated to 5 node length with 2.5 µM N6-benzyladenine were rooted on full-strength MS basal medium. The rooted plants were successfully established with 80% survival. About 400 such plants were transferred to the field.

KEYWORDS: *bioprospecting; conservation; organogenesis; plant tissue culture.*

INTRODUCTION

The genus *Uraria*, a member of the family Fabaceae has more than 11 species known from India, of which *Uraria lagopus* is well known for its medicinal properties. Though this species was widely distri-buted, it is increasingly becoming rare and endemic. *U. lagopus* is a Annual herb, commonly found in dry grasslands, growing densely and producing poorly viable seeds. The whole plant is of medicinal importance and is used by certain Adivasis and native tribes (Jain and Defilipps, 1991). The pulverized leaves of this plant are used medicinally in Southern Nigeria as a remedy for gonorrhea; and in India the plant is used as an antidote against the bites of certain vipers (Allen and Allen, 1981). The plant is better known for healing fractures, which is essentially credited to its property of accumulation of phos-phorous and deposition of calcium. The root has aphrodisiac properties, and the decoction is prescribed for cough, chilis, and fever (Anonymous, 1976). Biopros-pecting studies using

medicinal plants is increasingly important both in terms of adding economic value for biological resources and also creating an economic stake in conservation (Feinsilver, 1995). However, with the growing need for medicinal plants, especially those that are rare and endangered in nature, propagation of these species in large numbers is a necessity. *In vitro* techniques offer a rapid means for multiplication of such elite and rare germplasm (Bajaj, 1986) and an alternative method for *ex situ* conservation (Kartha, 1985). Plantlet regeneration from the mature nodal segments and competent nodal callus in U. lagopus is reported here for the first time. This paper envisages the effective use of micropropagation in conservation of a medicinal plant.

MATERIALS AND METHODS

Plant material. Uraria lagopus, a annual herb was collected from the Thane, Nagzari Nagpur district, and was grown under glasshouse conditions. Profusely growing mature segments were collected and single nodal segments were cut into 3-cm pieces. These were washed thoroughly in teepol solution of extran and placed under running tap water for 30 min. Explants were pretreated with 70% ethanol before surface sterilizing with 0.1% (wt/ vol) HgC12 for 5 min, followed by three to five rinses with sterile distilled water. The surface sterilized explants were trimmed to 0.5-1-cm pieces, enclosing a single node, before initiating the cultures in culture media. *Culture media, initiation, and multiplication.* Murashige and Skoog (MS) basal salts, with 3 % sucrose and 2 % Clarigel was used as basal medium for all the experiments. Media were adjusted to pH 5.8 before adding clarigel and dispensed evenly into 50 X 300 mm rimless culture bottles, with capped and autoclaved at 121 0C for 15 min. The explants were initiated on combinations of (x-naphthalene acetic acid, NAA (0.5-2.5 µM), and N 6 benzyladenine, BA (0.5-2.5 µM'), for bud break. The percentage of explants producing single shoots was assessed at the end of 3 wk incubation. In some of these combinations, callus induction was also observed and their percentage recorded.

The induced shoots (with one node) were removed, cut terminally, and subcuhured on MS basal medium and combinations of phytohormones were tried for induction of multiple shoots. The different cytokinins tried for multiple shoot induction were BA (2.5 µM), adenine sulphate, AS (2.0 µM), and kinetin, Kn (2.5 µM/), in the basal medium singly and in combinations. The cultures were maintained on the same media for three to four subcultures. The number of newly emerging green axillary buds per explant were assessed at the end of 4 wk of incubation. Their average and standard error per treatment were computed. *Plant regeneration from callus.* Some explants showed callusing at the cut ends of the internodal segment, which were transferred to basal medium, with different combinations of BA for assessing the morphogenetic potential of the callus. Calli that had undergone three to four subcultures on the initiation medium with NAA (2.5 µM) and BA (2.5 µM) were transferred to the above combinations. At the end of 2 wk incubation in light, elongated shoots were observed. The average number of shoots per cm -2 of caulogenic callus was assessed. Shoot *elongation, rooting, and acclimatization.* The emerging new shoots were terminally cut and transferred on basal medium with different combinations of BA (0.5-2.5 µM) for elongation. The average number of nodes per explant was calculated at the end of the second subculture. Subculturing was carried out routinely at 3-wk intervals on fresh media. All cultures were incubated in the culture room at 25 + 2 0C and illuminated by cool white Jam bottles for 16 h photoperiod. After 5 wk, ell-grown plants with an average of five nodes were washed free of medium and transferred to full strength basal medium for rooting. The rooting response was 100%. The rooted plants were washed free of medium and transferred to pots with soil and cocopith in 1:1 ratio for hardening. These plants were acclimatized under controlled environment greenhpuse and glasshouse daylight conditions. After about 4 wk, the acclimatized plants were transferred to a misting unit and subsequently to normal garden soil with 80% survival on field transfer. All experiments were carried on independently and repeated at least twice. The average response and their standard error were computed using the formula;

$$SE = \acute{\alpha}/\sqrt{n}$$

ά = Standard deviation
n = Number of replicates per treatment

RESULTS AND DISCUSSION

Establishment of *in vitro* cultures of U. lagopus posed considerable problems with bacterial contamination in primary cultures, which reappeared even after three to four subcultures. This problem could be considerably reduced by treating the washed explants with 70% ethanol for 3 min and on subsequent initiations. By this method, 70% healthy cultures could be established. No bud break was seen in explants cultured only on basal salts or with 2.22 ~tM BA. NAA (4.02 ~tM) alone in the basal medium without cytokinins induced bud break in about 25% of the explants, but these microshoots remained stunted. Addition of BA (2.22 ~tM-4.4 ~tM) in combination with NAA (2.68 gtM-5.37 jiM) improved the percentage bud break response. The best growth regulator combination for bud break (82%) was recorded on MS basal medium supplemented with 2.68 gtM NAA and 2.22 ~ BA (Fig. 1). This combination was recorded as optimal concentration for bud break. With 1.61 gtM NAA, minimal callusing (5%) was observed, while with 5.37 pM NAA as high as 72% of the explants showed callusing. NAA concentration above the optimum level in combination with BA (4.4 ~tM) showed reduction in response to bud break (30%). The observation thus confirmed that the combined effect of phytohormones NAA and BA was better for axillary bud sprouting. The increasing concentration of NAA induced callusing and the sprouted new shoots were stunted, with no further growth. Experiments substituting NAA with either indole 3-acetic acid (IAA) or indole 3-butyric acid (IBA) had no profound effect on induction (data not presented). The percentage of uuinodal shoots producing multiple buds on combinations of BA, AS, and Kn is presented in Fig. 1. Pinching and tipping of primary shoots were found to be effective, when the plant material was subcultured on a fresh medium. Similar observations were reported in rose (Bressan et al., 1982). About 60-66% of the explants produced multiple shoots when cultured on medium with AS and Kn. Although as high as 68% of explants responded in the combination of BA (4.4 p-M), the average number of new buds initiated were just two buds/node, which was relatively less than that recorded on control (three buds/node). The maximum numbers (seven multiple buds/node) in 63% of the explants were recorded on medium containing 2.47 p-M AS. The efficacy of this cytokinin at this low concentration has so far not been demonstrated for micropropagation of leguminous, medicinal plants. We observed better response to multiple shoot induction on medium containing combinations of AS and Kn (1.24 ~tM-2.47 ~tM + 2.32 p-M-9.3 p-M) in comparison to any other combinations of cytokinins (Table 1). Howevel, there are no reports to confirm this in legumes, even though the cytokinin AS has been used at higher concentrations for shoot induction with reference to tree species. The percentage induction of nodal callus on MS basal medium with NAA and BA is presented in Fig. 1. The callus initiated on the basal medium with 5.37 p-M NAA and 2.22 ~tM BA when repeatedly subcultured on the same medium (three to four times) gave competent organogenetic callus (Fig. 2). The callus was tested for morphogenesis on media with BA (0-0.22 p-M). The average number of shoots per centimeter square of the callus is presented in Table 2. In low or no concentrations of BA (0-0.04 p.M), callus regenerated into four to seven shoots confirming its autotrophic nature. This confirms the earlier reports of cytokinin autotrophic nature of callus in other systems (Huetteman and Preece, 1993). Addition of BA (0.089 p-M- 0.133 p-M) increased the number of shoots per callus from 7.50 to 17.35. The higher BA concentration (0.22 p-M) reduced this induction by almost four times and BA at concentrations of 0.133 p-M gave the best response to shoot regeneration (17.35 cm -2 callus) (Fig. 3). After three to four subcultures, the shoots were excised with the node and cultured on MS basal medium with different concentrations of BA for elongation. Average number of nodes recorded per shoot on 3 wk incubation in light was around five on 1.11 ttM BA (Table 3), which was significantly better than other combinations. The new shoots from both axillary buds and callus were elongated in the above medium and were rooted on half-strength basal medium with almost 100% response (Fig. 4). The rooted plants were established in the field with over 80% success (Fig. 5). No response was seen when the nodal callus was repeatedly subcultured on basal medium with single cytokinins (BA, Kn) singly and in combinations for somatic embryogenesis. Further experiments with higher and lower concentrations of carbohydrate (0.5-5% sucrose), and decreased strength of basal medium, yielded no response to embryogenesis. In legumes, multiple shoot proliferation has been described from apical meristem (Kartha et al., 1981), cotyledonary node segment (Cheng et al., 1980), intact seedlings (Malik and Saxena, 1992), the seedling shoot tip, and cotyledonary nodes (Vaquero et al., 1993). In all these studies, either young cotyledonary tissue or immature nodal segments from young seedlings were used

for shoot multiplication.

Table: 1. Effect Of Different Phytohormones On Multiple Shoot Induction From nodal Segments Of *Urania lagopus*.

Me dia	PGR mg /l	Days to shoot initiation	Response	No. of Shoot	L ength of Shoot (cm)
MS full strength	**Control**	25-30	**2 0%**	**4 .2 ± 0.2**	**4.5 ± 0.7**
3 % Sucrose	0.5 BAP	25-30	20%	**7 .2 ± 0.1**	**4.9 ± 0.9**
1.5 % C larigel (Gelrite)	1.0 BAP	25-30	60%	**6 .4 ± 0.1**	**6.3 ± 0.3**
	1.5 BAP	25-30	70%	**5 .8 ± 0.3**	**6.5 ± 0.2**
	2.0 BAP	25-30	40%	**4 .0 ± 0.2**	**7.9 ± 0.7**
	2.5 BAP	25-30	20%	**5 .4 ± 0.3**	**6.4 ± 0.9**
	0.5 Kn	25-30	66%	**7 .2 ± 0.4**	**3.5 ± 0.6**
	1.0 Kn	25-30	88%	**8 .9 ± 0.1**	**2.5 ± 0.4**
	1.5 Kn	25-30	88%	**11.2 ± 0.3**	**2.8 ± 0.3**
	2.0 Kn	25-30	77%	**3 .8 ± 0.1**	**3.7 ± 0.1**
	2.5 Kn	25-30	77%	**4 .2 ± 0.2**	**5.9 ± 0.7**
	0.5 BAP+ 0.5 NAA	25-30	20%	**7 .2 ± 0.2**	**5.2 ± 0.1**
	1.0 BAP+ 1.0 NAA	25-30	60%	**6 .4 ± 0.5**	**4.3 ± 0.6**
	1.5 BAP+ 1.5 NAA	25-30	70%	**5 .8 ± 0.4**	**8.3 ± 0.6**
	2.0 BAP+ 2.0 NAA	25-30	40%	**4 .0 ± 0.3**	**7.5 ± 0.9**
	2.5 BAP+ 2.5 NAA	25-30	20%	**8 .4 ± 0.3**	**3.7 ± 0.8**
	0.5 Kn + 0.5 NAA	25-30	88%	**11.2 ± 0.1**	**6.6 ± 0.8**
	1.0 Kn + 1.0 NAA	25-30	99%	**7 .9 ± 0.5**	**2.3 ± 0.5**
	1.5 Kn + 1.5 NAA	25-30	77%	**3 .2 ± 0.2**	**3.8 ± 0.5**
	2.0 Kn + 2.0 NAA	25-30	66%	**3 .8 ± 0.1**	**6.4 ± 0.1**
	2.5 Kn + 2.5 NAA	25-30	66%	**3 .8 ± 0.1**	**4.1 ± 0.1**

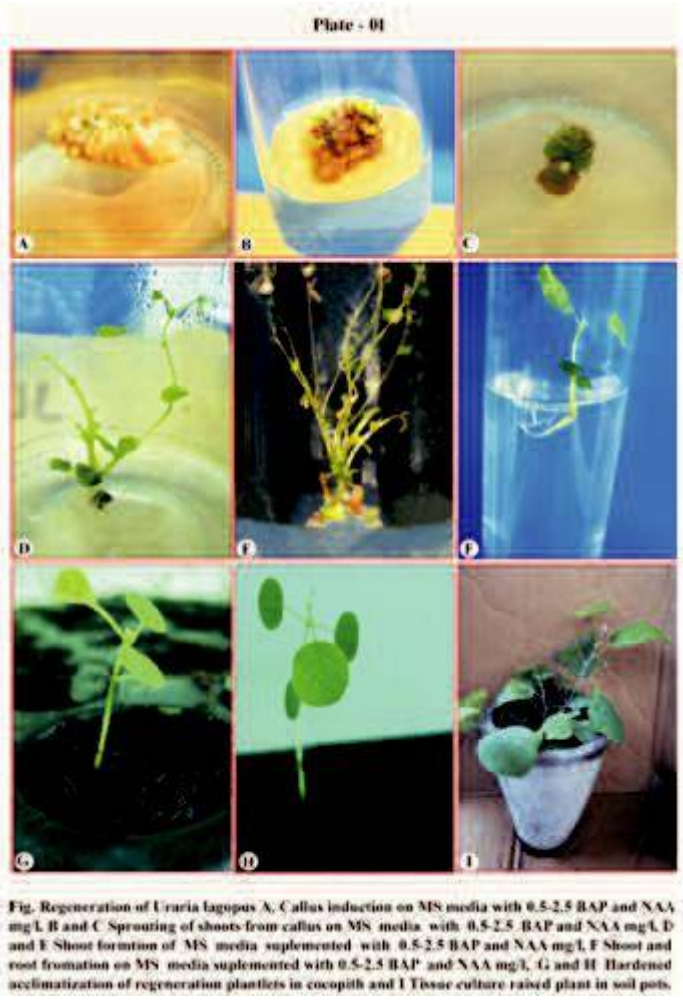

Fig. Regeneration of Uraria lagopus A. Callus induction on MS media with 0.5-2.5 BAP and NAA mg/L B and C Sprouting of shoots from callus on MS media with 0.5-2.5 BAP and NAA mg/L D and E Shoot formtion of MS media suplemented with 0.5-2.5 BAP and NAA mg/L F Shoot and root fromation on MS media suplemented with 0.5-2.5 BAP and NAA mg/L G and H Hardened acclimatization of regeneration plantlets in cocopith and I Tissue culture raised plant in soil pots.

However, the application of this method has limitation in shrubs and tree species with low seed setting, viability, and percentage germination. Kartha et al. (1981) reported that multiple shoot budding from apical meristems in legumes, adversely affected development of complete plantlets. With this protocol, both directly differentiated as well as callus-mediated shoot buds developed normally and were established with 80% survival under normal conditions. The morphogenetic potential of the mature uninodal segment is demonstrated here and these explants were organogenic as well. The efficacy of shoot multiplication with low concentrations of cytokinins, especially with AS in the absence of auxin, is against the general practice in legumes and is thus significant. The protocol developed may have immense value in conservation and multiplication of this important, but little known, medicinal plant.

Table -. Effect of auxins in root formation of Uraria lagopus.

Media	PGR mg/l	Days to root initiation	Result	No. of Root	Length of Root (cm)
MS full strenth	**Control**	20 -25	**20%**	**1.2 ± 0.1**	**0.8 ± 0.3**
3 % Sucrose	0.5 IBA	20 -25	20%	**4.2 ± 0.4**	**0.7 ± 0.2**
1.5 % Clarigel (Gelrite)	1.0 IBA	20 -25	60%	**3.8 ± 0.3**	**0.5 ± 0.2**
	1.5 IBA	20 -25	70%	**2.5 ± 0.6**	**1.4 ± 0.1**
	2.0 IBA	20 -25	40%	**4.8 ± 0.7**	**1.5 ± 0.5**
	2.5 IBA	20 -25	20%	**1.2 ± 0.1**	**1.4 ± 0.1**
	0.5 IAA	20 -25	20%	**4.2 ± 0.4**	**0.9 ± 0.1**
	1.0 IAA	20 -25	60%	**3.8 ± 0.3**	**0.5 ± 0.2**
	1.5 IAA	20 -25	70%	**3.5 ± 0.6**	**0.9 ± 0.3**
	2.0 IAA	20 -25	40%	**4.8 ± 0.7**	**0.6 ± 0.3**
	2.5 IAA	20 -25	20%	**1.2 ± 0.1**	**2.5 ± 0.2**
	0.5 NAA	20 -25	20%	**4.2 ± 0.4**	**2.3 ± 0.2**
	1.0 NAA	20 -25	60%	**3.8 ± 0.3**	**1.0 ± 0.2**
	1.5 NAA	20 -25	70%	**3.5 ± 0.6**	**2.5 ± 0.5**
	2.0 NAA	20 -25	40%	**2.8 ± 0.7**	**1.4 ± 0.7**
	2.5 NAA	20 -25	20%	**4.8 ± 0.7**	**1.7 ± 0.3**

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