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In vitro regeneration potential of patchouli shoot tip and leaf explants

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ABSTRACT

An effective, reproducible and *in vitro* rapid regeneration protocol for Patchouli shoot tip and leaf explants was studied for mass multiplication of plants. The shoot tip cultured on MS (Murashige and Skoog's) medium regenerated directly in to shoots and the medium supplemented with 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA exhibited higher percentage of proliferation and registered maximum shoot weight (15.90 g). Callus was established from the leaf explants on the same medium supplemented with varying concentration of 2, 4-D and BAP. 2.0 mg l^{-1} 2, 4-D and 0.1 mg l^{-1} BAP produced maximum callus weighing 55.30 g. Regeneration from the callus was observed on the same hormonal concentration as for direct regeneration. The regenerated shoots were readily rooted on half strength MS medium and IAA has had greater influence on number and length of roots. Plantlets readily adapted to greenhouse conditions with 98% of success.

KEYWORDS

Patchouli;
2, 4-D;
NAA;
BAP;
Direct regeneration;
Callus induction;
Shoot formation;
Root formation;
Hardening media;
Survival rate.

INTRODUCTION

Patchouli (*Pogostemon patchouli* Pellet) belongs to the family Laminaceae, native to Philippines, grown within Malaysia, Indonesia, Singapore and India. The dry leaves of patchouli on steam distillation yields "Oil of Patchouli" known to possess a warm, sweet herbaceous aromatic, spicy fragrance due to the presence of sesquiterpene and oxygenated sesquiterpene and has been largely used in perfume industries.

Currently India is not self sufficient in its requirement and has been importing 200 tonnes annually, valued at 4 million USD and the establishment of this crop in India will save the exchequer valuable foreign exchange, not forgetting the export potential in excess of 20 million USD. Commercial and systematic cultivation of elite strain of patchouli will, therefore, help meet the demand for the oil. The profitable cultivation, however, has not been successful owing to a range of constraints. The foremost being the unavailability of bulk planting material of desired types.

The rapid means of propagation through *in vitro* culture has thus become imperative. However, these efforts have been seriously constrained due to absence

of well characterized plantlet regeneration for augmenting the need of gene pool in the genetic improvement programs. Hence, there is a need to develop in vitro micropropagation and this technique may provide the only feasible alternative to meet the large scale demand for elite planting material and conservation of economically valuable resources for further use. For this reason, the development of a micropropagation protocol will be of great significance for the production of planting material to conserve this plant species. The aim of this work was to attempt for an effective and reproducible in vitro regeneration system for patchouli.

EXPERIMENTAL

Plant material and preparation of explants

The shoot tip and leaf of five-month old greenhouse grown Patchouli variety Johore were collected as explants from the Aromatic Garden at Division of Horticulture, UAS, GKVK, Bangalore. The explants excised from the mother plant were thoroughly washed in tap water for 30 minutes to remove the soil and dust adhering to it. Later these explants were washed with 2 – 3 drops of Tween – 20 (adjuvant) for 5 minutes, followed by tap water and then were rinsed in distilled water 3-4 times. Further, they were disinfected with Bavistin (Fungicide) and Streptomycin (Bacterioside) for 10 min followed by washing with distilled water. The explants were sterilized with 0.1 % Mercuric Chloride for 2 minute and then washed thoroughly with sterile water 3 to 4 times under aseptic condition in laminar air flow chamber before inoculation.

Media and culture conditions

MS medium was composed of MS salts and vitamins supplemented with sucrose (30 g l^{-1}), solidified with 0.8% (w/v) agar and adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 24 ± 2 °C under a 16 h light/8 h dark photoperiod with light provided by cool white fluorescent lamps (25 µmol m^{-2} s^{-1}) in a growth chamber.

Shoot induction and multiplication

MS basal medium supplemented with various concentrations (0.5, 1.0 to 2.5 mg l^{-1}) of different cytokinins *viz*., 6-BAP, kinetin and 2-ip [6- (-?, ?-dimethylallylamino purine)] either singly or in combination with (0.1 mg l^{-1}) indole-3- acetic acid (IAA) or a-naphthalene acetic acid (NAA) or without any plant growth regulator was used to evaluate the morphogenic potential of nodal explants. All the cultures were sub cultured to the fresh medium of same composition every 24 days.

Rooting and hardening

Rooting of shoots was carried out on half-strength and full strength MS medium supplemented with different concentrations of IAA and NAA (alone each at 0.5 mg l^{-1}) either individually or along with 0.1% activated charcoal. Rooted shoots were gently extracted from the culture vessels and washed thoroughly with tap water to remove adhered agar and traces of the medium in order to avoid contamination. The plantlets were given a final wash in distilled water for 5 min and then were treated with Bavistin (0.1%) for 5 minutes to avoid fungal infection. Later, plantlets were planted in thumb pot trays filled with sterilized media and transferred to primary hardening chamber, with 90 per cent relative humidity by adopting frequent misting, and were maintained for about 7 days. Later, these plants were transferred to secondary hardening chamber with 60 per cent humidity and reduced frequency of watering from daily to once in 3 days till 15 days of secondary hardening; the plants were ready for planting in main field.

Callogenesis, shoot and root induction

Leaves and stem segments excised from *in vitro* regenerated plantlets, were aseptically inoculated on to MS medium fortified with varying concentrations of auxins (IAA, NAA and 2,4-D; (0.5-4.0 mg l^{-1} each) either alone or in combination with 6-BAP were used for callus induction. Subcultures were carried out for every 24 days. Fresh callus tissue weighing approximately 200 to 250 mg was inoculated onto regeneration and rooting medium (as described above for shoot induction, multiplication and rooting).

OBSERVATIONS RECORDED

Shoot weight

Patchouli produced clumps of shoots during shoot proliferation and made counting of individual shoots very

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difficult. Therefore, weight was taken by removing the clumps of proliferated shoot from media and weighed by using electronic balance and expressed in milligrams.

Callus weight

The callii obtained in different treatments were removed from the culture bottles and fresh weight was taken and expressed in milligrams. The visual observations on type of callus and colour of callus were also made.

Root parameters

Number of roots was counted and length of individual root was measured using scale and expressed in centimetre.

Percent survival

The assessment of per cent survival of *in vitro* grown plants was also made on subjecting them to different media like sand, organic manure, sand and organic manure, soil-rite, and sand and soil-rite. Number of plants survived and number of plants lost was recorded and per cent survival was calculated. It is required to assure that plants were sufficiently acclimatized for outside environment before taking them directly in to main field.

RESULTS AND DISCUSSION

Shoot proliferation has been employed using shoot tip as a tool for clonal multiplication in many horticultural crops[15]. A spectacular success using this technique has been achieved in many herbaceous horticultural species [9, 11, 16, 20, 22] opined that the success of this technology is partially due to the weak apical dominance and strong root regenerating capacity of many herbaceous plants.

In tissue culture, cytokinins appear to be necessary for cell division. Cytokinins are very effective in promoting direct and indirect shoot initiation. Its successful treatment initiates several shoot buds from each explant over a period of four to six weeks. The effect of cytokinin on tissue / organ culture can vary according to the particular compound used, the type of culture, the variety of plant from which it was derived and whether the explants was taken from juvenile or mature tissue and the concentration of the cytokinin used[8].

Organogenesis was observed in nodal segments cultured on MS medium supplemented with all the concentrations of 6-BAP/ kinetin/ 2-ip alone or in combination with 0.2 mg l^{-1} IAA/NAA in both the cultivars of *Mucuna*. The morphogenic response was observed to be better with aminopurine class of cytokinins (6-BAP and 2-iP) than furfuryl amine class of cytokinin (kinetin). Although morphogenic response was observed in both the aminopurine (6-BAP and 2-iP) class of cytokinins and auxin (NAA/IAA) combinations, among these better response was observed only in case of 6-BAP, the other aminopurine cytokinin (2-iP) and even kinetin (furfurylamine) failed to show remarkable response. As the 6-BAP has been evaluated as the best suitable cytokinin for plant regeneration, hence for further studies in combination with auxins only 6-BAP has been used as choice of cytokinin. Hence the results of 2-iP and kinetin have not been discussed.

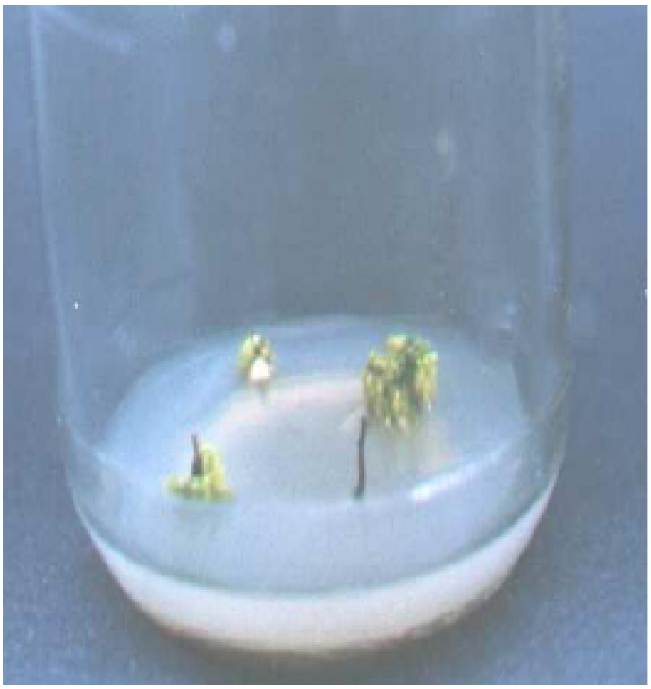

Plate 1. Direct regeneration of of Patchouli in MS basal (Control).

Direct regeneration

Direct shoot bud differentiation was observed on MS basal (Plate 1) as well as on MS medium supplemented with various concentrations of BAP alone and in combination with NAA. The maximum shoot weight (15.9 g) was observed in the MS media supplied with BAP (2.0 mg/l) and NAA (0.1 mg/l) (Plate 2) while, BAP (2 mg/l) alone, produced the shoot weight of 14.80

Plate 2. Direct regeneration Patchouli in MS basal medium supplemented with BAP (2mg/l) and NAA (0.1mg/l).

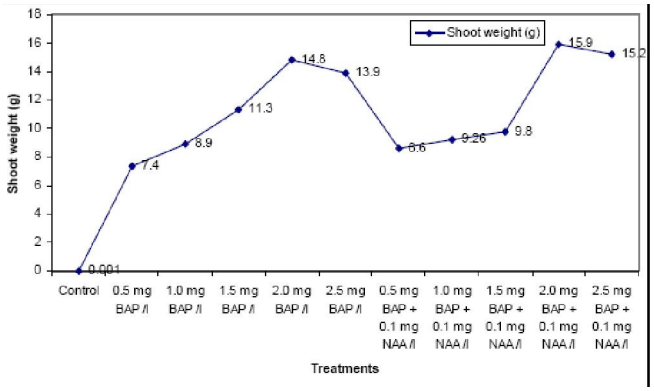

Figure 1 : Effect of BAP and NAA on shoot weight (g) of Patchouli

grams (Figure 1). However, with lower level of BAP (0.5 mg/l), the shoot weight was minimum (7.04 g). It was observed that as the concentration of BAP increased, the shoot weight also increased. Such an observation is in conformity with the results found by various authors in various species. Shoot tip of patchouli cultured on MS medium supplemented with BAP (2.0 mg/l) exhibited direct multiple shoot proliferation[2, 12, 18, 24] opined that shoot tip of patchouli cultured on MS medium supplemented with BAP (2.0 mg/l) and IAA (1.0 mg/l) produced the maximum shoot as compared to other treatments. It is very important to note that there was a decrease in shoot weight in the presence of BAP at lower level and BAP above 2.0 mg/l. Similar findings of regeneration 6-BAP and IAA/NAA combination were also reported by[1, 4-6, 17, 23, 26, 27].

Indirect regeneration

The efficiency of different levels of auxins like, NAA, 2, 4 – D and cytokinin like BAP for the initiation of callus from leaf explants was assessed in this experiment. In the treatments, where NAA was used alone, it produced brownish white callus and it was compact in nature whereas, NAA in presence of BAP produced compact light green callus (TABLE 1). Similar observation was noticed in tomato, by[10]. However in the treatment combination of 2, 4 – D and BAP the callus produced was light green in colour and it was compact and hard as well. It was known that BAP being a strong cytokinin and naturally occurring, tend to promote the formation of chlorophyll in callus and suspension culture, whereas, the auxin can be inhibitory[7].

TABLE 1: Effect of BAP and NAA on shoot weight in Patchouli

	Treatment details	Shoot weight (g)
T_1	Control	0.001
T_2	0.5 mg BAP /l	7.40
T_3	1.0 mg BAP /l	8.90
T_4	1.5 mg BAP /l	11.30
T_5	2.0 mg BAP /l	14.80
T_6	2.5 mg BAP /l	13.90
T_7	0.5 mg BAP + 0.1 mg NAA /l	8.60
T_8	1.0 mg BAP + 0.1 mg NAA /l	9.26
T_9	1.5 mg BAP + 0.1 mg NAA /l	9.80
T_{10}	2.0 mg BAP + 0.1 mg NAA /l	15.90
T_{11}	2.5 mg BAP + 0.1 mg NAA /l	15.20
	Mean	10.46
	F-test	*
	S. E m±	0.059
	CD @ 5%	0.17

The callus was initiated at the cut surface of the explants and at the petiole tip in all the treatments when placed on the media. This can be due to increase in the endogenous auxin levels, which might have activated the cell to divide rapidly to produce a mass of cells at the cut ends. The exposure of cells at the cut surface to excessive nutrients also might have enhanced the callus production at the cut ends[14]. Gradual increase in the weight of callus was observed with increased concentration of growth regulators. The maximum fresh weight (55.30 g) of callus was observed in MS medium supplemented with 2, 4 – D (2.0 mg/l) along with BAP (0.1 mg/l) (Plate 3), whereas, basal MS medium without growth regulators, did not produce any callus. It was observed that, 2, 4 – D in different concentrations pro-

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Plate 3. Induction of callus in MS basal medium supplemented with2.4-D (2mg/l) and BAP(0.1mg/l).

duced more callus as compared to NAA. These results are in line with the findings of[24] in patchouli, where she observed that callus was induced at the cut end of the leaf segments on basal MS medium supplemented with 2, 4 – D (2 mg/l) and it was poor in MS medium supplemented with NAA. This finding is also supported by[19] and[21] in patchouli. Auxin is known to enhance the cell division in *in vitro* condition, but its major effect is stimulation of cell enlargement while cytokinin mainly causes cell differentiation. The increase in callus weight with the increased concentration of NAA, 2, 4 – D along with BAP was observed in the present investigation which could be attributed to the rapid cell division by auxin and rapid cell differentiation by cytokinin. Callus induction through an auxin–cytokinin combination has also been reported in several plants [3, 13, 25, 28].

The weight of the callus appears to be a useful data in quantification and purification of secondary metabolites like terpenes or aroma compounds of patchouli, which could be extracted from the callus via suspension culture.

Root formation

The role of auxin in inducing rooting is well established. All available auxin was shown to induce the rooting with varying degrees. In the present experiment, NAA and IAA in general had a better influence on the overall rooting in patchouli, particularly IAA at 1 mg/l (Figure 2). It produced more number of roots as well as longer roots. But, the results showed non-significant differences among the treatments. With respect to cost of production, half strength MS medium was proved enough to get better rooting (Plate 4). These results are in conformity with the ones reported by[2, 24] in patchouli. The growth regulators present endogenously in the plantlets and carry over effect of growth regulators in multiplication medium might be sufficient to put forth the roots formation and development.

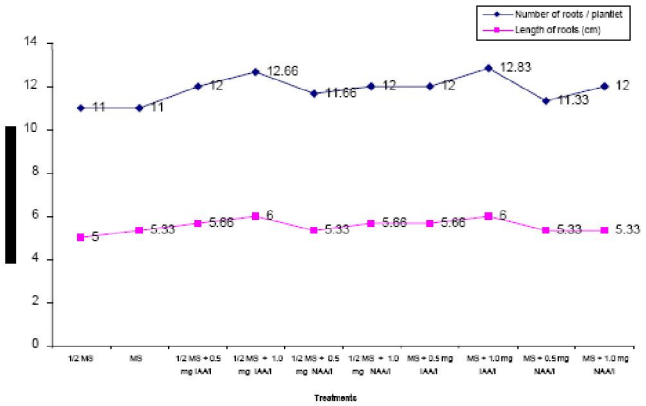

Figure 2 : Effect of strength of MS medium on rooting of Patchouli.

Plate 4. *In vitro* rooting of Patchouli at half strength MS basal medium.

Hardening off and survivability

The *in vitro* rooted patchouli plantlets were subjected to hardening chamber to acclimatize the plant-

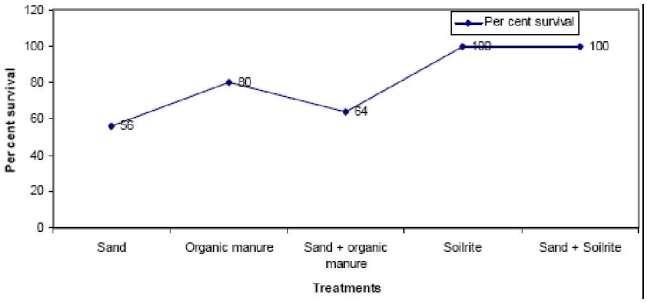

Figure 3 : Percent survival of Patchouli plantlets on different types of hardening media

lets to outside environmental conditions. The different hardening media were tried to get the maximum survivability of plantlets. Among the media tried, soilrite alone and in combination with sand (1:1 ratio) was found the best with 100 per cent survivability (Figure 3). The suitability of these substrates (Soilrite and sand @ 1:1 ratio) may be ascribed to their water holding capacity and aeration. This observation indicates that gradual acclimatization with decreasing levels of humidity may be ideal for plantlets survival during transferring period from culture bottles to pots and then to field. These observations confirm the findings of[2, 12, 19] in patchouli. A period of acclimatization is essential for the plantlets to adapt to the outside environment during which the plantlets undergo morphological and physiological adaptation enabling them to develop typical terrestrial plant having water control mechanism.

TABLE 3 : Effect of strength of MS media, IAA and NAA on production of number and length of roots in Patchouli

	Treatments	Number of roots / plantlet	Length of roots (cm)
T_1	Half strength MS	11.00	5.00
T_2	Full strength MS	11.00	5.33
T_3	Half strength MS + 0.5 mg IAA /l	12.00	5.66
T_4	Half sstrength MS + 1.0 mg IAA /l	12.66	6.00
T_5	Half strength MS + 0.5 mg NAA /l	11.66	5.33
T_6	Half sstrength MS + 1.0 mg NAA /l	12.00	5.66
T_7	Full strength MS + 0.5 mg IAA /l	12.00	5.66
T_8	Full strength MS + 1.0 mg IAA /l	12.83	6.00
T_9	Full strength MS + 0.5 mg NAA /l	11.33	5.33
T_{10}	Full strength MS + 1.0 mg NAA /l	12.00	5.33
	Mean	11.80	5.53
	F-test	NS	NS
	S. E m±	0.55	0.71

TABLE 2 : Effect of NAA and 2, 4 – D alone and in combination with BAP on type of callus, colour of callus, fresh weight of callus in Patchouli.

	Treatments	Colour of callus	Type of the callus	Fresh wt. (g)
T_1	Control	-	-	0.001
T_2	0.5 mg NAA /l	Browinsh white	Compact	40.20
T_3	1.0 mg NAA /l	Browinsh white	Compact	42.50
T_4	1.5 mg NAA /l	Browinsh white	Compact	43.40
T_5	2.0 mg NAA /l	Browinsh white	Compact	44.60
T_6	0.5 mg NAA + 0.1mg BAP/l	Light green	Compact	41.70
T_7	1.0 mg NAA + 0.1 mg BAP/l	Light green	Compact	42.20
T_8	1.5 mg NAA + 0.1 mg BAP/l	Light green	Compact	43.20
T_9	2.0 mg NAA + 0.1 mg BAP/l	Light green	Compact	44.50
T_{10}	0.5 mg 2, 4 - D + 0.1 mg BAP/l	Light green	Hard & compact	45.70
T_{11}	1.0 mg 2, 4 - D + 0.1 mg BAP/l	Light green	Hard & compact	48.60
T_{12}	1.5 mg 2, 4 - D + 0.1 mg BAP/l	Light green	Hard & compact	52.70
T_{13}	2.0 mg 2, 4 - D + 0.1 mg BAP/l	Light green	Hard & compact	55.30
	Mean	-	-	41.89
	F-test	-	-	*
	S. E m ±	-	-	0.54
	CD @ 5%	-	-	1.59

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TABLE 4 : Effect of different types of media on hardening of Patchouli

Sl. No.	Type of medium	No. of plants planted	No. of plants survived	Per cent survival
1.	Sand	25	14	56
2.	Organic manure	25	20	80
3.	Sand+organic manure	25	16	64
4.	Soil-rite	25	25	100
5.	Sand+Soil-rite	25	25	100

CONCLUSION

The ability of patchouli for *in vitro* regeneration resulted interesting outcome that it could be generated directly as well as through induction of callus. Thus the outcome is beneficial for the plant propagators to produce more number of plantlets through direct regeneration on MS medium supplemented with BAP (2.0 mg/l) and NAA (0.1 mg/l), in less time. The cost of production can be reduced since patchouli has got the ability to produce roots at half the concentration of MS medium. Survivability also reported best with cent per cent in soil rite and sand at equal proportion. It has also got significance in quantifying secondary metabolites from the callus as well as through suspension culture apart from regeneration via callus induction.

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