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Effect of explant types and plant growth regulators on direct regeneration in medicinal plant *Pogostemon cablin*

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Abstract

The present work was conducted to develop a high frequency and rapid *in vitro* regeneration protocol for *Pogostemon cablin* (Blanco) Benth. In order to improve the regeneration efficiency of this crop, the effects of explant types and plant growth regulators (PGRs) on *in vitro* shoot proliferation, and also the subsequent rooting of shoots were examined. Of the explant types, nodal stem with a single node (the second or third node of *in vitro* plantlets) was the most responsive explant, with 100% explants producing 129.7–138.1 shoots on the optimal medium, and also leaf and petiole possessed a high regenerative capacity. Of the tested cytokinins, 6-benzyladenine (BA) was most effective on shoot regeneration. BA at 0.1–0.2 mg·L^{-1}, High number and length of shoots per explant were achieved on Murashige and Skoog (MS) medium containing 0.1–0.2 mg·L^{-1} BA. Combinations of BA and 1-napthaleneacetic acid (NAA) resulted in slower shoot development and growth as compared to BA alone. For regenerated shoots rooting, half-strength MS medium supplemented with 0.2 mg·L^{-1} indole-3-butyric acid (IBA) was most effective with maximum number (49.3 roots per plantlet) and length (1.45 cm per root) of roots respectively. Regenerated plantlets were successfully transferred into pots with soil and over 90% of them grew into healthy mature plants. The current study provided information toward commercial *in vitro* propagation of *Pogostemon cablin*.

Keywords: patchouli, *in vitro* culture, culture material, cytokinin, auxin.
Abbreviations: BA_6-benzyladenine; IAA_indole-3-acetic acid; IBA_indole-3-butyric acid; Kn_kinetin; MS_Murashige and Skoog; NAA_1-naphthaleneacetic acid; PGR_plant growth regulator; Zn_zeatin.

Introduction

Pogostemon cablin (Blanco) Benth. (Patchouli) is an aromatic and erect herb, which belongs to the family Lamiaceae. Patchouli oil extracted from its dried leaves by steam distillation is showing great commercial interest in perfumery industry (Singh and Ganesha Rao, 2009). Patchouli oil also has marked pharmacological activities as antiseptic, anti-insecticidal, antifungal, anti-inflammatory, aphrodisiac and antidepressant (Kukreja et al., 1990; Sharma et al., 1992; Bunrathep et al., 2006). In China, patchouli is a traditional herbal medicine widely used against indigestion, cold, headache, fever, vomiting and diarrhea (Chinese Pharmacopoeia Commission, 2010). Patchouli is native to tropical regions of Asia, and is now extensively cultivated in China, Indonesia, the Philippines, Malaysia, India and even Brazil (Mahanta et al., 2007; Arpana et al., 2008). The genetic variability within patchouli is relatively limited; therefore, breeding potential for resistance to biotic and abiotic stresses is also limited.

Patchouli is propagated by stem cuttings, which further limits its available genetic pool. And stem cuttings can harbor the pathogens, thus allowing the disease to be perpetuated through the vegetative propagation. Also, the propagation method has a very low multiplication rate and requires a large number of stock plants. Tissue culture techniques have been widely used for the rapid production of high-quality, pathogen-free and uniform plants on a commercial scale. It has been played an important role in rapid propagation of many plant species. In recent years, *in vitro* plant regeneration of patchouli via direct organogenesis from stem tip (Kukreja et al., 1990; Sugimura et al., 1995), leaf segment (Paul et al., 2010) and nodal segment (Kumara Swamy et al., 2010) has been established, but in most cases evidence for plant regeneration were restricted to only very few explant types. There are also some reports on callus induction and plant regeneration in patchouli (Misra, 1996; Santos et al., 2011). Some of these methods gave a lot of little shoots, but these might need more time period to develop plantlets. Hence, there is certainly a need for more research to meet commercial-scale vegetative propagation of patchouli. In the present study, the comparison of shoot induction ability of different explants (leaf, nodal stem, petiole, internodal stem segment and root tip) was conducted, and the effects of plant growth regulators (PGRs) on shoot and root induction were studied. The objective of this study was to establish a high frequency and rapid regeneration protocol for patchouli.

Results

Comparison of shoot regeneration ability of different explants

The regeneration ability of each explant type was scored every week for five weeks periods. Shoots initiated from nodal stem segments within one week of culture, and shoots formed from leaf segments and multiple little buds sprouted from petiole segments and internodal stem segments after two weeks of culture, and then only a few shoots were observed from root tips until the third week of culture (data

not shown). The data after 35 days of culture were showed in Table 1. Among the tested explants, leaf segments and nodal stem segments were most responsive with the highest shoot regeneration frequency (100%), followed by petiole segments and internodal stem segments with the shoot regeneration frequency 90.1% and 65.5%, respectively. While, only 12.9% of root tips regenerated shoots. The maximum shoot number (129.7 shoots per explant) and shoot length (1.09 cm) per explant were obtained from nodal stem segments. About 100 shoots (greater than 0.68 cm in length) were regenerated from petiole segments and internodal stem segments. All shoots regenerated from nodal stem, leaf, petiole and internodal stem segments were vigorous with green leaves and robust stems (Fig. 1A-D). Shoots formed from root tips were much smaller than those regenerated from other segments (Fig. 1E). The results suggest that nodal stem is the best explant for shoot regeneration, and also leaf and petiole possess good regeneration capacity in patchouli.

Effect of different cytokinins on shoot regeneration

Data in table 2 showed that nodal stem segments were more regenerable than petiole segments. On PGR-free medium (control), the shoot formation frequencies from nodal stem segments and petiole segments were 92.1% and 77.7%, respectively. Addition of cytokinins to MS medium enhanced the frequency of shoot regeneration and greatly promoted multiple shoot formation in both types of explants. BA was the most effective cytokinin for multiple shoot induction from nodal stem segments, followed by Zn, giving an average of 133.1 and 81.8 shoots per explant, respectively. BA and Zn were equally effective in multiple shoot induction from petiole segments; both shoot regeneration frequency (over 95%) and shoot number (more than 109 shoots per explant) in the treatments were significantly higher than that of control. While, Kn in the medium was the least effective for multiple shoot induction from nodal stem as well as petiole explants. Moreover, based on observations, shoots from explants on PGR-free medium and media containing BA or Zn appeared morphologically normal (with typical leaves and stems), while some regenerated shoots in Kn treatment became stunted or hyperhydric. Overall, these results indicate BA and Zn are both effective cytokinins among the cytokinins tested for shoot regeneration of patchouli.

Effect of different concentrations of BA and combinations of BA with NAA on shoot induction

Nodal stem segments from patchouli were cultured on MS media containing BA alone or in combination with NAA for shoot induction. The explants cultured on PGR-free medium or media containing BA alone produced adventitious shoots directly from node region and then around cut ends of most explants without intervening callus formation. At high BA concentrations (0.5–1.0 $mg \cdot L^{-1}$), both adventitious shoots and a small amount of callus appeared simultaneously on the cut surfaces of some explants. The explants cultured on media containing BA and NAA induced white friable callus on the cut surfaces in the first week. Then, the callus became green and compact and the subsequent shoots were initiated from the callus. The contribution of BA or both BA and NAA on multiple shoot induction from nodal stem explant with regard to the percentage of response, mean number of shoots and average shoot length was examined and the results after 5 weeks of culture was shown in Table 3. On PGR-free medium (control), 95.8% of explants produced 3.0 shoots. The explants cultured on MS medium supplemented with 0.1 or 0.2 $mg \cdot L^{-1}$ BA gave a perfect shoot formation (100%) and great shoot multiplication (132.3–138.1 shoots per explant). BA up to 0.5 and 1.0 $mg \cdot L^{-1}$ resulted in reduction in shoot number and length. In the presence of NAA along with various levels of BA (0.1–1.0 $mg \cdot L^{-1}$), the shoot number and height decreased compared with the BA alone treatments. Increasing NAA concentration up to 0.5 $mg \cdot L^{-1}$ caused a significant decline in shoot production. Moreover, many regenerated shoots were stunted and did not elongate further, and few of these could form well-developed plants (Fig. 1F). These results suggested that higher level of NAA stimulated callus formation and slowed down the subsequent plant formation. Low concentrations of BA alone were found suitable for patchouli shoot initiation and further multiplication.

Root regeneration and acclimatization

For root induction, excised microshoots were inoculated on full and half-strength MS basal medium supplemented with different auxins. Rooting percentage, number and length of roots per plantlet were recorded after 3 weeks of culture (Table 4). All microshoots produced roots. The number and length of roots per plantlet on the media containing half-strength MS salts were significantly higher than those cultured on the media containing full-strength MS salts. It was observed that IBA was the best auxin for root induction of patchouli compared to NAA and IAA. Highest number (49.3 roots per plantlet) and length (1.45 cm per root) of roots were both obtained on half-strength MS medium with 0.2 $mg \cdot L^{-1}$ IBA (Fig. 1G). Plantlets with well-developed shoots and roots were transferred to pots containing sterile soilrite for hardening. Hardened plants were transferred to pots filled with normal garden soil and maintained under shade conditions. Well acclimatized plants were finally planted in the field. No detectable variation in morphology was observed among the acclimatized plants. Over 90% of the plantlets grew up into healthy mature plants in several weeks (Fig. 1H).

Discussion

The explant is the most important factor in plant tissue culture. The successful regeneration is based on an appropriate choice of explant. For most micropropagation work, the explant of choice is either apical bud or nodal segment (Bhojwani and Dantu, 2013). In this experiment, regeneration potential of different explants i.e. leaf, petiole, root tip, nodal and internodal stem segments from patchouli have been tested. Shoot buds could be initiated from all the explants tested, however the explant regeneration capacity was varied. Shoots were first initiated from nodal stem explants within one week of culture, followed by leaf, petiole and internodal stem explants after two weeks of culture, while root explants began to form shoots after three weeks of culture**.** Regenerated shoots formed directly at cut edges or around cut ends of explants, these damaged tissues provoked a rapid cell division and provided an adequate target tissue for further manipulations eventually. Besides, shoot regeneration also occurred from node region of nodal stem explants due to meristems there having a high capability for cell division and differentiation. After 35 days of culture, both the percentage of explants producing shoots and the mean number of shoots per explant in all the types of explants

Table 1. Shoot regeneration from different explants (from *in vitro* plantlets at 8-10 leaf stage) cultured on MS medium supplemented with 0.2 mg·L^{-1} BA.

Explant	Shoot regeneration (%)	Mean no. of shoots per explant	Mean length of shoots (cm)
Leaf segment	100.0 ± 0.0^{a}	63.8 ± 0.9^{c}	0.96 ± 0.04^{a}
Petiole segment	90.1± 1.3^{b}	102.4 ± 2.3^{b}	0.68 ± 0.01^{b}
Nodal stem segment	100.0 ± 0.0^{a}	129.7 ± 1.6^{a}	1.09 ± 0.12^{a}
Internodal stem segment	65.5 ± 2.7^{c}	103.2 ± 7.9^{b}	0.75 ± 0.07^{b}
Root tip	12.9 ± 0.2^{d}	2.4 ± 0.8^{d}	0.39 ± 0.04^{c}

Data were recorded after 35 days of culture; Values represent the mean ± SE; Means within each column followed by the same letter are not significantly different at P≤0.05 according to Duncan's Multiple Range Test.

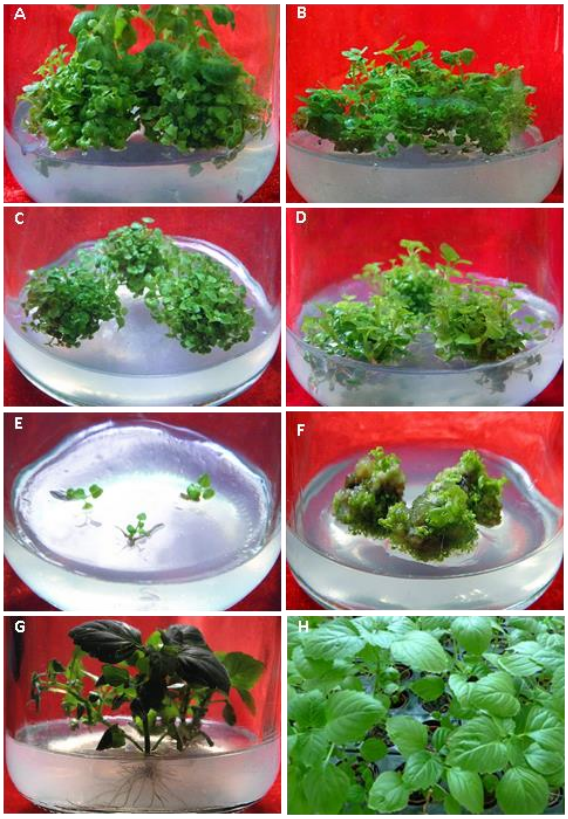

Fig 1. *In vitro* plant regeneration of *Pogostemon cablin*. Shoots from nodal stem explants (A), leaf explants (B), petiole explants (C), internodal stem explants (D) and root tips (E) on MS medium with 0.2 mg·L^{-1} BA after 5 weeks of culture; (F) Shoots from nodal stem explants on MS medium with 0.2 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA after 5 weeks of culture; (G) Rooted shoots on 1/2MS medium with 0.2 mg·L^{-1} IBA after 3 weeks of culture; (H) Regenerated plants 2 months after cultivation in the greenhouse.

tested were significant different with varying from 12.9% to 100.0% and from 2.4 to 129.7 respectively. Maximum shoot regeneration frequency (100%), mean shoot number (129.7 shoots per explant) and mean shoot length (1.09 cm per shoot) were all achieved from nodal stem explants. Leaf and petiole also had high regeneration efficiencies. The different regeneration conditions among the five types of explants might be attributed to different endogenous hormonal levels in various parts of the plant. Although plant regeneration from leaf or nodal stem explants of patchouli has been reported in previous studies (Paul et al., 2010; Kumara Swamy et al., 2010; Wan Nurul Hidayah et al., 2012), this is, to our knowledge, the first report of direct shoot regeneration from five different types of explants in patchouli.

Cytokinins have main functions on releasing axillary buds from suppression by apical dominance and promoting division of plant cells, thus initiating shoot proliferation (Haberer and Kieber, 2002; Shirin and Rana, 2007). Three types of cytokinins were tested in direct shoot regeneration for patchouli. On control medium (PGR-free) over 90% of nodal stem explants and 77.7% of petiole explants could produce shoots, however the number of regenerated shoots per explant were generally low. Addition of cytokinins to MS medium enhanced the frequency of shoot regeneration and greatly promoted multiple shoot formation of explants. Of the tested cytokinins, BA was most effective on shoot proliferation. Using nodal stem explants, shoot production and height were obviously greater in the presence of BA at lower concentrations than higher levels. Similar results were also obtained from Wan Nurul Hidayah et al. (2012). While Kn was the less effective one which was in accordance with the report by Misra (1996).

Several studies have reported the use of BA alone or in combination with NAA for plant *in vitro* regeneration of patchouli. Misra (1996) reported that callus could be obtained from leaf and nodal stem segments on the medium supplemented with 0.5 mg·L^{-1} BA and 2 mg·L^{-1}

Table 2. Effect of different cytokinins on shoot induction from nodal stem segments and petiole segments.

Plant growth regulator ($mg \cdot L^{-1}$)	Nodal stem segment		Petiole segment	
	Shoot regeneration (%)	Mean no. of shoots/explant	Shoot regeneration (%)	Mean no. of shoots/explant
PGR-free	92.1 ± 1.6^{b}	3.5 ± 0.5^{d}	77.7 ± 2.7^{b}	10.9 ± 2.4^{c}
BA (0.2)	100.0 ± 0.0^{a}	133.1 ± 1.0^{a}	95.5 ± 4.5^{a}	110.1 ± 1.8^{a}
Zn (0.2)	100.0 ± 0.0^{a}	81.8 ± 3.3^{b}	97.8 ± 1.1^{a}	109.0 ± 2.7^{a}
Kn (0.2)	100.0 ± 0.0^{a}	32.3 ± 1.5^{c}	90.9 ± 3.9^{a}	66.8 ± 3.9^{b}

Data were recorded after 35 days of culture; Values represent the mean ± SE; Means within each column followed by the same letter are not significantly different at P≤0.05 according to Duncan's Multiple Range Test.

Table 3. Effect of different concentrations of BA and combinations of BA with NAA on shoot induction.

Plant growth regulator ($mg \cdot L^{-1}$)		Shoot regeneration (%)	Mean no. of shoots per explant	Mean length of shoots (cm)
BA	NAA			
0	0	95.8 ± 4.2^{abc}	3.0 ± 0.4^{f}	2.08 ± 0.14^{a}
0.1	0	100.0 ± 0.0^{a}	132.3 ± 6.6^{a}	1.28 ± 0.02^{b}
0.2	0	100.0 ± 0.0^{a}	138.1 ± 6.0^{a}	1.00 ± 0.05^{c}
0.5	0	100.0 ± 0.0^{a}	108.1 ± 2.5^{b}	0.68 ± 0.04^{de}
1.0	0	100.0 ± 0.0^{a}	92.7 ± 3.0^{c}	0.44 ± 0.04^{fg}
0.1	0.1	96.7 ± 3.3^{ab}	95.2 ± 4.3^{c}	0.57 ± 0.03^{ef}
0.1	0.5	88.9 ± 6.4^{c}	18.0 ± 0.4^{e}	0.35 ± 0.06^{gh}
0.2	0.1	100.0 ± 0.0^{a}	130.7 ± 2.6^{a}	0.73 ± 0.02^{d}
0.2	0.5	91.7 ± 4.2^{bc}	39.7 ± 2.0^{d}	0.22 ± 0.01^{hij}
0.5	0.1	100.0 ± 0.0^{a}	99.0 ± 2.3^{bc}	0.40 ± 0.02^{g}
0.5	0.5	96.3 ± 3.7^{ab}	25.7 ± 0.7^{e}	0.19 ± 0.01^{ij}
1.0	0.1	100.0 ± 0.0^{a}	91.8 ± 7.3^{c}	0.33 ± 0.01^{ghi}
1.0	0.5	96.7 ± 3.3^{ab}	24.8 ± 3.3^{e}	0.18 ± 0.01^{j}

Data were recorded after 35 days of culture; Values represent the mean ± SE; Means within each column followed by the same letter are not significantly different at P≤0.05 according to Duncan's Multiple Range Test.

Table 4. Effect of medium salt strength and auxins on rooting.

Treatment ($mg \cdot L^{-1}$)	Root formation (%)	Mean no. of roots/plantlet	Mean length of roots (cm)
MS	100.0 ± 0.0	20.9 ± 0.9^{e}	0.94 ± 0.01^{d}
MS + IBA (0.2)	100.0 ± 0.0	36.6 ± 1.2^{b}	1.06 ± 0.05^{c}
MS + IAA (0.2)	100.0 ± 0.0	22.6 ± 0.9^{de}	0.49 ± 0.04^{e}
MS + NAA (0.2)	100.0 ± 0.0	25.8 ± 1.8^{cd}	0.85 ± 0.02^{d}
1/2MS	100.0 ± 0.0	28.1 ± 1.4^{c}	1.30 ± 0.03^{b}
1/2MS + IBA (0.2)	100.0 ± 0.0	49.3 ± 0.7^{a}	1.45 ± 0.03^{a}
1/2MS + IAA (0.2)	100.0 ± 0.0	28.7 ± 1.3^{c}	0.83 ± 0.00^{d}
1/2MS + NAA(0.2)	100.0 ± 0.0	39.9 ± 1.0^{b}	1.16 ± 0.06^{c}

Data were recorded after 21 days of culture; Values represent the mean ± SE; Means within each column followed by the same letter are not significantly different at P≤0.05 according to Duncan's Multiple Range Test.

NAA after 4–6 weeks of culture, and another 30 days later shoots were regenerated from the callus on the medium with Kn or BA. Paul et al. (2010) suggested that for leaf explants, maximum shoot regeneration frequency of 82.2% with 81.3 shoots per explant was obtained on medium containing BA and NAA, and some explants formed callus. Additional studies suggested that 80% and 100% of cultured node explants (nodal segments) produced 17.1 and 32.9 shoots per explant on medium containing BA alone after 40 days and 4 weeks of culture, respectively (Hermbrom et al., 2006; Wan Nurul Hidayah et al., 2012). The results of the present study suggested that low concentrations of BA (0.1–0.2 $mg \cdot L^{-1}$) alone could promote shoot development and growth as compared to BA in combination with NAA, with highest shoot regeneration frequency (100%) and shoot number (more than 130 shoots per explant) within 35 days of culture. Furthermore, in this study, although high shoot number was also obtained on medium supplemented with 0.2 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ NAA, many regenerated shoots were smaller than those on medium with BA alone. Increasing NAA concentration up to 0.5 $mg \cdot L^{-1}$, most of regenerated shoots developed slowly or even poorly to vital plants. In summary, we managed to obtain a high frequency and direct regeneration without undesirable callus formation in patchouli, and thus to improve shoot regeneration, shorten the time period needed for regeneration and reduce the possibility of somaclonal variability.

Materials and Methods

Collection of plant material and establishment of culture

Healthy 1-year-old plants of patchouli were collected from Guangdong province, China and used as the source of explants. Shoot tips and nodal explants were used for this purpose. The explants were all dipped into 70% (w/v) ethanol for 30 s after being washed with running tap water thoroughly. They were then sterilized with 0.1% (w/v) $HgCl_2$ for 5 min under agitation, followed by five rinses with sterile distilled water. The surface sterilized explants were cut into 1–1.5 cm length containing a single node with an axillary bud or a shoot tip with an apical bud and then were placed vertically on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The *in vitro* regenerated plantlets were further used as explant sources for adventitious shoot regeneration.

MS basal medium was used for shoot regeneration and both half and full strength MS medium were used for *in vitro* rooting. All media were supplemented with 3.0% (w/v) sucrose and 0.7% (w/v) agar. The pH of all media was adjusted to 5.8 before autoclaving at 1.05 $kg \cdot cm^{-2}$ and 121°C for 15 min. All cultures were incubated in a culture room at 26 ± 2°C with a 16 h photoperiod under a fluorescent light.

Multiple shoot induction

Different types of explants as nodal stem with a single node (the second or third node), internodal stem, petiole, root tip (0.5–1 cm) and leaf (1 cm^2) from micropropagated plantlets at 8-10 leaf stage were cultured on MS medium supplemented with 0.2 $mg \cdot L^{-1}$ 6-benzyladenine (BA) to find suitable explants for multiple shoot induction. Effect of plant growth regulators (PGRs) on shoot induction was conducted in two separate sets of experiments. In the first experiment, using petiole and nodal stem segments as explants, 0.2 $mg \cdot L^{-1}$ BA, kinetin (Kn) or zeatin (Zn) were supplied into MS media, to select the best cytokinin for the response of shoot induction. In the second set, BA at different concentrations (0.1, 0.2, 0.5 and 1.0 $mg \cdot L^{-1}$) alone or in combinations with 1-napthaleneacetic acid (NAA) (0.1 and 0.5 $mg \cdot L^{-1}$) were assessed for shoot multiplication from nodal stem explants. Petiole, nodal and internodal stem explants were inserted with their basal ends down into the medium. Leaf explants were placed horizontally with the abaxial sides in contact with the medium. Root tips were placed horizontally on the medium. Shooting frequency, mean number of shoots per explant, and mean length of shoots were recorded once a week.

Root regeneration and acclimatization

Regenerated shoots (2–3 cm long) were excised and transferred to full strength as well as half strength MS medium supplemented with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and NAA, respectively, to allow root organogenesis. Rooting frequency, mean number of roots per plantlet and mean length of roots were recorded once a week. Plantlets with well-developed shoots and roots were washed free of agar and then transferred to pots containing sterile soilrite in a moisture saturated glass chamber with 80% relative humidity for four weeks. Subsequently hardened plants were transferred to pots filled with normal garden soil under shade conditions and gradually exposed to full sunlight.

Statistical analysis

Each treatment was repeated three times and each replicate consisted of at least 20 explants. All the experiments were repeated at least twice and completely randomized. Data were analyzed using the SPSS version 17. Significance differences were assessed by using Duncan's Multiple Range Test at the 5% probability level. All the data were expressed as mean ± standard error (SE).

Conclusion

Pogostemon cablin (patchouli) is a medicinal and aromatic plant widely used for cosmetics and medicinal purposes. *In vitro* culture techniques offer a practical option for large scale, commercial production of patchouli. In this study, nodal stem, leaf and petiole were found to be alternative sources of explants for *in vitro* propagation of patchouli. BA was superior to Zn and Kn on shoot regeneration, and low concentrations of BA alone could lead to increased shoot initiation and further multiplication. Half-strength MS medium supplemented with 0.2 $mg \cdot L^{-1}$ IBA was most effective for root formation. Over 90% of *in vitro* propagated plantlets survived in the field after acclimatization. These results may potentially benefit commercial production of high-quality and uniform seedlings in patchouli.

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