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Effect of cytokinin combined elicitors (L-phenylalanine, salicylic acid and chitosan) on *in vitro* propagation, secondary metabolites and molecular characterization of medicinal herb – *Coleus aromaticus* Benth (L)

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PAL gene expression

Abstract This study investigated the effect of cytokinin combined elicitors (L-phenylalanine, Salicylic acid and chitosan) on *in vitro* propagation of *Coleus aromaticus*. Furthermore, we determined the elicitor induced changes on genetic stability through Random amplified polymorphic DNA (RAPD), secondary metabolites and Phenylalanine ammonia lyase (PAL) gene expression. In *in vitro* propagation, the explant source of shoot tips and nodal explants were cultured on Murashige and Skoog (MS) medium with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l of cytokinins (Benzyl amino purine (BAP), Kinetin (KIN)) for shoot bud induction. Further, nodal explants derived shoots cultured on the combination of 1.0 mg/l of BAP with 0.5, 1.0, 1.5, 2.0, 2.5 mg/l of L-Phe, 0.2, 0.4, 0.6, 0.8, 1.0 mg/l of SA and 20, 40, 60, 80 of mg/l of Ch individually. The 79.81% of shoot bud induction was obtained with 1.0 mg/l of BAP supplemented nodal explants. In multiple shoot development, 87% of multiple shoot development was obtained in the combination of 1.0 mg/l BAP and 40 mg/l chitosan supplemented culture. The developed shoots were rooted with 0.5 mg/l of Naphthalene acetic acid (NAA) and transplanted to greenhouse condition with 75% survival rate. *In vitro* regenerated plants were subjected to RAPD profiling and it was exposed a similar banding pattern with that the mother plant. In secondary metabolites, compared to *in vivo* control the higher percentage of alkaloids (16), flavonoids (24), saponins (7.5), terpenoids (6.2), total phenolic content (24.4) and tannins (8) with increased *PAL* gene expression was analyzed in *in vitro* regenerated plants. Hence, this study shows that the choice of cytokinin combined elicitors supplementation

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during tissue culture noticeably influences not only multiple shoot development, but also the production of secondary metabolites without genetic modification.

1. Introduction

In current scenario the herbal product demand is growing exponentially throughout the world. The major pharmaceutical and herbal product industries are currently conducting extensive research on plant material for their potential medicinal values. The World Health Organization (WHO) estimated that the international market of herbal products is around $6.2 billion, which will be grown to $5 trillion by the year 2050 (Ramesh Kumar and Janagam, 2011). The worldwide increasing demand for plant derived medicines is that there has been a concomitant increase in the demand for raw herbal materials. However, the increasing human and animal populations affected the status of wild plants, particularly those used in herbal medicine. To overcome this increasing demand and elevated level of secondary metabolite synthesis, *in vitro* propagation methods offer a powerful tool for its rapid multiplication and large scale production.

Coleus aromaticus is a perennial succulent aromatic herb, belonging to the family Lamiaceae. It is widely distributed all over the world, especially in tropical Africa, Asia and Australia (Lukhoba et al., 2006). It contains protein, vitamins, minerals, trace metals and fibers containing edible nutritive plant (Gupta et al., 2005). In addition *C. aromaticus* is used in Indian folklore medicine as an antioxidant, nephroprotectant (Palani et al., 2010), antimicrobial (Devi and Yogyarti, 2006), anti-leishmania (Tempone et al., 2008), antitumor, antiepileptic activity (Gurgel et al., 2009) and in the treatment for cough, throat infection, nasal congestion, asthma, cold and epilepsy (Khare, 2007). The essential oil (terpenes) and phytochemicals of *C. aromaticus* such as flavones, salvigenin, 6-methoxygenkwanin, quercetin, chrysoeriol, and luteolin (Sahaykhare et al., 2011) play a major role in pharmaceutical and industrial products.

Large scale propagation of *C. aromaticus* depends on the selection of explants and hormones. The majority of study was carried out on *in vitro* propagation with different combination of cytokinin and auxins. A few papers have dealt with the regeneration of *C. aromaticus* with different combinations of BAP, IAA and NAA (Peter Arulanandam et al., 2011; Rajasri and Sabita, 2001). The elicitors are substances that are supplemented to the culture for development and elicitation of secondary metabolites and it can classify as biotic and abiotic compounds. The enhanced plant growth and secondary metabolites were achieved in elicitors supplied tissue cultured plant (Karwasara et al., 2011; Kamonwannasit et al., 2008).

L-Phenylalanine is an aromatic amino acid and a substrate of phenylalanine ammonia lyase (PAL) that catalyzes the L-phenylalanine into transcinnamic acid as the first step of the biosynthesis of plant phenolic compounds (Kubota et al., 2001). Phenylalanine ammonia lyase (PAL) involved in phenylpropanoid production of plants (Achnine et al., 2004). Plant cell and tissue culture were being used for the production secondary metabolites (Verpoorte et al., 2002). The metabolites produced from phenylalanine through PAL activity such as phenolics, coumarins, essential oils, flavonoids, lignin and tannins are depending on the plant growth factors and exogenous stimulant (Creasy, 1987; Bidlack and Buxton, 1995). Salicylic acid is an endogenous growth regulator and it can modulate *in vitro* growth of plants by influencing their physiological and biochemical functions (Agami and Mohamed, 2013). The promontory role of salicylic acid in callus growth, multiple shoot development, rooting and hardening of *in vitro* derived plantlets has been observed in *Ziziphus spina-christi* (Galal, 2012), *Hibiscus acetosella* and *Hibiscus moscheutos* (Sakhanokho and Kelley, 2009). Chitosan is a chitin derived linear polysaccharide. The positive effect of chitosan on shoot growth and leaves has been observed in Orchids (Nge et al., 2006) and Gerbera (Sukwattanasinitt et al., 2001). The usage of hormones and elicitors induces alterations in sensitive regions of plant genome such as DNA methylation, amplification or activation of transposable elements (Brar and Jain, 1998) and also induces somaclonal variations in a number of crops. It is necessary to establish plants without somaclonal variations when using lower and higher concentration of elicitors or growth regulators. The molecular markers such as Random Amplified Polymorphic DNA (RAPD), Random Fragmented Length Polymorphism (RFLP) and Inter Simple Sequence Repeat (ISSR) were used to analyze the genetic stability. The RAPD molecular markers were effectively used for genetic stability analysis (Carvalho et al., 2004; Venkatachalam et al., 2007).

However, there is no report in cytokinin combined elicitors on *in vitro* propagation of *C. aromaticus* and its effect on secondary metabolite content and related *PAL* gene expression. Hence, the present study aimed to develop a rapid plant regeneration protocol using cytokinin combined elicitors for large scale propagation of genetically stable *C. aromaticus*, investigation of its effect on major secondary metabolites and *PAL* gene expression. It may provide us a new approach in the application of elicitors in metabolic engineering.

2. Materials and methods

2.1. Preparation of explants

C. aromaticus plants were collected from the ABS botanical garden, Salem, Tamil Nadu, India, and maintained in the greenhouse, Department of Biotechnology, Periyar University, Salem. For shoot bud induction, shoot tips and nodal explants were excised from 3 months old plant and surface sterilized using fungicide (Bavistin, 0.2%) for 20 min, 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2 min followed by 4 rinses with sterile distilled water. Sterilized shoot tips and nodal explants were further used for *in vitro* propagation studies.

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2.2. Culture media and conditions

The MS (Murashige and Skoog's, 1962) medium fortified with different concentrations of activated carbon (Charcoal: 2.5, 5.0, 7.5 and 10 mg/l) and antioxidants (ascorbic acid: 0.5, 1.0, 1.5 and 2.0 mg/l) to protect the explants from phenolic exudation. The pH of all media was adjusted to 5.7 with 0.1 N NaOH/HCl before adding agar and 30 ml medium was poured into the culture bottles then autoclaved at 121 °C for 15 min. After sterilization, shoot tips and nodal explants were placed in culture bottles and incubated for a week at 24 ± 2 °C under 16/8 h (light/dark cycle) photoperiod to observe the blackening of tissues. The survival of explants against phenolic exudation was calculated by the following equation:

$$\text{Healthy explants } (\%) = \text{No. of healthy explants} / \text{No. of inoculated explants} \times 100.$$

2.3. In vitro propagation

2.3.1. Effect of BAP and KIN on shoot bud induction

Surface sterilized shoot tip and nodal explants were cultured on optimized MS medium supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l of BAP and KIN separately for shoot bud induction.

2.3.2. Effect of BAP and elicitors on multiple shoot development

For multiple shoot development, the *in vitro* derived nodal explants were cultured on MS basal medium containing 1.0 mg/l of BAP with 0.5, 1.0, 1.5, 2.0, 2.5 mg/l of L-Phe, 0.2, 0.4, 0.6, 0.8, 1.0 mg/l of SA and 20, 40, 60, 80 of mg/l of Ch. The elicitors were added to the culture medium before adjusting pH 5.7 prior to autoclaving. The number of responding explants, produced shoots and length of the shoots per treatment was recorded 20 days after inoculation. Further, regeneration was carried out on MS medium supplemented with the combination of 1.0 mg/l BAP and 40 mg/l Ch at 15 days interval where nodal explants showed fast growth with more number of shoots.

2.4. Rooting and acclimatization

The 2–2.5 cm of elongated shoots was cultured on MS medium with 0.5, 1.0, 1.5 and 2.0 mg/l of NAA and IAA in combination with 0.5 mg/l BAP for root induction. Plantlets with well developed roots were removed from the culture bottles and smoothly washed to remove adhering medium. Subsequently, the rooted plantlets with expanded leaves were successfully transferred into plastic cups containing sand and soil in the ratio of 1:2 and covered with polythene bags. The plantlets were initially kept in culture room for 8–12 days and then transferred to the greenhouse. After one month, the plantlets were transferred to the earthen pots containing garden soil and the survival rate was estimated by the following equation:

$$\% \text{ of survival} = (\text{No. of survival plantlets} / \text{No. of plantlets transplanted}) \times 100$$

2.5. Analysis of genetic stability by RAPD

Genomic DNA from control and *in vitro* regenerated plants were isolated by Doyle and Doyle (1987) developed a protocol. RAPD was carried out with primer OPA-13 (5′-CAGCACCCAC-3′), OPA-18 (5′-AGGTGACCGT-3′), OPA-20 (5′-GTTGCGATCC-3′), OPB-07 (5′-GGTGACGCAG-3′), OPB-14 (5′-TCCGCTCTGG-3′), OPC-05 (5′-GATGACC GCC-3′) OPD-03 (5′-GTCGCCGTCA-3′) OPE-04 (5′-GTGACATGCC-3′) OPW-06 (5′-AGGCCCGATG-3′) OPW-07 (5′-CTGGACGTCA-3′) in total volume of 20 µl, containing 2 µl of genomic DNA, 2 µl of primer, 10 µl of reaction mixture (dNTP mix, Taq DNA polymerase) and 0.6 µl of sterile distilled water. The amplification was performed in thermo cycler using the following PCR program: 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 5 min, annealing at 34 °C for 1 min, and extension at 72 °C for 10 min. The PCR products were separated on a 1.2% agarose gel using 1×TAE buffer and stained with ethidium bromide (4 µl). A size of marker used as 1 kb GeneRuler™ DNA ladder Mix. The reproducibility of amplified product was tested twice. The band appearances in RAPD profiles, as compared to the control profile, were scored by their presence (1) or absence (0).

2.6. Quantification of secondary metabolites

The secondary metabolite variations such as Total alkaloids (Harborne, 1973), Total flavonoids (Kumaran and Karunakaran, 2007), Terpenoids and Saponins (Obadoni and Ochuko, 2001), Total phenolic content (McDonald et al., 2001), Tannins (Van-Burden and Robinson, 1981) of the control and *in vitro* regenerated plants were quantified.

2.7. Analysis of PAL gene expression

2.7.1. RNA extraction and cDNA synthesis

The total RNA from control and *in vitro* regenerated plants was isolated using TRiZol reagent. Reverse Transcription was carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The RNA/primer mixture was prepared using Total RNA (5 µg), random hexamers (50 ng/µl to −3 µl), 10 mM dNTP mix (1 µl), 10 µl Diethylpyrocarbonate (DEPC) H_2O then the samples were incubated at 65 °C for 5 min and kept on ice for 1 min. The master mixture was prepared for each reaction with 10× RT buffer- 2 µl, 25 mM $MgCl_2$- 4 µl, 0.1 M DTT- 2 µl, RNaseout- 1 µl. The master mixer was added to the RNA/primer mixture, mixed and placed at room temperature for 2 min. One microliter (50 units) of SuperScript II RT added to each tube mixed and incubated at 25 °C for 10 min, then incubated the tubes at 42 °C for 50 min, heat inactivated at 70 °C for 15 min, and chilled on ice. One micromole of RNase H was added and incubated at 37 °C for 20 min, then the 1st strand cDNA was stored at −20 °C until use for real-time PCR.

2.7.2. Primers and real time PCR

The reactions were carried out in a total volume of 25 µl that contained 12.5 µl SYBR Green Mix (2×), 0.2 µl cDNA, 1 µl

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primer pair mix (5 pmol/μl each primer) and 11.3 μl H_2O. The primer sequence was 5′-CACAACTGAAGCACCACCC-3′ (forward), 5′-GAGTTCACGTCCTGGTTGTG-3′ (reverse) for PAL and 5′-GAGTTGACCTGCCATTT-3′ (forward), 5′-TTTCACGATTAGCCTTT-3′ (reverse) for β-actin. Amplifications were performed under the following conditions: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. The products subjected to agarose gel electrophoresis and the band intensity stained with ethidium bromide and it was measured using UV Transilluminator (Bio Doc System). The RT-PCR results are shown from one experiment that was repeated three times for the confirmation.

2.8. Statistical analysis

The cultures were periodically observed and frequency of responses in shoot bud initiation, multiple shoot regeneration and rooting were recorded. All the experiments were analyzed by one way ANOVA and means were compared with the Tukey's test using Graphpad prism 5.01.

3. Results and discussion

The development of an efficient regeneration in plant system plays a major role in crop improvement. In a similar way, the production of medicinal plants and its secondary metabolites can be achieved by developing suitable regeneration protocols. In this study, an efficient, commercially feasible multiple shoot regeneration protocol was evolved for *C. aromaticus* using cytokinin combined elicitors and its efficacy on genetic stability, secondary metabolites and related *PAL* gene expression was analyzed.

While initiating micropropagation of *C. aromaticus*, using shoot tips and nodal segments as explants, the tissue blackening and death were observed within 4–6 days of inoculation because of phenolic exudation by the explants in medium. To overcome this problem, the culture medium was optimized with different concentrations of activated charcoal and ascorbic acid. In our study, 7.5 g/l activated charcoal containing MS basal medium showed absolute protection for the explants against polyphenols compared to ascorbic acid (Fig. 1a and b). The hydrogen bonds of activated charcoal tend to absorb and bring down the synthesis of polyphenols and thereby prevent the browning of explants (Raghu et al., 2010). Ascorbic acid could reduce phenolic compounds through oxidation reactions catalyzed by Cu(II) and Fe(III), which are the components of MS medium (Elmore et al., 1990) and decrease explant browning. Similarly, the previous reports of Sharada et al. (2003) and Prajapati et al. (2003) revealed that the activated charcoal protected the explants by preventing the effect of leached phenolics. Supplementation of ascorbic acid to the medium inhibited the exudation of phenols in banana tissue culture (Strosse et al., 2004) as well as increased the percentage of multiple shoots in *Lawsonia inermis* (Rout et al., 2001). Nevertheless, the complete prevention of phenolic exudation was not observed in ascorbic acid as activated carbon because of the instability nature of ascorbic acid due to temperature, pH (Meucci et al., 1985) and dissolved oxygen (Tramell et al., 1986).

3.1. In vitro propagation

3.1.1. Effect of BAP and KIN on shoot bud induction

Among the five different concentrations tested in shoot bud induction 1.0 mg/l of BAP has given maximum level shoot bud breaking and development from nodal explants with the frequency of 79% (Table 1, Fig. 2a). Cytokinin supposed to play a key role in DNA synthesis, cell division, shoot proliferation and also regulating the protein synthesis responsible for the formation of mitotic spindle (Jouanneau, 1975). Earlier, the excellence of BAP on shoot bud regeneration over other cytokinins has been reported in *Pterocarpus marsupium* (Singh, 2004), *Portulaca grandiflora* (Srivastava and Joshi, 2009), and *Bambusa balcooa* (Sharma and Sarma, 2011). George (1993) reported that the enhanced shoot formation and lateral bud release from dormancy. The shoot bud initiation was declined with further increasing the BAP and KIN above 1.0 mg/l. In previous study, the higher concentrations of BAP on inhibition of shoot formation in *P. marsupium* were reported by Anis et al. (2005).

3.1.2. Effect of BAP and elicitors on multiple shoot development

In order to evaluate the multiple shoot development, *in vitro* nodal derived explants were cultured on 1.0 mg/l of BAP containing MS medium supplemented with 0.5–2.5 mg/l of L-Phe,

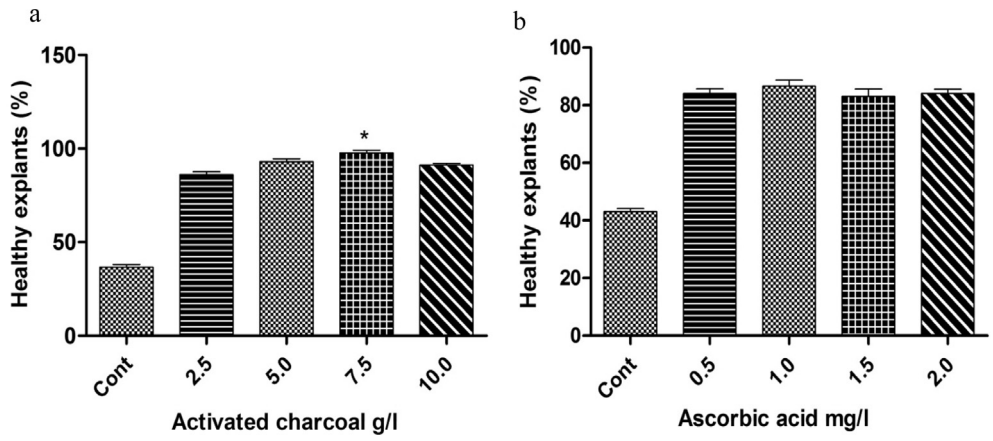

Figure 1 Effect of activated charcoal (a) and ascorbic acid (b) on Phenolic exudation inhibition. * indicates the higher percent of healthy explants.

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Table 1 Effect of cytokinin on shoot bud induction from shoot tip and nodal explants of *C. aromaticus*.

Cytokinin (mg/l)	Percent of explants induction		Number of shoots	
	Shoot tips	Nodes	Shoot tips	Nodes
BAP				
0.5	64.21 ± 1.01[b]	67.40 ± 1.01[b]	1.10 ± 0.14	1.30 ± 0.12
1.0	73.17 ± 1.23[a]	79.64 ± 1.66[a]	1.70 ± 0.21	2.20 ± 0.20
1.5	64.24 ± 1.16[b]	68.31 ± 2.15[b]	1.30 ± 0.10	1.70 ± 0.10
2.0	59.41 ± 1.39[c]	61.32 ± 1.05[b]	1.10 ± 0.11	1.10 ± 0.13
2.5	43.92 ± 1.72[d]	48.33 ± 1.52[d]	1.00 ± 0.10	1.00 ± 0.12
KIN				
0.5	61.76 ± 2.02[b]	68.11 ± 1.12[b]	1.06 ± 0.13	1.11 ± 0.18
1.0	66.21 ± 1.87[b]	71.21 ± 2.02[a]	1.40 ± 0.24	1.36 ± 0.24
1.5	57.31 ± 1.76[c]	61.00 ± 1.21[b]	1.21 ± 0.16	1.02 ± 0.12
2.0	49.42 ± 1.09[d]	52.00 ± 1.02[c]	1.00 ± 0.04	1.00 ± 0.12
2.5	32.31 ± 1.17[e]	37.25 ± 1.02[e]	1.00 ± 0.09	1.00 ± 0.09

Note: Results are expressed as the means of three replicates ±SE. Mean values followed by different letters are significantly different at $P < 0.05$ level.

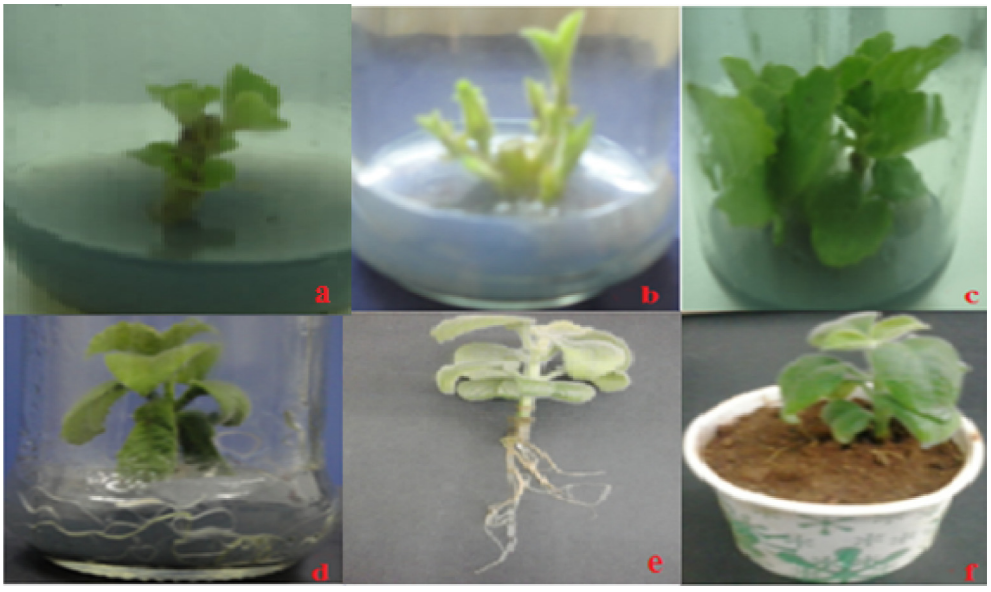

Figure 2 *In vitro* propagation of *C. aromaticus* (a) shoot bud induction, (b) multiple shoot bud development from *in vitro* derived nodal explants, (c) multiple shoots (1.0 mg BAP + 40 mg/l Ch), (d) root initiation and (e, f) rooted plant growing in the plastic cup with soil and sand in the ratio of 1:1.

0.2–1.0 mg/l of SA and 20–80 mg/l of Ch. Of these, the combinations of 1.0 mg/l BAP and 40 mg/l Ch showed a synergistic effect and produced high rate of shoot multiplication and elongation (9.37 shoots/explant) (Fig. 2b and c and Table 2). The better shoot length was also obtained in BAP and Ch combinations over other combinations. The present study clearly suggested that the combination of 1.0 mg/l BAP and 40 mg/l Ch was found to be the best for shoot multiplication. The positive effect of BAP combined Ch on plant growth may be attributed to increased uptake of essential nutrients through adjusting osmotic pressure and key enzyme activities of nitrogen metabolism (nitrate reductase, glutamine synthase) induced plant growth and development (Mondal et al., 2012). Chitosan is a polysaccharide and used as a growth stimulator in tissue culture (Chandrkrachang, 2006). Chandrkrachang (2002) stated that the chitosan increases the growth of orchids and flower production, and increases the Protocorm Like Bodies (PLB) fresh weight in *Dendrobium* species. The associated function of cytokinin and chitosan may have induced multiple shoot development rapidly in *C. aromaticus*. The limited concentration of elicitors had favored the *in vitro* multiplication but when it exceeded, morphological abnormalities and decreased biomass were seen. Our findings were also supported by Ait Barka et al. (2004) in grapevine culture, *Panax ginseng* (Jeong and Park, 2005) and *Artemisia annua* (Putalun et al., 2007). Further, *in vitro* regenerated shoot buds were subcultured subsequently on MS medium supplemented with the combinations of 1.0 mg/l of BAP and

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Table 2 Combined effect of BAP and elicitors on shoot bud multiplication from *in vitro* derived nodal explants of *C. aromaticus*.

Elicitors (L-Phe, SA, Ch)	Percent of shoot regeneration	No. of shoots/explants	Length of shoots (cm)
Combination of L-Phe with 1.0 mg BAP/l			
0.5	57.0 ± 1.4[d]	2.40 ± 0.86[e]	3.75 ± 3.43[d]
1.0	61.0 ± 1.30[c]	3.30 ± 0.32[d]	5.02 ± 1.22[b]
1.5	43.0 ± 1.05[e]	3.61 ± 0.14[d]	2.50 ± 2.26[e]
2.0	36.0 ± 1.02[f]	2.32 ± 0.30[e]	3.35 ± 2.17[d]
2.5	32.0 ± 1.92[f]	2.65 ± 0.14[e]	3.50 ± 0.40[d]
Combination of SA with 1.0 mg BAP/l			
0.2	61.0 ± 1.16[c]	3.59 ± 0.23[d]	3.10 ± 1.02[d]
0.4	64.0 ± 1.02[c]	4.43 ± 0.22[c]	4.20 ± 3.43[c]
0.6	59.0 ± 1.03[d]	3.49 ± 0.17[d]	3.60 ± 0.75[d]
0.8	52.0 ± 1.03[d]	3.22 ± 0.17[d]	4.50 ± 2.62[c]
1.0	38.0 ± 1.03[f]	1.67 ± 0.12[f]	3.83 ± 1.64[d]
Combination of Ch with 1.0 mg BAP/l			
20	82.0 ± 1.12[a]	4.36 ± 0.27[c]	4.03 ± 1.17[c]
40	87.0 ± 0.72[a]	9.14 ± 0.20[a]	6.33 ± 2.44[a]
60	71.0 ± 2.21[b]	5.41 ± 0.77[b]	3.40 ± 1.34[d]
80	67.0 ± 1.15[c]	3.42 ± 0.19[d]	3.50 ± 1.17[d]

Note: Results are expressed as the means of three replicates ±SE. Mean values followed by different letters are significantly different at $P < 0.05$ level.

Table 3 Effect of different concentrations of NAA and IAA on root formation.

Auxin Conc. (mg/l)	Percent of rooting	No. of roots/shoots	Root length
NAA			
0.5	86 ± 2.03[a]	8.3 ± 1.28[a]	9.13 ± 1.10[a]
1.0	73 ± 2.88[b]	5.2 ± 0.09[b]	6.43 ± 0.84[b]
1.5	56 ± 4.04[d]	4.8 ± 1.32[c]	3.54 ± 0.64[e]
2.0	38 ± 2.02[e]	2.5 ± 1.06[e]	2.03 ± 0.34[f]
IAA			
0.5	79 ± 2.30[b]	5.9 ± 1.32[b]	5.40 ± 0.49[c]
1.0	60 ± 5.01[c]	3.4 ± 0.35[d]	4.40 ± 0.49[d]
1.5	54 ± 1.10[d]	2.4 ± 0.05[e]	3.50 ± 0.20[e]
2.0	31 ± 2.10[e]	2.1 ± 0.21[e]	2.13 ± 0.56[f]

Note: Results are expressed as the means of three replicates ±SE. Mean values followed by different letters are significantly different at $P < 0.05$ level.

40 mg/l Ch for multiple shoot regeneration. After three subcultures the maximum number of shoots was obtained (28 shoots/explant) (Fig. 2d).

3.1.3. Rooting of shoots and acclimatization

Elongated shoots were transferred to different concentrations of NAA and IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) supplemented MS medium for rooting. The roots developed within two weeks of culture and data were recorded. The higher frequency (86%) and maximum number of roots (8.0 roots/shoot) were observed in 0.5 mg/l of NAA (Fig. 2e, Table 3). Among the two auxins (NAA, IAA) used, the maximum percent of rooting was obtained in MS medium fortified with NAA which was found to be superior for rooting over IAA. Peeters et al. (1991) reported in tobacco plant that the rate of root formation by NAA is six times faster than IAA. Lin et al. (2000) documented that NAA is a more effective regulator in root formation. The rate of root formation was declined when increasing the concentration. Similarly, Seyyed et al. (2013) obtained higher number of roots in micropropagation of *Alstroemeria* at 0.5 mg/l of NAA and it was decreased when increasing the concentration. IAA is photo-oxidized rapidly by the plant tissues (Epstein and Ludwig-Müller, 1993). Smulders et al. (1990) stated that the NAA is not destroyed by auxin oxidase so it might be the preferable auxin in tissue culture. Using NAA at lower concentration, the rate of root formation is faster within a short period. Roots induced on MS medium with NAA were found to be thick and long whereas the roots were thin and short on MS medium augmented with IAA.

The rooted plantlets were transferred into 1:2 of sand and soil containing plastic cups. The plastic cubs were covered with polythene bags to ensure high humidity. The plantlets were kept in the controlled environment for two weeks. The poly bags were removed and acclimatized the plantlets under greenhouse conditions. Subsequently, they were transferred to garden soil with 75% of survival rate (Fig. 2f).

3.2. Analysis of genetic stability by RAPD

The prolonged exposure of BAP combined Ch, the chances of somaclonal variation induction increased in regenerated plant-

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Table 4 Oligonucleotide sequences and RAPD amplification results of *C. aromaticus*.

Oligonucleotide primer	Sequences (5′-3′)	No. of scored bands	No. of polymeric bands	Percent of polymorphism (%)
OP A-13	CAGCACCCAC	7	–	0
OP A-18	AGGTGACCGT	6	–	0
OP A-20	GTTGCGATCC	5	–	0
OP B-07	GGTGACGCAG	4	–	0
OP B-14	TCCGCTCTGG	4	–	0
OP C-05	GATGACCGCC	6	–	0
OP D-03	GTCGCCGTCA	5	–	0
OP E-04	GTGACATGCC	5	–	0
OP W-06	AGGCCCGATG	5	–	0
OP W-07	CTGGACGTCA	6	–	0

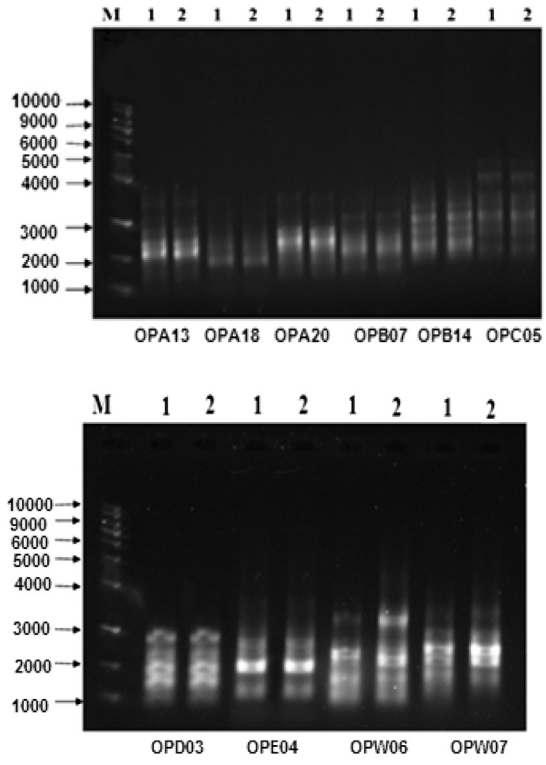

Figure 3 RAPD band profiles generated by the primers OPA 13, OPA 18, OPA 20, OPB 07, OPB 14, OPC 06, OPD 05, OPE 04, OPW 06 and OPW 07. Lane M: 1 kb DNA ladder, 1: *in vitro* regenerated plant, 2: control plant.

lets. In this study, RAPD marker analyzes the genetic stability of *in vitro* regenerated plants. A total of ten selected primers OPA-13 (5′-CAGCACCCAC-3′), OPA-18 (5′-AGGTGACC GT-3′), OPA-20 (5′-GTTGCGATCC-3′), OPB-07 (5′-GGT GACGCAG-3′), OPB-14 (5′-TCCGCTCTGG-3′), OPC-05 (5′-GATGACCGCC-3′) OPD-03 (5′-GTCGCCGTCA-3′) OPE-04 (5′-GTGACATGCC-3′) OPW-06 (5′-AGGCCCGA TG-3′) OPW-07 (5′-CTGGACGTCA-3′) generated a total of 110 bands within 600 to 1000 bp size and the number of formed bands in each primer ranged from 4 to 8 (Table 4, Fig. 3). The amplification products were found monomorphic bands across the samples produced by all RAPD primers. This confirmed the *in vitro* regenerated *C. aromaticus* authenticated that genetically stable and free from clonal variations. Similarly, the absence of genetic variation using RAPD has been reported in several cases of micropropagated shoot tips and auxiliary buds of *Terminalia arjuna* (Gupta et al., 2014), axillary bud of chestnut root stock hybrids and almond plantlets (Carvalho et al., 2004; Martins et al., 2004). In contrast, somaclonal variations were reported in micropropagated plants of *Populus tremuloides* (Rahmann and Rajora, 2001) and *Actinidia deliciosa* (Palombi and Damiano, 2002). In the present study, similar banding pattern was observed in *in vitro* regenerated and control plants in RAPD profiles which indicate the absence of clonal variations.

3.3. Quantification of secondary metabolites

The total alkaloids, total flavonoids, terpenoids, saponins, total phenolic contents and tannins are working as a powerful antioxidant and playing a beneficial role in various disorders of inflammatory, cancer and diabetes. Being an important medicinal plant biochemical constitution of *C. aromaticus* is essential. Variations were observed between control and *in vitro* propagated plants as Total alkaloids (16%), Total flavonoids (24%), Terpenoids (6.2%), Saponins (7.5%), Total phenolic content (24.4%) and Tannins (8%) were determined in *in vitro* regenerated plant (Fig. 4). Bourgaud et al. (2001), Ramachandra Rao and Ravishankar (2002) reported the higher quantity of secondary metabolite (Bakuchiol) determined in aerial parts of *in vitro* derived *Psoralea drupacea*. The variations in metabolites may have the differences in media supplementation. Our results suggested that the elicitor supplementation may have influenced the secondary metabolite synthesis. Similarly, the increased quantity of secondary metabolites were observed in tissue cultured *Polygonum multiporum* (Lin et al., 2003), *Gentiana daviddi var. femosana* (Chueh et al., 2001) and *Salacia chinensis* (Chavan et al., 2012). The possible reason for enhanced level of secondary metabolites in tissue cultured plants varies because grown under controlled condition with optimum supply of nutrients and elicitors.

3.4. PAL gene expression

The level of *PAL* gene expression from *in vitro* regenerated plants were determined and compared to control plant. The higher level of gene expression was found in *in vitro* regenerated plant than control plant (Fig. 5). Our results suggested

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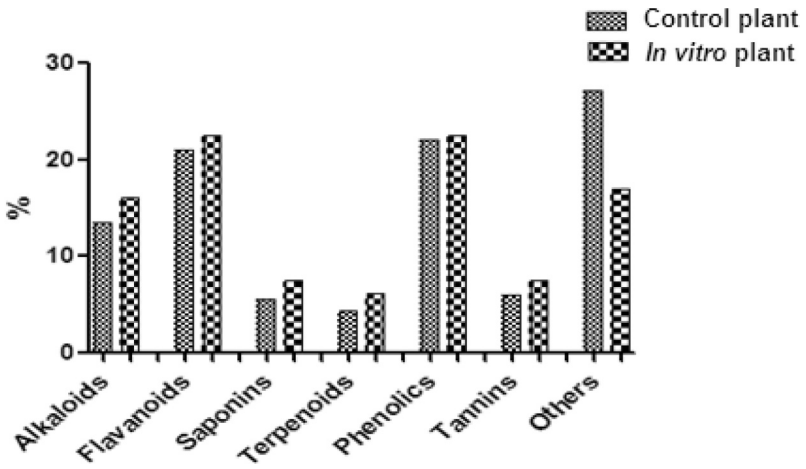

Figure 4 Quantification of secondary metabolites from control and *in vitro* regenerated plant leaf extract of *C. aromaticus*.

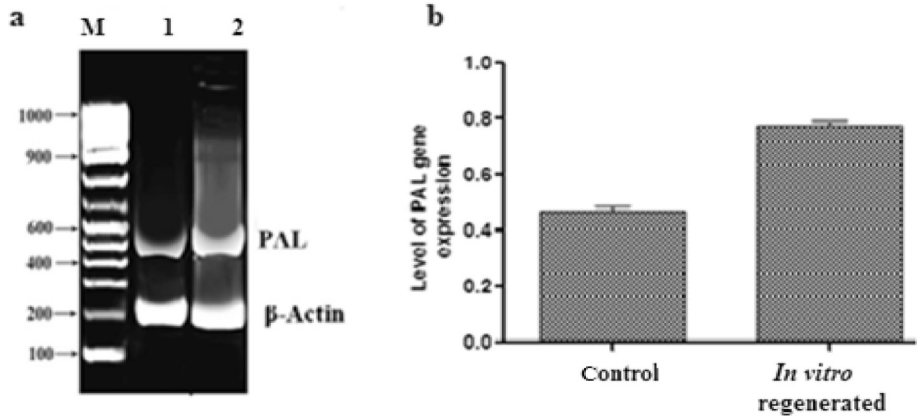

Figure 5 (a) Expression of *PAL* mRNA from control and *in vitro* regenerated *C. aromaticus*. (b) Band intensity of *PAL* gene expression. M – Ladder, 1 – *in vitro* regenerated plant, 2 – control plant.

that the accumulations of secondary metabolites are directly related to nutrient supplementation and level of gene expression. Dixon and Paiva (1995) reported that PAL catalyzes the conversion of L-phenylalanine to trans-cinnamic acid in phenylpropanoid pathway. In this concern, Bate et al. (1994) reported that the severe and small suppression of *PAL* activity in tobacco transgenic plants resulted in drastically reduced metabolite accumulation, whereas enhanced level of phenylpropanoid products were found with increased *PAL* activity (Liu et al., 2006). These results indicated that PAL is a key enzyme in the regulation of overall flux into metabolite biosynthesis. Kostenyuk et al. (2002) reported that the overexpression of AtPAL in citrus transgenic roots substantially altered the growth and phenolic production. The activation of PAL under plant growth condition was considered as a part of defense mechanism and production of secondary metabolites (Peiser et al., 1998).

4. Conclusion

This study provides an efficient protocol for higher frequency of genetically stable multiple shoot development and complete plant regeneration system through nodal segments by supplementation of cytokinin combined elicitors. The present investigation provides an efficient micropropagation method which could be commercially feasible for producing uniform plants with high multiplication rate. The enhanced major secondary metabolites with *PAL* gene expression were determined by supplementation of elicitors in a relative short time. The combination of BAP and Ch showed positive influence on multiple shoot induction than cytokinin alone. To the best of our knowledge, this is the first report on the efficacy of cytokinin combined elicitor on genetically stable multiple shoot development from nodal explants, and enhanced secondary metabolite synthesis with upregulated gene expression of *C. aromaticus*. Further, this protocol can be used to engineer metabolic pathway genes for producing industrially important secondary metabolites.

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