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RESEARCH ARTICLE

INDUCTION OF MULTIPLE SHOOTS AND IN VITRO FLOWERING FROM NODAL EXPLANT OF MOLLUGO PENTAPHYLLA L A POTENT NUTRACEUTICAL HERB

Anju G Nagannawar* and M. Jayaraj

P. G. Department of Botany, Karnatak University Dharwad, Karnataka state-580003, India

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ABSTRACT

Mollugo pentaphylla L. is an nutraceutical herb, and is used by traditional practitioners for curing whooping cough, treatment of eye diseases, to treat sprue, mouth infections. The leaves are used to make a soup that is said to promote the appetite, used to treat stomachache, earache, skin disorders and abdominal pains. This plant in nature propagated itself through seeds only during the rainy season and not available in other seasons. To overcome these constraints *in vitro* propagation protocol for *Mollugo pentaphylla* L. has been developed using nodal explants. Induction of nodal callus observed with Murashige and Skoog's (MS) media containing 0.5 mg/L of BAP and NAA with mass 2829.11±1.82 (mg). MS media containing 0.5 mg/L of BAP and 0.5 mg/L of Kn was found to be suitable for multiple shoot induction 26±1.95 with length 15.82±0.97 and MS media with 0.5 mg/L of BAP and 0.5 mg/L of NAA induced multiple shoots 20.03±2.17 with length 14.08±1.97 from the nodal callus. Roots were induced on half-strength MS medium containing 0.5 mg/L of NAA and 0.5 mg/L of IBA subsequently, *in vitro* flowering observed after 3 weeks on the same culture.

INTRODUCTION

Tissue culturing of medicinal plants is widely used to produce bioactive compounds. It is also used to multiply and conserve of genetic material of many threatened medicinal plants. At present, there are many well established protocols for multiplication and conservation of herbal and medicinal plants. Many herbal practices which are popular in many parts of the world (Ahmed *et al.*, 2010; Muthukumar *et al.*, 2016; Utami *et al.*, 2017 and Lattoo *et al.*, 2006; Nagesh and Shanthamma, 2011; Sahay and Varma, 1999; Castillo *et al.*, 2000). Hence, herbal medicines are being proved as effective as synthetic drugs with lesser side effects and are in line with nature, with less hazardous reactions (Carmona-Martin and Petri, 2020; Uma *et al.*, 2016; Purohit *et al.*, 2015). Plant biotechnology plays an important role in the mass multiplication and conservation and also to produce number of plant based drugs. The biotechnological tools are important to select, multiply and conserve the plants. Most of the medicinal plants either do not produce seeds, geminate, or seeds with low viability.

In vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines, mass propagation and conservation of medicinal plants (Sidhu, 2011). *Mollugo pentaphylla* L., of Molluginaceae is an annual, glabrous pubescent, diffuse weed found throughout India (Cooke, 1958). It is found on wet rocks, sandy soil, roadsides and certain waste land. This plant is used as medicine for the treatment of eye diseases, to treat sprue and mouth infections, the leaves are used to make a soup that is said to promote the appetite, used as a stomachic and in earache, skin disorders and abdominal pain. It is also used as antipyretic (Sahu *et al.*, 2011), antiseptic, appetizer, antidiabetic (Maharana *et al.*, 2012) anticancerous, antitoxic, hypoglycemic (Bhubanesar, 2010), antioxidant (Valamathi *et al.*, 2011) and diuretic agent. As there are no reports on micropropagation studies on this medicinal plant, the present study was focused on the development of protocol for direct and indirect regeneration and mass propagation (Fig. 1).

MATERIALS AND METHOD

Plant Material and Disinfection: *Mollugo pentaphylla* L., belonging to family Molluginaceae is an annual, glabrous pubescent, diffuse weed found throughout India. It is found in wet rocks, sandy and roadside footpaths (Fig. 2A).

*Corresponding author: Anju G Nagannawar,
P. G. Department of Botany, Karnatak University Dharwad,
Karnataka state-580003, India.

It is an erect slender annual herb up to 10-20 cm. high and arid areas. Leaves are whorled or opposite, linear-lanceolate to obovate. This plant characterized with pentangular stem and five leaves with different size at the node. Flowers are white in terminal compound cymes. Stamens three, style three, capsules sub-globose with numerous seeds and are roundish rainy form, compressed covered with raised tubercular point, dark brown (Fig. 2 A). *Mollugo pentaphylla* L. was collected from Kamatak University Campus, Dharwad, State Karnataka, India during the month of September and authenticated by one of the authors in the Department of Botany, Kamatak University Dharwad. Healthy explants like nodal explants were selected and washed thoroughly under running tap water for 15 mins to wash off the dirt and microbes present on the surface. The explants were cut (1-2cm) separately and they were washed with two drops of Tween 20 detergent solution for 10 mins. After, that they were thoroughly washed under running tap water until the traces of Tween 20 was removed and then rinsed with distilled water. The standard steps of surface sterilization were carried out under aseptic conditions in the Laminar airflow chamber. The plant was then subjected to 70% ethanol treatment for 30 sec and again washed with distilled water at least three to four times. After washing with distilled water, surface sterilization was done with mercuric chloride (0.1%w/v HgCl₂) solution for 2 min and rinsed four to five times with sterilized distilled water. Sterilized nodal explants were inoculated on Murashige and Skoog (MS) medium for direct and indirect regeneration.

Culture Media and Conditions: Murashige and Skoog's medium (1962) used to test the response of nodal explants using different concentrations and combinations of growth regulators. The nutrient medium containing inorganic and organic compounds was stabilized with 0.4 % Taxim (antibiotic), the pH of media was adjusted to 5.6 - 5.8 with NaOH solution (1N) or HCl solution (0.1 N) before autoclaving at 120°C for 20 mins under pressure of 1.5±0.1 kg. cm². Inoculations of explants into culture tubes (150 X 25 mm) containing 15 to 20 ml of MS medium and plugged tightly with non-absorbent cotton done under aseptic conditions in Laminar air-flow cabinet. All the cultures were incubated under tube light provided by cool white fluorescent tube provided by 40W (Philips, Mumbai, India) at a photoperiod of 16 hr at 25±2°C and 70-80% humidity.

Induction of nodal callus on MS with 0.5mg/L BAP And NAA: Nodal explants were inoculated on MS media supplemented with 0.5 mg/L of BAP and NAA for the induction of nodal callus.

Response of nodal explants on MS medium with different concentrations and combinations of cytokinins: Nodal explants were inoculated on to the different concentrations of BAP (0.2 to 2 mg/L) and Kn (0.2 to 2 mg/L) for the induction of multiple shoots through both indirect and direct organogenesis.

***In vitro* root induction:** Regenerated plantlets from *in vitro* raised multiple shoots (both from indirect and direct organogenesis) were separated and individual shoots (8-14 cm) were transferred to MS medium consisting of 0.5 mg/l of NAA (α -naphthaleneacetic) and 0.5 mg/l of IBA (Indol-3-butylric acid) for root induction. All the cultures were

incubated under the same conditions was maintained during plantlet regeneration.

Hardening and Acclimatization: The well-rooted *in vitro* plantlets were removed from the culture bottles and washed with slightly warm sterile distilled water to remove the agar traces and dipped in fungicides (taxim) for a few minutes. Then the plantlets were transferred to Vermiculite: Sand: Garden soil (1:1:1) in polystyrene (50.28 cc) and covered with polythene bags having small holes and maintained under culture room tube light provided by cool white fluorescent tube lamps at a photoperiod of 16h at 25±2°C and 70-80% humidity for one week. The pots were irrigated with quarter strength of MS basal salts solution and when planted, then plants produced new leaves, after few days they were transferred from culture room to greenhouse and eventually to field conditions. Survival percentage was recorded for two weeks transferring to an open field.

Data collection and statistical analysis: Data on the percentage response per explant on MS medium with different combinations of plant growth regulators (i.e., number of shoots, shoot lengths, number of roots and root lengths) were recorded after 5 weeks of culture. The data were analyzed statistically using IBM SPSS Statistics v 20 software. The significance of differences among the means was calculated using Duncan's Multiple Range Test (at $P \leq 0.05$).

RESULTS

Experiment was conducted to test the response of nodal explants on different types of plant growth regulators such as BAP, Kn individually and in the combination of BAP and Kn on Murashige and Skoog's medium with different concentrations and combination (Graph-1).

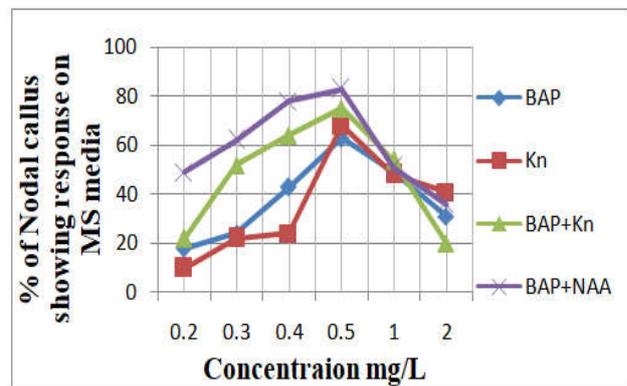
Induction of nodal callus: Nodal explant cultured on MS medium fortified with 0.5 mg/L of BAP and 0.5 mg/L of NAA induced compact green callus with mass 2829.11±1.82 (mg). (Table 1: Fig. 2B).

Induction of multiple shoots and *in vitro* flowering: Nodal explant cultured on MS medium fortified with different concentrations and combinations of cytokinins (BAP and Kn) for multiple shoot induction. Induction of multiple shoots 20.03 ±2.17 with length 14.08 ±1.97 from nodal callus on MS medium with 0.5 mg/L of BAP and 0.5 mg/L of NAA has been observed (Fig.-2 C and 2 D). However, highest number of shoots buds (26.08 ±1.95) produced on medium with 0.5 mg/L of BAP and 0.5 mg/L of Kn with length (15.82 ±0.97) within 4 weeks of incubation (Fig.-2 E). Subsequently, *in vitro* flowering observed after three weeks from nodal callus derived shoots on MS+0.5 mg/L of BAP and Kn (Fig.-2 E). Number of shoot buds with leaves have been recorded. Whereas, on MS medium with individual growth regulators, BAP (0.5 to 2mg/L) has produced (13.42±1.40) shoots with average length (7.90±0.48) and Kn (0.5 to 2mg/L), (15.08 ±1.34) shoots with average length (10.12 ±0.55) produced less number of shoots compared within a combination of BAP and Kn (Fig.-2 D). The present investigation revealed that the combination of BAP with 0.5 mg/L and Kn with 0.5 mg/L were most effective for the induction of multiple shoots. Whereas MS medium with BAP and Kn individually found to be less effective for the

induction of multiple shoot buds than in combination. Thus obtained shoots subjected to induction of root and subsequently transferred to soil (Table-2; Fig. 2 F).

Induction of *in vitro* root: *In vitro* shoot buds were transferred to MS half-strength liquid medium with different concentrations and combinations of 0.5 mg/L of IBA and 0.5 mg/L of NAA produced *in vitro* roots, (Table-3, Fig-2 F).

Hardening and Acclimatization: *In vitro* grown plantlets were gently removed from culture tubes and washed with slightly warm (35°C) distilled water to remove all traces of nutrient medium (Pattar and Jayaraj, 2012). Subsequently they were transferred to polystyrene cups containing a 3:1 (v/v) mix with sterile vermiculite and sand and maintained by covering the cups with polythene bags. The plantlets were irrigated with MS basal salt solution (quarter strength) (Bhattacharyya *et al.*, 2016) and regularly sprayed with distilled water for one week and maintained at room temperature 25±2°C with 70-80% humidity. The polythene bags were removed after four days and plantlets were acclimatized for one week in an aseptic culture room condition. Further, the plantlets were exposed gradually to sunlight for acclimatization and were maintained in a garden. The well-established plantlets were transferred to pots containing Vermiculite: Sand and Soil (1:1:1). These plants were gradually transferred from culture room condition to greenhouse and eventually to the field condition and their survivability rate was 90% (Fig. 2 G).



Graph 1. Morphogenic response of nodal callus of *Mollugo pentaphylla* L. on MS medium containing different concentrations of cytokinins and auxin

DISCUSSION

Present study revealed that MS media with 0.5 mg/L of BAP and 0.5 mg/L Kn was found more effective for induction of multiple shoots from nodal callus of *Mollugo pentaphylla* L. Similar report on multiple shoot induction from shoot tip of *Mollugo nudicaulis* Lam. (Nagesh and Shanthamma, 2011) on MS media supplemented with combination of 2 mg/L of BAP and 1 mg/L of IBA. Leaf and nodal explants of *Aristolochia indica* L. (Pattar and Jayaraj, 2012). induced multiple shoots on MS media with BAP (0.8 mg/L) NAA (0.5 mg/L), similarly both leaf and nodal explants cultured on MS medium supplemented with 0.8 mg/L BAP developed into mass of callus and subsequently developed into shoots and roots on MS medium supplemented with 0.8 mg/L BAP + 0.5mg/L NAA. Nodal explants of *Sida cordifolia* L. (Pattar and Jayaraj, 2012). cultured on MS medium supplemented with 0.5 mg/L Kn. Subsequently this nodal calli differentiated into multiple shoots on MS medium supplemented with 0.5 mg/L Kn and 0.5 mg/L NAA. The effect of induction of multiple shoots of the combination of BAP and NAA observed in present study was also reported for *Bauhinia vahlii* (Upreti and Dhar, 1996) from nodal explant with 1.0 µM of BAP and 1.0 µM of NAA. Present investigation indicated that the nodal callus of *Mollugo pentaphylla* L. was found to be more suitable for induction of multiple shoots on different concentrations and combinations of BAP (0.5 mg/L) and Kn (0.5 mg/L).

The stimulating effect of BAP and Kn on induction of multiple shoots has been reported earlier for several medicinal and aromatic plants namely, *Hemidesmus indicus* (Patnaik and Debata, 1996) from nodal explant with 4.44 µM of BAP and 0.05 of NAA and *Hypeicum androsaemum* (Dias *et al.*, 2000) from stem explant with 4.5 µM NAA and 2.3 µM on MS medium. *In vitro* root induced in the present work with different concentrations and combinations of 0.5 mg/L of IBA and 0.5 mg/L of NAA, similarly nodal callus of *Reseda pentagyna* (Al-Qurainy *et al.*, 2018) with 1mg/L of NAA and 0.8 mg/L of IAA, nodal callus *Spilanthes acmella* (Nabi *et al.*, 2018) with 1 mg/L of NAA and 0.8 mg/L of IAA and leaf and stem callus of *Heliotropium indicum* L. induced roots in the combination of auxin 2-4, D, NAA and BAP at 1.0 mg/L to 3.0 mg/L and cytokinin Kn and BAP 2 mg/L (Bagadekar and Jayaraj, 2011).

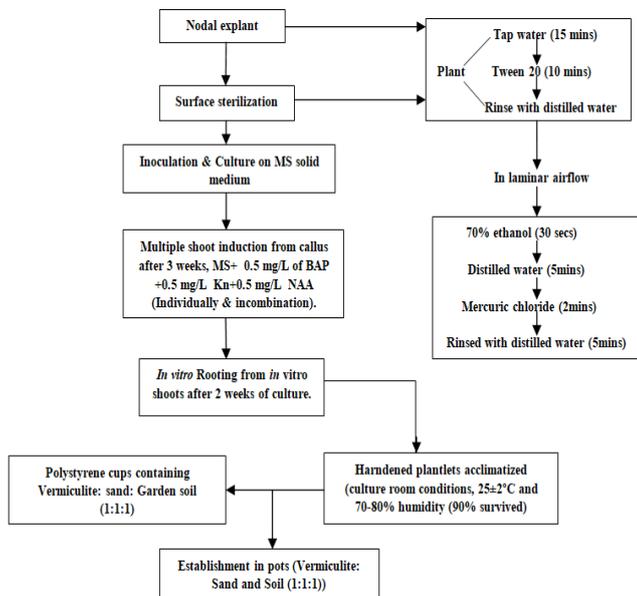


Figure 1. Schematic representation of the protocol for induction of multiple shoots from nodal explant of *Mollugo pentaphylla* L.

Table-1: Induction of callus from nodal explant of *Mollugo pentaphylla* L.

Sl. No.	MS medium supplemented with combination and concentrations of hormones (mg/L)		Mass of nodal callus (mg)
	BAP	NAA	
1.	0.2	0.2	119.08±0.82 ^a
2.	0.3	0.3	347.11±1.20 ^b
3.	0.4	0.4	1821.28±0.31 ^b
4.	0.5	0.5	2829.11±1.82 ^c
5.	0.8	0.8	2250.24±2.03 ^c
6.	1.0	1.0	724.22±0.03 ^d

Table 2. Induction of multiple shoots from nodal callus of *Mollugo pentaphylla* L. on MS media supplemented with different growth regulators

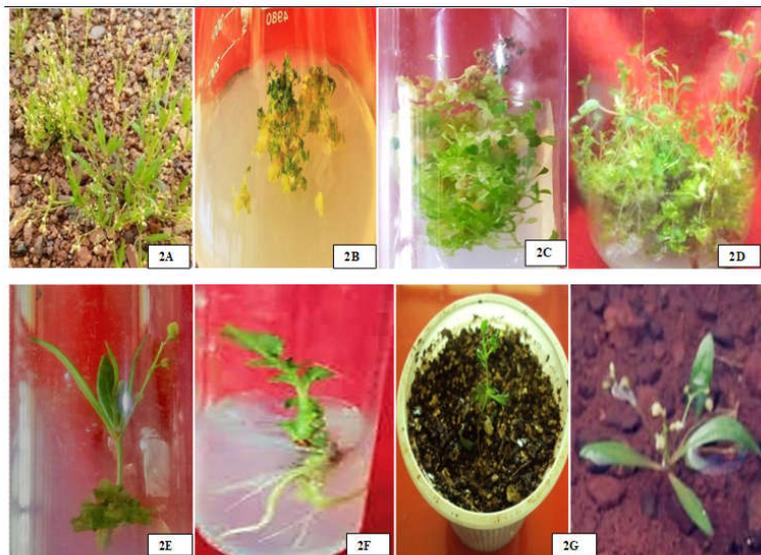
Plant growth regulators (mg/L)				% of shoot response	Number of shoots (mean±SE)	Shoot length (cm) (mean±SE)
BAP	Kn	BAP+Kn	NAA			
	0.2			18.06±1.73 ^{ab}	2.48±0.22 ^a	2.64±0.31 ^a
	0.3			24.79± 3.09 ^{ab}	3.22± 0.31 ^a	3.80±0.46 ^a
	0.4			43.33± 6.66 ^{bc}	19.10±1.85 ^{bc}	6.78±0.71 ^b
	0.5			63.32 ±5.00 ^d	23.42±1.40 ^c	11.90±0.48 ^c
	1.0			49.99± 3.33 ^c	18.72 ±0.63 ^a	5.12±0.86 ^b
	2.0			31.53± 1.95 ^{ab}	4.42±0.99 ^a	2.04±0.21 ^a
			0.2	10.11 ±2.95 ^{ab}	3.04 ±0.51 ^a	1.62 ±0.20 ^a
		0.2		22.26 ±1.46 ^{ab}	8.72 ±0.70 ^{ab}	2.52 ±0.23 ^a
		0.3		24.99 ±7.45 ^{bc}	11.78 ±1.22 ^c	8.52 ±0.42 ^b
		0.4		68.33 ±6.23 ^c	21.08 ±1.34 ^c	10.12 ±0.55 ^c
		0.5		58.99 ±4.55 ^{ab}	9.80 ±1.15 ^c	7.86 ±0.12 ^a
		1.0		41.66 ±4.24 ^{ab}	2.76 ±0.18 ^a	1.74 ±0.28 ^a
		2.0		22.19±3.84 ^e	8.0±0.63 ^a	4.20 ±0.28 ^{ab}
			0.2+0.5	52.74±3.59 ^a	14.0±1.04 ^a	10.74 ±0.08 ^a
			0.3+0.5	64.99±5.89 ^a	20.8±0.86 ^a	13.70 ±0.81 ^{bc}
			0.4+0.5	75.69±10.50 ^c	26.08 ±1.95 ^d	15.82 ±0.97 ^c
			0.5+0.5	54.99±7.72 ^b	19.20±1.35 ^c	9.50 ±0.70 ^d
			1.0+0.5	20.09±11.44 ^a	11 ±1.44 ^b	6.50±0.57 ^c
			2.0+0.5	49.02 ±1.67 ^a	1.61±0.30 ^b	1.70 ±0.24 ^a
			0.2	62.16 ±2.03 ^a	1.91 ±0.73 ^a	2.0 ±0.34 ^a
0.2			0.2	78.19 ±4.20 ^b	4.06 ±0.52 ^a	4.60 ±0.46 ^c
0.3			0.3	83.05 ±5.49 ^c	20.03 ±2.17 ^c	14.08 ±1.97 ^{ab}
0.4			0.4	51.38 ±4.22 ^b	11.0 ±4.63 ^b	7.05 ±0.89 ^b
0.5			0.5	36.80 ±1.29 ^a	4.36 ±0.46 ^a	2.73 ±0.57 ^a
1.0			1.0			
2.0			2.0			

Each value represents the mean ± Standard error of triplicates, followed by superscript letters through columns that differ significantly at P<0.005 level when subjected DMRT followed by SPSS. BAP, benzy lhm inopurine; Kn, kinetin; NAA, naphthaleneacetic acid.

Table 3. Induction of *in vitro* roots from *in vitro* shoots of *Mollugo pentaphylla* L. on MS medium with 0.2- 2.0 mg/L of NAA and IBA

MS medium + NAA(mg/L)	IBA (mg/L)	% of plantlets producing roots	Number of roots/shoot	Root-length (cm)
0.2	-	10.02 ±2.81 ^{ab}	3.60± 0.87 ^a	1.24± 0.11 ^a
0.3	0.5	21.66±4.24 ^{bc}	12.40± 1.36 ^b	2.76 ±0.21 ^{ab}
0.4	0.5	31.66±4.85 ^c	17.80±0.86 ^c	4.20 ±0.75 ^{bc}
0.5	0.5	66.66±4.56 ^d	18.80± 1.95 ^d	9.08 ±1.02 ^d
1.0	0.5	26.66±9.99 ^c	8.0 ±1.30 ^a	4.88 ± 0.50 ^c
2.0	0.5	5.11 ±3.04 ^a	4.0 ±1.87 ^a	2.62 ± 0.40 ^{ab}

Each value represents the mean ± Standard error of triplicates, followed by superscript letters through columns that differ significantly at P<0.005 level when subjected DMRT followed by SPSS. NAA, naphthaleneacetic acid; IBA, indole-3-butyric acid.



2A-Habit- *Mollugo pentaphylla* L., 2B-Induction of nodal callus with 0.5mg/L of BAP and NAA, 2C-Multiple shoot induction from nodal callus, 2D-Combined effect of BAP and Kn and high frequency of shoots were produced on medium with 0.5 mg/L of BAP and Kn with NAA of 0.5 mg/L within 4 weeks of incubation, 2E-*In vitro* flowering from nodal explants on MS 0.5 mg/L of BAP and Kn , 2F-*In vitro* root induction from nodal explant with NAA and IBA 0.5 mg/L., 2G-Hardening in polystyren cup and Acclimatized plant.

Figure 2. A-G. Induction of nodal callus and multiple shoots from nodal explants

Conclusion

Induction of multiple shoots and *in vitro* flowering from nodal callus of *Mollugo pentaphylla* L. was achieved on MS medium supplemented with plant growth regulators namely BAP, Kn and NAA. These plantlets were regenerated roots in presence of NAA and IBA. 90% of regenerated plantlets survived when acclimatized in a mixture of soil with vermicompost. This micropropagation protocol of *Mollugo pentaphylla* L. is more effective, reproducible and commercially viable and may be used to make the plant available throughout year both for pharmaceutical uses and *ex-situ* conservation.

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Conflict of interest: The authors declare that there is no conflict of interest.

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