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In vitro multiplication of *Peganum harmala* - An important medicinal plant

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The frequency of shoot regeneration and multiplication of *P. harmala* was influenced by the type of explant and kind and concentration of hormones. Of the various seedling explants, cotyledonary node exhibited maximum shoot regeneration frequency from axillary region on MS medium supplemented with 5 μ*M* BAP. Addition of 0.1 μ*M* NAA enhanced the efficacy of BAP for multiple shoot regeneration as well as improved the growth of shoots. BAP (5 μ*M*) in combination with NAA (0.1 μ*M*) was found to be the optimal for inducing an average of 4-5 shoots per explant in 75% of the cultures within 5 weeks. Replacement of BAP with other cytokinins at equimolar concentration of BAP i.e. 5 μ*M* was not effective in inducing multiple shoots. Regenerated shoots were rooted on MS medium containing IBA (8 μ*M*) with 80% efficiency. The plantlets were successfully established in soil where 80% of them developed into morphological normal plants.

Peganum harmala L., a perennial herb of Rutaceae is commonly known as "harmal" or "Isband" or "Syrian Rue". It is distributed over semi arid areas of North-West India, North-Africa and Central Asia. The important chemical constituents of *P. harmala* are the alkaloids harmaline, harmine, harmalol and vasicine, an essential oil and flavonoid glucosides[1,2]. The greatest amount of alkaloids is found in seeds, fruits and roots while least in the leaves[3] though all the parts of plant are used. The whole plant is used as abortifacient, aphrodisiac, emmenagogue, galactogogue and diuretic; it enriches the blood and is useful in weakness of muscles and brain[1,2,4]. Seeds are used as narcotic, hypnotic, anodyne, anthelminthic, antipyretic, antispasmodic, nauseant and emetic[1,2,4]. Leaves are useful in asthma, colic, dysmenorrhoea, hiccup, hysteria, neuralgia and rheumatism[1,2,4]. The plant has also been used as antimicrobial[5,6], in curing malaria[4] and has insecticidal potential[7].

Peganum is propagated by seeds. One of the constraints of this conventional propagation is the very short span of seed viability[8,9]. Moreover, owing to increasing exploitation of the natural population for its wide uses in traditional medicine and since, plant grows as wild only and not cultivated, it is facing the problem of extinction. There is no alternative mode of multiplication to propagate and to conserve the genetic stock of this useful plant[8,9]. Therefore, tissue culture offers an alternative method for rapid multiplication of desirable clones. Reports on the establishment of callus, cell suspension and rhizogenic cultures of harmala are available [10-13]. But, till date no reports on its *in vitro* multiplication are there. For the first time an efficient protocol for regeneration and multiplication of *Peganum harmala* using various seedling explants for obtaining healthy plants for drug and also for developing *in vitro* strategies for its conservation has been described here.

Materials and Methods

The seeds of *Peganum harmala* were obtained from a local ayurvedic medicine shop and were also collected during July 1998 from the natural population near Hissar, India. Healthy and uniform seeds were surface sterilized by agitating them thoroughly in 70% (v/v) alcohol for 1-2 min and then sterilized with 0.2% (w/v) aqueous $HgCl_2$ solution for 6 min, followed by 4 or 5 rinses in sterile distilled water. These seeds were germinated aseptically on MS basal medium[14] containing 3% (w/v) sucrose and 0.8% (w/v) agar in petriplates (9cm diam). Various explants, entire mature cotyledons, hypocotyls (10 mm) and cotyledonary nodes along with shoot tips were excised from 4-day-old-seedlings and cultured on MS medium supplemented with different concentrations of BAP either alone or in combination with NAA (0.1 and 2 μ*M*) in upright position with cut end slightly embedded in medium. The *p*H of the medium was adjusted to 5.8 before adding 0.8% (w/v) agar. Molten medium (20 ml) was dispensed into each culture tube (25x150 mm). The tubes were plugged with cotton and then autoclaved, at 1.06 kg/cm^2 pressure and 121°C for 15 minutes. At least 20 cultures were raised for each treatment and all experiments were repeated

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at least twice. The cultures were maintained under a 16hr/day photoperiod (80 μE m^{-1} s^{-1}) of cool-white fluorescent light at 25°±2°C.

The shoots (> 2cm long) were excised from the explants and transferred to half or full strength of MS medium supplemented with different concentrations of IBA (1-8μ*M*) for rooting.

The plantlets with well developed roots were thoroughly washed in tap water to remove agar medium from roots and transferred to plastic cups containing autoclaved vermiculite and sand in 1:1 ratio. The cups were then covered with a polythene bag to ensure high humidity and were kept in the culture room. Plants were watered after every 3 days with water containing ¼ of MS salts. After 12 days the plants were transferred to earthen pots containing sand and garden soil in 1:3 ratio.

Results and Discussion

The seeds of *Peganum harmala* obtained from the local market showed only 8% germination on MS basal medium in about 14 days. Treatment of H_2SO_4(25% v/v) to seeds for 5 min and/or their germination on GA_3 (0.5 μ*M*) containing MS basal medium could not improve the germination significantly. The low germination may be due to the loss of seed viability during storage. On the other hand, freshly harvested seeds showed 63% germination on basal medium in 14 days. A four-day-old-seedling had a pair of cotyledonary leaves, a long hypocotyl and a tap root.

The dose of cytokinin is known to be critical in shoot organogenesis. Therefore, the response of different seedling explants to various concentrations of BAP was compared. MS basal medium was not effective in inducing shoot buds/shoots. Incorporation of BAP to basal medium induced green nodular callus at the proximal and on the basal cut ends of the cotyledon and cotyledonary node explants, respectively. This was followed by multiple shoot formation at the proximal embedded end in cotyledon (Fig.1a) and directly in the axil of the cotyledonary node explants (Fig.1b) after 28 d of culture. BAP induced multiple shoots at the uncallused morphological upper end of the hypocotyl explants (Fig.1c). The frequency of regeneration varied with the dose of cytokinin and the type of explant. The highest frequency of shoot regeneration was observed from cotyledonary node explants followed by cotyledons and hypocotyls (Table 1). The regeneration frequency of cotyledonary node and hypocotyl explants decreased with increase in BAP concentration while that of cotyledons increased with increase in the concentration of BAP. BAP at 5μ*M* concentration was found to be the optimal for maximum frequency of shoot formation (i.e. 85%) from cotyledonary node explants. However, a maximum number of shoots (5-6) were obtained from cotyledon explants in 35% cultures on 20μ*M* BAP. The average length of shoots on cotyledon explants increased with increase in BAP concentration while that on cotyledonary node and hypocotyl explants decreased with increase in BAP concentration. The amount of basal callus formed on cotyledon and cotyledonary node explants was directly proportional to the concentration of BAP. The basal callus formed on cotyledon explant on BAP (20μ*M*) medium on transfer to the same fresh medium produced multiple adventitious shoots (Fig.1d).

Addition of NAA at low concentration (0.1μ*M*) to BAP containing medium increased the regeneration frequency and the number of shoots per explant in cotyledon and cotyledonary node cultures (Table 1). Presence of NAA in medium also proved to be beneficial for shoot elongation. On the contrary, addition of NAA to BAP medium completely inhibited the shoot forming response of hypocotyl explant due to its complete callusing. This may be attributed to the differences in endogenous level of hormones in various seedling explants. The cotyledonary node explants on BAP (5μ*M*) and NAA (0.1μ*M*) produced a maximum of 4-5 shoots in 75% of the cultures (Table 1). The effect of different cytokinins (kinetin, zeatin, 2-ip) and AdS in MS medium containing NAA (0.1μ*M*) was compared at an equimolar concentration of BAP (5μ*M*). All cytokinins and AdS increased the regeneration frequency but decreased the number of shoots per explant as compared to BAP (Table 2). AdS and kinetin in combination with NAA induced roots also at the base of shoots. Of all cytokinins tested, BAP in combination of NAA was found to be the most effective in inducing multiple shoots. Similar observations were made in other plants[15].

Well developed shoots (> 2 cm long) regenerated from cotyledonary node explants on BAP were transferred to MS medium containing IBA (1-8 μ*M*) for rooting. The number of shoots forming roots increased with increase in IBA concentration. IBA (8 μ*M*) was found to be optimal for inducing branched and thick roots at the base of 80% of shoots (Table 3). IBA at low concentrations (1-5 μ*M*) induced a small amount of callus at the cut ends of the shoots and subsequently, roots arose from the callus. In such

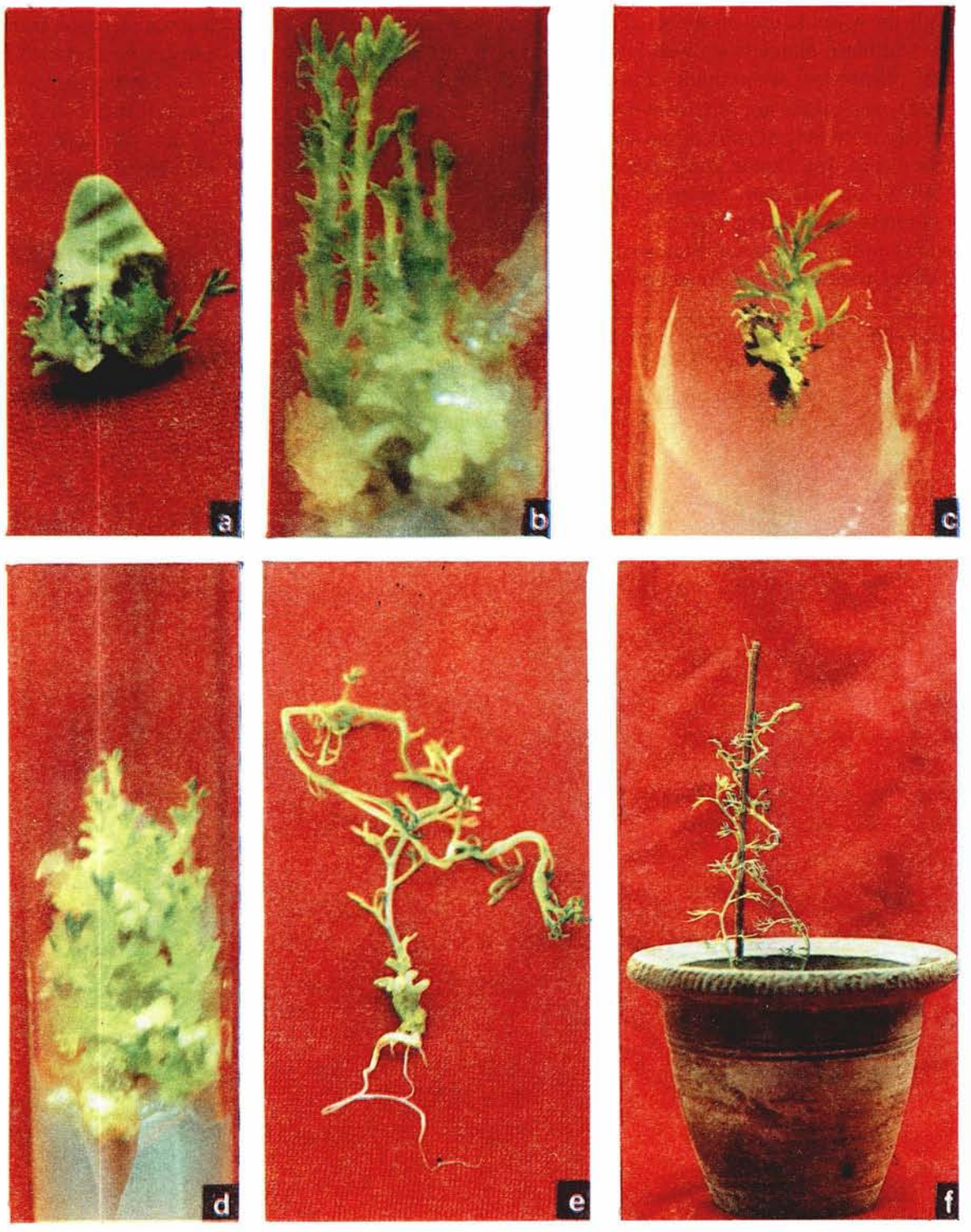

Fig. 1 (a-f)—*In vitro* regeneration of *P. harmala*. a, Regeneration of multiple shoots from cotyledon explant on MS + 10μ*M* BAP; b, shoots developing from cotyledonary node explant on MS + 5.0 μ*M* BAP + 0.1 μ*M* NAA; c, Regeneration of shoot from hypocotyl explant on MS + 5 μM BAP; d, Regeneration of shoots from subculture of callus derived from cotyledon on MS + 20 μM BAP; e, Induction of roots on shoots on MS + 8 μ*M* IBA; f, A potted plant in garden soil and sand (photographed after 3 weeks of transplantation).

plantlets, the vasculature of roots are not directly connected with the vasculature of shoots. Therefore, on transfer to soil these plantlets showed low percentage of survival. However, when high concentration of IBA (8 μM) was added to MS, root formation occurred directly from the cut ends of the shoots without any callusing (Fig. 1e). Hence, these plantlets showed higher (80%) survival and resumed growth upon transplantation to the soil (Fig.1f).

In conclusion, the present study showed for the first time the direct multiple shoot induction from cotyledonary node and hypocotyl explants of *P. harmala*. The direct shoot regeneration from cotyledonary nodes is efficient and can be exploited to multiply elite genotypes and also for developing *in vitro* strategies for the conservation of this useful medicinal plant. The regeneration of plants from cotyledon callus may help in selection of desirable somaclones.

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Table 1—Effect of different concentrations of BAP either alone or in combination with NAA on multiple shoot regeneration from different seedling explants of *P. harmala* after 7 weeks of culture.

Explant	Plant growth regulator (μM)		Culture regenerating shoot (%)	Numberm of shoots/explant (mean±SE)*	Mean length of shoot (cm)	Basal callus
	BAP	NAA				
Hypocotyl	0	0	0	0	—	—
	1	—	0	0	0	—
	5	—	14.2	2±0.17^a	0.8	—
	10	—	9.5	1±0.00^b	0.6	—
	15	—	9.5	3±0.21^c	0.5	—
	20	—	0	0	0	—
	5	0.1	0	0	0	+
	10	0.1	0	0	0	+
	5	2	0	0	0	++++
Cotyledon	0.5	—	0	0	0	+
	1	—	10	1.5±0.11^a	0.5	+
	5	—	10	2±0.18^a	0.5	+
	10	—	15	2.3±0.21^{ac}	0.9	+
	15	—	20	1.5±0.20^a	1	++
	20	—	35	5.1±0.22^b	2.7	+++
	10	0.1	45	5±0.25^b	3	+++
	5	0.1	10	1±0.00^{ad}	0.6	++
	5	2	0	0	0	++++
Cotyledonary	5	—	85	2.5±0.23^a	2.3	+
node with	10	—	75	3±0.26^a	2.1	+
shoot tip	15	—	60	4±0.27^a	1.9	++
	20	—	50	3±0.26^a	1.8	++
	5	0.1	75	4.3±0.29^a	3.3	++
	10	0.1	50	2±0.19^a	3.2	+++

Number of (+) signs indicates the degree of callusing.

*For different explants separately, the mean values followed by the same letter are not significantly different at P=0.05 (Newman-Keul's test).

Table 2—Effect of different cytokinins with NAA on shoot regeneration from cotyledonary node explants of *P. harmala**.

Plant growth regulators conc.(μM)	Culture regenerating shoots (%)	Number of shoot/explant (mean±SE)	Mean length of shoot (cm)	Shoot Cultures with root (%)	Basal callusing in explant (%)	Basal callusing Intensity
2ip+NAA (5) (0.1)	94	1.4±0.22[a]	4.8	0	100	++++
Kinetin+NAA (5) (0.1)	100	1.2±0.19[a]	3.4	40	80	++
AdS + NAA (5) (0.1)	94	1±0.00[a]	2.8	58.8	41.1	+
Zeatin + NAA (5) (0.1)	100	2.2±0.46[b]	3	0	100	++++

* Data recorded after 8 weeks of culture.
Mean values followed by the same letter are not significantly different at P=0.05 (Newman-Keul's test).
Number of (+) sign indicates the degree of callusing.

Table 3—Effect of different concentrations of IBA on rooting of shoots. Data recorded after 3 weeks of culture

IBA Conc. (μM)	Percentage of shoots rooted	Basal callusing (%)
1	25	+
2.5	25	+
5	55	+
8	80	–
5*	65	+

* ½ strength MS medium supplemented with 2% sucrose.
+/– indicates presence/absence of callusing.

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