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Effect of different plant growth regulators on *in vitro* propagation of *Clausena heptaphylla* (Roxb.): An aromatic and medicinal shrub

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Abstract
An efficient and reliable micropropagation technique for *Clausena heptaphylla* (Roxb.) was developed using different explants and media. Shoot apex and nodal explants of field grown plants were aseptically cultured on Murashige and Skoog (MS) medium supplemented with different concentration and combinations of four plant growth regulators (PGRs,) namely IAA, NAA, BAP and Kn. Direct multiple shoot buds developed within 20 days in case of all explants in most media tested. The highest number of multiple shoot buds (4.20 ± 0.18) was obtain from nodal explants on MS medium containing 2.0 mg/l BAP+1.0 mg/l IAA. Multiple shoot buds underwent rapid elongation (5.60 cm) on MS media fortified with 1.5 mg/lBAP+1.0 mg/l IAA. Elongated shoot buds produced strong and stout roots in rooting media. Half strength MS media supplemented with1.0 mg/l IBA + 0.5 mg/l IAA was better for induction and proliferation (3.56 ± 0.10) of roots. 91% of plantlets were successfully acclimatized to *ex vitro* condition, exhibiting a normal development.

Keywords: *Clausena heptaphylla***,** micropropagation, explants, shoot buds, *In vitro.*

1. Introduction
Clausena heptaphylla (Roxb.) commonly known as Panbahar, belongs to the family Rutaceae. It is an important aromatic and medicinal shrub. The leaves of the species emit a most agreeable fragrance, arise seed. Occasionally grows wild in Chittagong and Chittagong hill Tracts. The plant distributed in South and Southeast Asia. Stem and roots of the plant contain carbazole alkaloids, calusines the coumarins clausenin. Leaves contain clausenlactum, safrole, glutinol, steroidal glycosides, rutin and a carbazole alkaloid, clausine-L. Previous phytochemical studies with *Clausena* led to the isolation of carbozole allkoids [1], clausmarin [2], lunamarin C [3], cumarins [4] and limnoids [5]. The sap of leaves is rubbed on all kinds of muscular pain and boil. The plant also possesses antibacterial activity. The new carbazole alkaloids showed significant inhibition of rabbit plantlet aggregation and caused vasocontraction. In Bangladesh, there is virtually no cultivation of medicinal plants on any significant scale [6] for commercial purpose. It is therefore important to take immediate steps for large scale propagation of this medicinal plant species, to save it as well as to meet the demand of traditional ayurvedic industries.
Plant tissue culture techniques have become powerful tools for studying and solving the basic and applied problems in plant propagation and genetic manipulation. In recent year these techniques have gained greater momentum in commercial application in the field of plant propagation, mainly in horticulture as well as for medicinal plant species [7-16].
A number of medicinally important plant species have been successfully propagated on a mass scale with the use of *in vitro* technique [17-20]. *In vitro* propagation helps in production of very large number of plants from a tiny explant. In the recent past over exploitation of the medicinal plant panbahar (*Clausena*) from the natural resources by traditional herbalists led to become a rare one. The herbal medicine companies of Bangladesh demand this species on a mass scale for commercial uses, which can only be achieved by tissue culture technique. Accordingly the present investigation was under taken with a view to develop a reliable and efficient protocol for mass scale propagation, and to compare the effect of different plant growth regulators (PGRs) on development of this plant species.

2. Materials and methods

2.1 Plant material and explant preparation

The shoot Apex and nodal segments of field grown plants of *Clausena heptaphylla* (Roxb.) were collected and thoroughly washed under running tap water for 10 minutes, treated with liquid detergent for 10 minutes, followed by dipping in 5% (v/v) savlon solution for 10 minutes. The materials were then washed 6-7 times with distilled water. After rinsing with 70% ethanol for less than 60Seconds, they were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 minutes and washed with sterile double distilled water 4-5 time after each surface disinfection treatment under aseptic conditions.

2.2 Preparation of explants for inoculation

In the laminar airflow cabinet the explants were kept on a sterile aluminium slab and were cut into pieces (0.5 - 1.0cm) with a sterilized surgical blade and then inoculated onto the culture media. MS basal medium supplemented with different concentration and combination of plant growth regulators (PGRs) such as BAP, Kn, NAA and IAA were used for induction of organogenesis or embryogenesis. In some cases the multiple shoot buds (MSBs) that developed from nodal explants or from shoot apex elongated on MS supplemented with different PGRs and for rooting, elongated shoots at a height of 2-4 cm were rescued aseptically from the cultured on rooting medium containing half strength MS medium fortified with different concentration and combinations of IBA, IAA and NAA. In all cases the media were solidified with 0.8% (w/v) agar and P^H was adjusted to 5.8 prior to autoclaving for 30 minutes at 121 ^{0}C under 1.1kg/cm^2 pressure.

2.3 Incubation of culture vessels with explants

The culture vessels with inoculated explants were then taken to the culture room for incubation where a cycle of 14 hours continuous light and 10 hours continuous dark phase was maintained. The room temperature was maintained at 25±2 ^{0}C.

2.4 Subculture for multiple shoots

Proliferated multiple shoots were rescued very carefully in aseptic conditions and divided into clusters of 2-3 shoots using a sterile sharp scalpel. Sub culturing was done on the same or different media for further response at an interval of 15-20 days and culture vessels were maintained in the culture room in the same light and temperature conditions.

2.5 Rooting of micro-shoots

Experiments of adventitious root formation on the shoots proliferated *in vitro* were conducted only after having sufficient amount of shoot cultures. Different rooting experiments were carried out with half strength MS medium with or without growth regulators to determine the suitable media composition, optimum growth requirements. After 10-20 days, the proliferated multiple shoots were separated and individual shoots were placed in rooting media.

The adventitious roots were produced from the cut ends of micro shoots within 2-3 weeks of culture on suitable medium. Elongated shoots at 2-4 cm height were rescued aseptically from the culture vessels and cultured on freshly prepared rooting medium.

2.6 Acclimatization and transfer of plantlets to soil

The well rooted plantlets were transferred to pots containing a mixture of soil and compost (2:1) following successive phases of acclimatization. For the purpose, the month of the culture vessels were kept open for one day in the culture room and they were then kept outside the culture room for 6 hours in the next day. Later on those were kept outside the culture room for 12 hours. Finally the seedling were taken out of the culture vessels and rinsed with running tap water for complete removal of medium attached to the roots.

2.7 Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explants and number of roots per shoot. Means and standard deviations were calculated for each treatment. The data means ± SD of at least three different experiments were represented.

3. Results and discussion

Both Shoot apex and nodal segments were aseptically grown on MS medium supplemented with different concentration and combinations of auxins and cytokinins (Table - 1). Both explants gave differential responses to different PGRs combinations. The shoot apex and nodal segments underwent direct organogenesis producing multiple shoot buds (Figure-1A&B) on MS medium supplemented with 0.5 - 2.0 mg/l BAP or Kn in combination with 0.5-1.0 mg/l IAA/NAA. The efficiency of the media was assessed based on the number of shoot buds produced per explants. The maximum number of shoot buds per explant was produced on MS with 2.0 mg/l BAP + 1.0 mg/l IAA. The shoot apex and nodal segments gave positive response and such differentiation was also noted in other medicinal plants including, *Eucalyptus tereticornis* [21], *Ocimum basilicum* [22], *Plumbago indica* [23], *Eclipta alba* [24], *Boerhaavia diffusa* [25], *Boerhaavia diffusa* [26], *Gentiana kurroo* [27], *Helicteres isora* [28].

The multiple shoot buds that developed directly from shoot apex and nodal segments underwent elongation when individually grown on different PGRs supplemented elongation media (Table-2). The efficiency of a medium was assessed in terms of enhancing elongation of shoots. The longest shoot buds (Figure-1C) were (5.60 cm) developed on MS medium supplemented with 1.5 mg/l BAP + 1.0 mg/l IAA. Such enhancement of elongation of *in vitro* induced shoot buds was noted in many other medicinal plants such as *Rauvolfia serpentine* [29], *Vitex negundo* [30], *Ruta graveolens* [31], *Drymaria cordata* [32], *Gentiana kurroo* [27].

Table 1: Development of multiple shoot buds from shoot apex and nodal segment of *C. heptaphylla* when grown on 0.8% (w/v) agar solidified MS medium supplemented with different PGRs.

PGRs combination (mg/l)	Explant	% of explant giving response	Time (d) required for induction of multiple shoot buds	Average* no. of multiple shoot buds sprouted from explants(mean ± SE)
BAP + IAA	NS**	45	20-25	1.90 ± 0.18
0.5 + 0.1	SA***	30	20-25	1.20 ± 0.21
0.5 + 0.5	NS	47	18-20	2.40 ± 0.15
	SA	32	20-25	1.80 ± 0.11
1.0 + 0.5	NS	57	15-20	2.60 ± 0.19
	SA	40	15-20	2.00 ± 0.13
1.0 + 1.0	NS	62	15-20	2.80 ± 0.13
	SA	43	15-20	2.10 ± 0.21
1.5 + 0.5	NS	85	10-15	3.60 ± 0.25
	SA	55	15-20	2.25 ± 0.10
2.0 + 1.0	NS	90	10-12	4.20 ± 0.18
	SA	62	10-15	3.12 ± 0.51
BAP + NAA	NS	38	15-25	2.10 ± 0.15
0.5 + 0.5	SA	29	15-25	1.00 ± 0.20
0.5 + 0.5	NS	46	15-25	2.80 ± 0.19
	SA	32	15-25	1.30 ± 0.51
1.0 + 0.5	NS	53	10-15	3.00 ± 0.22
	SA	40	10-18	1.50 ± 0.42
1.0 + 1.0	NS	66	10-15	3.80 ± 0.26
	SA	42	10-18	2.00 ± 0.47
1.5 + 0.5	NS	72	10-12	5.00 ± 0.18
	SA	50	10-15	2.50 ± 0.07
1.5 + 1.0	NS	79	10-12	4.20 ± 0.10
	SA	55	10-12	2.80 ± 0.21

Note: *Values are the mean of three replicates each with 15 explants. **NS- Nodal segment, *** SA- Shoot apex

Table 2: Data on the elongation of directly produced multiple shoot buds of *C. heptaphylla* when grown on MS based agar solidified medium supplemented with different PGRs.

PGRs combination (mg/l)	Average* initial length (cm) of individual Shootbud (mean ± SE)	Average* length(cm) of multiple shoot bud after 30d of culture (mean ± SE)
BAP + IAA		
0.5 + 0.5	1.18 ± 0.13	2.00 ± 0.31
0.5 + 1.0	1.21 ± 0.17	2.10 ± 0.33
1.0 + 0.5	1.60 ± 0.13	2.80 ± 0.24
1.5+ 1.0	2.30 ± 0.13	5.60 ± 0.28
1.5 + 0.5	1.50 ± 0.31	3.10 ± 0.23
2.0 + 0.5	1.80 ± 0.47	3.20 ± 0.62
BAP + NAA		
0.5 + 0.5	1.00 ± 0.18	1.60 ± 0.20
0.5 + 1.0	1.10 ± 0.11	2.01 ± 0.15
1.0 + 0.5	1.20 ± 0.25	2.10 ± 0.31
1.5+ 1.0	1.28 ± 0.28	2.50 ± 0.33
Kn +IAA		
1.0 + 0.5	1.90 ± 0.32	4.15 ± 0.13
1.5 + 1.0	1.22 ± 0.41	2.01 ± 0.07
2.0 + 0.5	1.15 ± 0.12	2.30 ± 0.42

Note:* Values are the mean of three replicates each with 15 explants.

In order to get complete plantlets, two to three cm long *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentration of auxins (NAA, IAA & IBA) was used for rooting experiment. The shoot buds failed to produce any root in these auxins supplemented MS media but half strength MS media supplemented with either IAA or IBA alone or IBA in combination with IAA or NAA, produced root within 10-15 days of culture. Data were recorded 6 weeks after inoculation. Response of shoots to rooting was very much dependent on the concentrations and combination auxins provided (Table-3). The highest mean number 13.01 ± 0.21 and mean length 3.56 (Figure1-D) of root per culture was noted in half strength MS medium supplemented with 1.0 mg/l IBA + 0.5 mg/l IAA. The influence of IBA and IAA for induction and proliferation of root growth has been reported in other medicinal plants *Vitex negundo* [33], *Abrus precatorius* [34], *Vitex negundo* [35], *Rauwolfia tetraphylla* L [36], *Talinum triangulare* [37] & *Viola pilosa* [38].

In vitro grown complete plantlets were than successfully transferred from the culture room to pots outside through a process of successive phase of acclimatization. The regenerated plants were finally transferred to earthen pots containing garden soil and compost in the ratio of 2:1. Most of the transferred plants survived, survival ratio 91% and the surviving plants have now grown to a mature stage.

Table 3: Data on the development of roots in elongated multiple shoot buds of *Clausena heptaphylla* when grown on 0.8% agar solidified rooting media.

PGR	mg/l	Half strength MS medium		
		Rooting %	Number* of roots per shoot	Length of root (cm) (mean ± SE)
IBA	0.1	60	9.60 ± 0.13	2.00 ± 0.17
	0.5	65	11.00 ± 0.22	2.10 ± 0.19
	1.0	78	12.00 ± 0.24	2.53 ± 0.17
IAA	0.1	52	8.80 ± 0.20	2.30 ± 0.16
	0.5	75	9.30 ± 0.27	2.10 ± 0.13
	1.0	55	8.40 ± 0.20	2.02 ± 0.17
IBA + IAA	0.5 + 0.5	70	11.40 ± 0.19	2.80 ± 0.18
	1.0 + 0.5	91	13.01 ± 0.21	3.56 ± 0.10
	1.0 + 1.0	65	10.90 ± 0.29	2.50 ± 0.17
IBA + NAA	0.5 + 0.5	72	13.00 ± 0.22	2.61 ± 0.13
	1.0 + 0.5	80	11.70 ± 0.27	2.42 ± 0.16
	1.0 + 1.0	75	10.21 ± 0.45	2.50 ± 0.13

Note:***** Values are the mean of three replicates each with 15 explants.

4. Conclusion

The overall result of this preliminary study revealed that mass scale micropropagation and conservation of *Clausena heptaphylla*, an important but rare medicinal plant species of Bangladesh, is possible through induction of direct organogenesis from nodal explants and shoot apex. MS medium containing 2.0 mg/l BAP + 1.0 mg/l IAA is the best for shoot proliferation and half MS medium supplemented with 1.0 mg/l IBA +0.5 mg/l IAA is the best root induction. Though the percentage survival of the transferred plantlets to the outside environment was not very high, this technique will encourage large scale micropropagation of this valuable medicinal and aromatic plant and the regenerates can be utilized for the extraction of the essential oil which is much in demand in the pharmaceutical, flavour and fragrance industry. Future experiments are in progress to pinpoint the factors related to improvement of this culture technique and the establishment procedure.

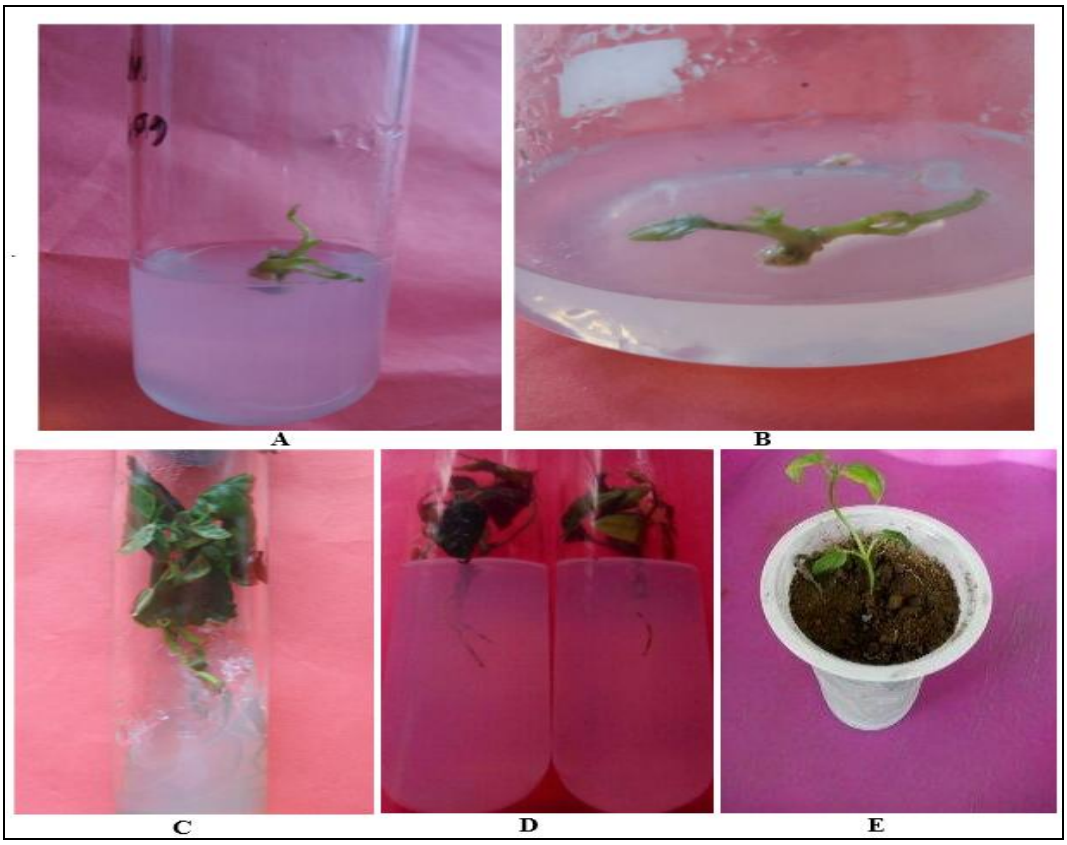

Fig 1: Different steps of direct shoot regeneration in *C. heptaphylla* A & B. Proliferation of multiple shoot buds, C. Elongation of multiple shoot buds, D. Elongated shoot buds produced root system, E. *In vitro* grown seedling established in outside pot.

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