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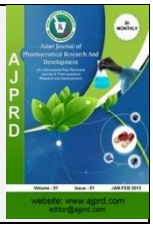

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Research Article

Micropropagation of *Pogostemon paniculatus*

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A B S T R A C T

Plant regeneration potential from node & leaf explants of *Pogostemon paniculatus* through direct shoot proliferation was studied on Murashige and Skoog medium supplemented with various concentrations of cytokinins & auxins. As the seed germinability of the plant is low *in vitro*, we have tried these explants for micropropagation. Among different concentrations of individual hormones tested for nodal explants, benzylaminopurine (BAP) at 5µM & kinetin (Kn) at 0.5µM showed 4 & 2 mean number of shoots/explant respectively & combination of Kn (0.5 µM) + BAP (1.5 µM) showed 15 mean number of shoots/explant. For leaf explant combination of Kn (0.5 µM) + BAP (1.5 µM) showed 20 mean number of shoots/explant. Other hormones tested at all concentrations showed callus formation without the shoot development. Roots were established on the isolated shoots on naphthaleneacetic acid (NAA) at 0.25µM. Rooted plants were acclimatized. This is the first report of *Pogostemon paniculatus* micropropagation.

Keywords: *Pogostemon paniculatus,* Micropropagation, benzylaminopurine, kinetin, Acclimatization.

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INTRODUCTION

Owing to the ill effects of chemical compounds both on the environment and on the health of humans, the world is witnessing increased demand for natural products. Because of this, almost every known medicinal and aromatic plant is being exploited to the maximum. Aromatic plants have been widely used since ages in pharmaceutical, cosmetic, perfumery, chemical, agricultural and food industries for various applications. Their properties are attributed to biochemicals including range of high valued volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components.

Pogostemon paniculatus (Willd.) Benth. belongs to the family Lamiaceae, is a much branched, erect and perennial herb growing wildly in Western Ghat regions of India. It is famously known as 'pache tene' or 'pache kadiru' in kannada. Also, it is known as wild patchouli. It is being substituted for the *Pogostemon cablin* which is the horticultural patchouli plant known for aromatic Patchouli oil. Patchouli oil is the oil preferred by many of the cosmetic industries which has a complex earthy, woody and leathery fragrance which is believed to increase olfactory quality after years of storage[1]. Annual production of patchouli oil has been reported to hit 2000 tons of which Indonesia alone contributes about 90% [2]. Patchouli oil has been approved as a natural additive for food flavouring for human consumption by Food and Drug Administration, USA, and included in the Generally Regarded As Safe (GRAS) list [3,4]. Similar status could be seen in European Union and in China [1].

It is used as antidepressant, anti-inflammatory, antiseptic, aphrodisiac, astringent, carminative, diuretic, febrifuge, fungicide, insecticide, sedative and tonic [5]. The essential oil of *P. paniculatus* reported to possess mainly 19 compounds which comprised more than 85% of essential oil. Of these, Patchouli alcohol (30.65%), α-guaiene (10.67%), β-guaiene (9.09%), caryophyllene (8.64%), eicosene (5.27%) were the major constituents reported to be present [5]. The essential oil of this plant is also characterized by a large number of other sesquiterpene hydrocarbons and sesquiterpenes which were also reported to be present in *P. cablin* in different concentrations [6,7]. Antimicrobial,

antioxidant and larvicidal properties of *P. paniculatus* essential oil was also reported [4,8] which simply authenticates plant's use in folk medicines[4,8]. It has been used for preparation of massage oil in traditional Indian medical system, Ayurveda. It is an important element in fine perfumes, soaps, cosmetic products & in food flavours too.

Vegetative propagation of pache tene plant is very difficult due to the climatic variation, mosaic virus & genotype variation of the plant. Rapidness in tissue culture method could be helpful for large scale production of the plant than the conventional method [9,10]. Reports of *in-vitro* regeneration of *Pogostemon cablin* are available but no report on micropropagation of *P. paniculatus* is available. Because of commercial and medicinal importance it is necessary to get multiple plants with less expense and within short time interval. For this purpose we have tried micropropagation of *P. paniculatus*, on MS media supplemented with different growth regulators.

MATERIALS AND METHODS

Plant material and aseptic culture

Young seedlings of *Pogostemon paniculatus* were collected from regions in and around Ujire, Dakshina Kannada, India & reared in the garden of the SDM College (Autonomous), Ujire, India. Plant tissues (seeds, leaves and nodes) were collected from well grown plants. All the tissues were initially washed with running tap water followed by 10 min treatment with 5% (v/v) aqueous solution of Teepol (Himedia Laboratories Ltd., India) and by 1% Bavistin (Fungicide) for 5 minutes each. Fungicide was washed off with 5 times rinsing in double-distilled water. The surface sterilization of the explants was carried out inside a laminar airflow cabinet with 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$, Himedia Laboratories Ltd, India) for 5 min followed by 5 rinses with sterile double-distilled water.

Culture Media and Explant preparation

Approximately 350 seeds were inoculated aseptically in the laminar air flow cabinet either on Murashige and Skoog's (MS) basal media[11] or in the baby jar bottles containing sterilized moistened filter paper for germination. Simultaneously, under aseptic conditions in laminar air flow chamber, the leaf explants with size of about 0.5 cm^2 and nodal explants (1-1.5 cm^2) were prepared and inoculated on to MS medium supplemented with different hormonal concentrations as shown below.

All the media used in this study were augmented with 30.0 g/l sucrose and gelled with 8.0 g/l of agar. The pH of the media was adjusted to 5.8 ± 0.1 and autoclaved at 121°C and 15 pounds per square inch pressure. After inoculation, all the cultures were maintained at 25 ± 1°C under the light intensity of 5000 Lux for 16 h photoperiod.

Establishment of shoot culture

Sterilised explants were inoculated to the MS medium supplemented with various concentrations of auxins and cytokinins. In this study different concentrations (0.5, 1, 2, 5 and 10 µM) of plant growth regulators such as 2, 4-dichlorophenoxyaceticacid (2,4-D), naphthalene aceticacid (NAA), benzyl aminopurine (BAP) and kinetin (KN) aloneand also combinations of KN (0.5 µM) + BAP (0.5, 1, 1.5and 2 µM) were used forthe establishment of adventitious shoots. Well developed shoots were rooted using either on basal MS medium or on media supplemented with NAA (0.25 µM). Each experiment comprised of 6 replicates & the experiments were repeated thrice.

Hardening / Acclimatization of plantlets

Well rooted, *in vitro* raised plantlets were removed from cultures and washed in distilled water thoroughly to remove gel. Later, they were transferred to plastic cups filled with coco peat, sand and soil (1:1:1 ratio) and are covered with transparent plastic cover to maintain humidity. The cultures were maintained at controlled environmental conditions (temperature 25 ± 2 °C under 16 h photoperiod) for a week. Afterwards, the plantlets were transferred to green house and maintained for *ex-vitro* hardening for 2-3 weeks. Finally, the hardened plantlets were transplanted to pots in the shade house for further growth.

RESULTS AND DISCUSSION

Seed germination: Seeds are produced in plenty in this plant. So, if they are germinated *in vitro*, it could source unlimited aseptic tissues. With this in mind, we tried to germinate the seeds. But none of the seeds germinated either on the MS media or on the moistened filter paper indicating problems associated with seed germinability. But this has not reported by anyone and further research in this regard is warranted.

Nodal culture of *P. paniculatus*

Effect of cytokinins and auxins alone and in combination

The nodal segments were cultured on MS medium supplemented with cytokinins BAP, KN and auxins NAA, 2,4-D at concentrations of 0.5, 1, 2, 5 and 10µM. The nodal explants cultured on BAP (2, 5µM) and KN (0.5µM) showed sprouting of axillary buds within 2 weeks (Table 1). The highest shoot length (3 cm) at the end of 8 weeks is obtained. Nodal explants cultured on other concentrations of BAP and KN did not show any response and became necrotic within 2 weeks of culture. The nodal explants cultured on MS medium supplemented with auxins also did not show any responses. To study the effect of cytokinins in combination, nodal explants were cultured on the medium supplemented with 0.5 µM KN and different concentrations of BAP (i.e. 0.5, 1, 1.5 & 2 µM). Theaxillary shoot induction was observed from the pre-existing axillary buds after 2 weeks of culture initiation.. Combination of 0.5 µM KN and 1.5 µM BAP showed direct shoot regeneration. Upto 15 shoots regenerated from a single node.

In a similar manner, Kumaraswamy et al. (2010) reported nodal explants of *P. cablin* induced multiple shoot formation on MS medium supplemented with 0.5 mg/l BAP alone whereas callus formation resulted from higher

concentration of BAP & KN [9,10]. In their study, MS medium supplemented with BA and KN (0.5 mg/l each) induced multiple shoots whereas concentrations beyond 0.5 mg/l of the same hormone induced callus with hyperhydric shoots and morphological abnormalities [10]. On contrary, our experiment produced multiple shoots without any morphological abnormalities. However, Hembromet al.(2006) used a higher concentration of BAP (2mg/l) for maximum shoot multiplication of P. heyneanus[12]. George *et.al* (2008) has reported that BAP alone is most effective for shoot [13]. Combination treatment (BAP along with KN) was found to exhibit highest frequency of shoot multiplication in *P. cablin* [10].

Table 1:- Effect of different concentrations of Hormones on regeneration of shoots from nodal explants

Hormonal concentration	% of response	Remarks	Mean number of shoots / explants
BAP (µM):- 0.5	0	No response	0
1.0	0	No response	0
2.0	33	Shoots produced without callus formation	1
5.0	16.66	Shoots produced without callus formation	4
10.0	0	No response	0
Kn (µM):- 0.5	33	Shoots produced without callus formation	2
1.0	0	No response	0
2.0	0	No response	0
5.0	0	No response	0
10.0	0	No response	0
2,4-D(µM):- 0.5	0	No response	0
1.0	0	No response	0
2.0	0	No response	0
5.0	0	No response	0
10.0	0	No response	0
NAA (µM):- 0.5	0	No response	0
1.0	0	No response	0
2.0	0	No response	0
5.0	0	No response	0
10.0	0	No response	0
BAP+Kn (µM):-0.5+0.5	0	No response	0
1.0+0.5	0	No response	0
1.5 +0.5	16.66	Shoots without callus	15
2.0+0.5	0	No response	0

Leaf culture of P.paniculatus

Effect of cytokinins and auxins alone and in combination

The leaf segments were cultured on MS medium supplemented with cytokinins BAP, KN and auxins NAA, 2,4-D at concentrations of 0.5, 1, 2, 5 and 10µM individually. The leaf explants cultured on BAP (0.5, 1, 5, and 10µM), KN (1µM) showed formation of callus without shoot regeneration after 2 weeks of culture (Table 2). On the other hand, the leaf explants cultured on the MS medium supplemented with cytokinins BAP (2µM) and KN (0.5, 2, 5& 10µM) did not show any response and became necrotic after 4 weeks of culture. Among the auxins tested only 2,4-D (10 µM) induced callus formation, the rest did not show any response. To study the effect of hormonal combination, leaf explants were cultured on the medium supplemented with 0.5 µM KN and different concentrations of BAP (i.e.,0.5, 1, 1.5 & 2 µM). Combination of 0.5 µM KN and 1.5 µM BAP showed callus formation initially which later gave multiple

shoots. Upto 20 shoots regenerated from a single leaf explants (Table 2; Fig. 1).

Table 2:- Effect of different concentrations of Hormones on regeneration of shoots from leaf explants

Hormonal concentration	% of response	Remarks	Mean number of shoots / explants
BAP (µM):- 0.5	16.66	Callus without shoots	0
1.0	16.66	Callus without shoots	0
2.0	0	No response	0
5.0	16.66	Callus without shoots	0
10.0	33.33	Callus without shoots	0
Kn (µM):- 0.5	0	No response	0
1.0	16.66	Callus without shoots	0
2.0	0	No response	0
5.0	0	No response	0
10.0	0	No response	0
2,4-D(µM):- 0.5	0	No response	0
1.0	0	No response	0
2.0	0	No response	0
5.0	0	No response	0
10.0	16.66	Callus without shoots	0
NAA (µM):- 0.5	0	No response	0
1.0	0	No response	0
2.0	0	No response	0
5.0	0	No response	0
10.0	0	No response	0
BAP+Kn (µM):-0.5+0.5	16.66	Callus without shoots	0
1.0+0.5	0	No response	0
1.5 +0.5	50	Callus with multiple shoots	20
2.0+0.5	33.33	Callus without shoots	0

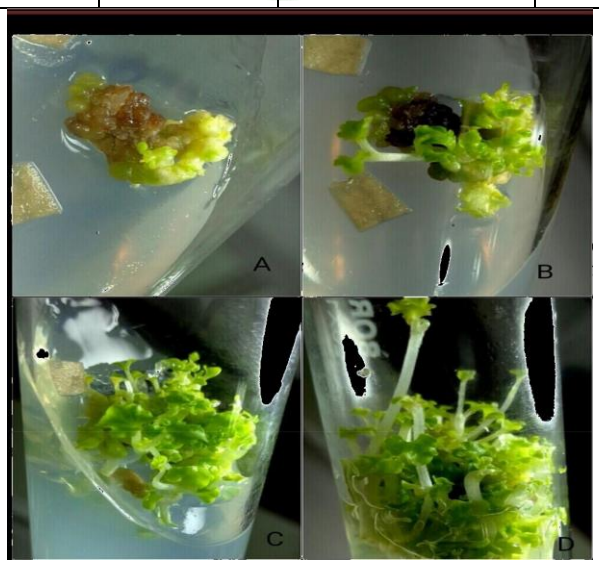

Figure 1: Shoot induction from leaf explants of *P. paniculatus* on MS medium supplemented with KN-0.5 µM + BAP 1.5 µM
A) 2 Week old culture showing sprouting of shoots on MS Medium supplemented with KN-0.5 µM +BAP 1.5 µM
B) 4 Week old culture showing sprouting of shoots on MS Medium supplemented with KN-0.5 µM +BAP 1.5 µM
C) 6 Week old culture showing sprouting of shoots on MS Medium supplemented with KN-0.5 µM +BAP 1.5 µM

D) 8 Week old culture showing sprouting of shoots on MS Medium supplemented with KN-0.5 µM +BAP 1.5 µM

Earlier report from Paul et al., (2010) suggests patchouli plants produced highest number of shoots (94.6/explants) from 96.2% of leaf explants on MS medium containing 2.5 µM BAP and 0.5 µM NAA [14]. Similarly, high Frequency of plant regeneration through adventitious shoots proliferation from leaf explant of *P. cablin* has been demonstrated [15]. They did obtain best response from the explants cultured on MS medium containing 0.5 mg/l BAP + 5 mg/l 2, 4-D. Maximum numbers of 74 adventitious shoots were observed and rooted on the medium containing 0.5 mg/l BAP and 0.5 mg/l NAA after six weeks of culture [15]. Misra (1996) reported that single leaf explant or nodal segment produced about 150-200 plants within 2-3 months by repeated culture of callus in shoot inducing media (2 mg/l NAA + 0.5 mg/l BA) with 79.3 shoots per gram of callus in regeneration of patchouli *via* callus culture from leaf segments [16]. Sailaja et.al., (2017) reported that MS media supplemented with 0.5mg/l of BAP and 0.5mg/l of kinetin showed multiple shoot generation in patchouli plant [17].

Rooting and acclimatization

The effect of auxin supplemented to full strength MS medium and basal MS medium was tested for rooting efficiency in regenerated shoots. NAA at 0.25 µM concentration was used. Both auxin supplemented and basal MS medium showed rooting. But rooting was profuse in NAA supplemented medium (Fig. 2). The rooted plantlets with expanded leaves and well developed roots were transferred to plastic cups containing coco peat and reared in green house. The survival rate was 55% after 2 weeks (Fig. 2). Survived plants were transferred to pots and maintained in shade house for 4 weeks. Plants after hardening were transferred to soil. Contrary to our findings, Paul et al., (2010) reported that almost every regenerated shoots rooted spontaneously on half-strength MS basal medium and were successfully hardened and transferred to nursery [14].

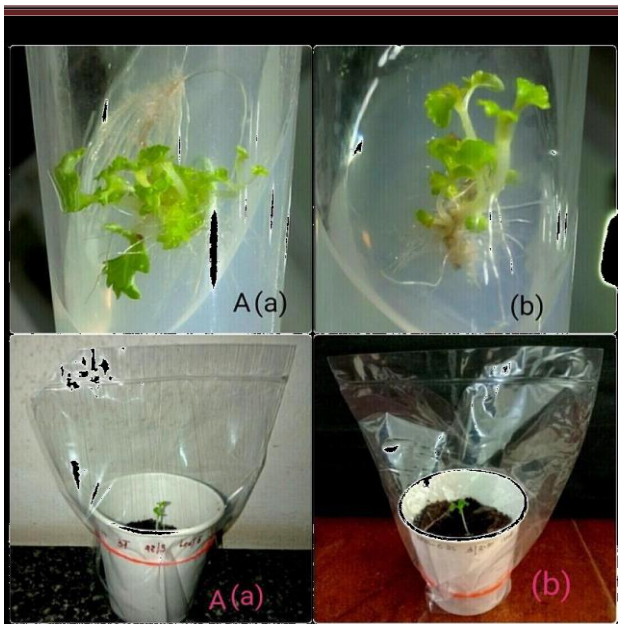

Figure 2: Rooting & Acclimatization of plantlets of *P.paniculatus*
Fig A (a, b): Eight week old cultures showing rooting of shoots on MS medium with 0.25µM/l NAA.
Fig A(a, b): Acclimatized plant after two weeks

CONCLUSION

It can be concluded that BAP 5 µM & 0.5 µM KN were found to be the best among the tested plant growth regulators in induction of axillary shoots from nodes in *P. paniculatus*. Combination of cytokinins like KN 0.5 µM + BAP 1.5 µM concentration showed best results for shoot regeneration from nodes and leaf explants. Rooting of regenerated shoots was achieved on MS medium supplemented with 0.25 µM NAA with optimum roots per shoot. All the plantlets showed a high survival rate *in vivo* on acclimatization. Thus, an efficient procedure was developed for direct & indirect shoot regeneration from the leaf & node explant of *P.paniculatus*.

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