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## Changes in Regeneration and Oil Accumulation of *Pelargonium nervosum* under Various Culture Conditions

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**Abstract:** When leaf and petiole explants of *P. nervosum* were cultured on Murashige and Skoog medium supplemented with various combinations of 6-benzylaminopurine and 1-naphthaleneacetic acid, callus was initiated, but the growth rate and shoot differentiation differ during 5 subcultures. In general, the highest shoot induction (97%) with maximum increase in fresh and dry weight of culture was obtained on medium supplemented with 0.5 mg L<sup>-1</sup> 6-benzylaminopurine + 0.2 mg L<sup>-1</sup> 1-naphthaleneacetic acid after 3 subcultures. On the other hand, benzylaminopurine at 5.0 or 2.0 mg L<sup>-1</sup> stimulated growth of callus with very little shoots and accompanied by an increase in fresh and dry weight till 4th and 5th subcultures, respectively. Moreover, when cytokinin (BAP) was added at 1 mg L<sup>-1</sup> and auxin (NAA) at 0.2 or 0.1 mg L<sup>-1</sup>, an increase in shoot production (80%) was obtained after 4 subcultures. Whole plantlets of *P. nervosum* were recovered following rooting of shoots in MS medium without hormones and successfully re-established in soil under controlled conditions. The relative amounts of the major component of the essential oil of the regenerated shoots, namely menthone, myrtenol, citral, neryl acetate,  $\alpha$ -muurolene, allo-aromadenderene,  $\beta$ -farnesene,  $\gamma$ -muurolene,  $\delta$ -cadiene were different from those present in the partially differentiated callus and parent plant however, citral was the main oil components present in all cultures.

**Key words:** Regeneration, essential oils, *Pelargonium nervosum*, shoot cultures

### INTRODUCTION

The genus *Pelargonium* (Family: Geraniaceae) contains a large number of scented variants which produce between them a wide range of essential oil components including mono and sesquiterpenes. These are commercially important plant products used widely in the perfume, pharmaceutical and flavour industries. Generally, monoterpene and other lipophilic compounds are sequestered in specialized cells or tissues, e.g. glandular hairs, resin canals and schizogenous glands. It has often been noted that these facets of differentiation are usually absent in non-producing cell lines and it has therefore been implied that there is an actual requirement for a level of differentiation in the culture before product synthesis can take place<sup>[1]</sup>. However the cultures in which these natural storage sites are absent, any essential oil that is synthesized has to be stored in the cell vacuole or extracted into the medium. There is ample evidence to show that free monoterpene are toxic to plant cells, acting by inhibiting respiration and photosynthesis and by decreasing cell membrane permeability<sup>[2]</sup>.

In general, undifferentiated callus or suspension of cells accumulate essential oil at a fraction of the concentration produced by the parent plant. Thus,

approximately half of all callus lines derived from 34 variants scented *Pelargoniums* accumulate less than 0.1% of the oil of the whole plant and only eight cultures accumulated oil at a level greater than 1% of that of the parent plant and even the most productive lines are derived from *P. Miss Australia* and *P. nervosum* accumulated a mere 5% and 3.5%, respectively of the oil of the intact plant<sup>[2]</sup>. *P. nervosum* is a robust perennial plant with showy-rose lavender flower and considered as one of the most refreshing and fragrant of the scented geranium.

Brown and Charlwood<sup>[3]</sup> have investigated the control of callus formation and differentiation within *Pelargonium* genus and have confirmed a correlation between monoterpene production and morphological differentiation *in vitro*. Further studies indicated that neither organogenesis nor the formation of specialized tissues is a prerequisite for monoterpene synthesis in plant cell cultures<sup>[4]</sup>. However, organized or differentiated cultures often accumulate high levels of secondary metabolites<sup>[2,5]</sup>.

The present investigation aim to establish the use of plant growth regulators to improve the essential oil accumulation and organogenesis in *P. nervosum*, also to obtain fully developed plants and transfer them to the soil.

## MATERIALS AND METHODS

**Initiation of shoot cultures:** Callus of *P. nervosum* was initiated from petiole and leaf explants as described

by Brown and Charlwood<sup>[6]</sup> and maintained on MS medium<sup>[7]</sup> containing 5 mg L<sup>-1</sup> 6-benzylaminopurine (BAP), 1 mg L<sup>-1</sup> 1-naphthaleneacetic acid (NAA), 3% sucrose and 1% agar adjusted to pH 5.5. Incubation was carried out under continuous light at 26°C. In order to determine the growth curve for the callus grown on maintenance medium, sample cultures (each of size 2.0±0.4 g fresh weight [W<sub>0</sub> at t<sub>0</sub>]) were harvested 5-7 days over 4 weeks culture period (W<sub>1</sub> at t<sub>1</sub>).

Again, callus on the initiation medium was transferred to solidified MS medium with varying plant growth regulator of the following concentrations as mg L<sup>-1</sup> and maintained under these conditions for 5 passages.

a-2.0 BAP: 0.1 NAA, d-5.0 BAP: 0.2 NAA

b-1.0 BAP: 0.1 NAA, e-1.0 BAP: 0.2 NAA

c-0.5 BAP: 0.1 NAA, f-0.5 BAP: 0.2 NAA

In order to determine the growth characteristics for each treatment, samples cultures (each of size 3.0±0.4 g fresh weight) were subcultured every 2 weeks and incubated under continuous light at 26°C. It was on these calli that the following tests were performed for each treatment. About 3.0 g of the tissue was used for subculture, another 3.0 g extracted with dichloromethane and the remain of cultures were treated with liquid nitrogen before placed on the freeze dryer till constant dry weight.

The fresh and dry weights were determined for 3 replicates per each treatment, standard error was determined and the doubling time was calculated using the equation  $t_d = (1/n2.(t_1-t_0))/\ln W_1/W_0$ .

**Essential oil extraction and analysis:** Essential oils from samples (3-4 g) of callus, shoot cultures or the leaves of the intact plant were extracted with dichloromethane and reduced to 0.1 mL under an atmosphere of nitrogen before analysis. Extracts were analyzed directly by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard model HP5890 series II chromatograph equipped with a BPI capillary column (25 m x 0.22 mm i.d.; film thickness 0.25 µm) the outlet of which was connected to a Jeol model AX505W mass spectrometer. The GC oven temperature program was 50°C held for 5 min, increased to 250°C at a rate of 3°C min<sup>-1</sup> and held for 4 min. The carrier gas was helium at a flow rate of 1.0 mL min<sup>-1</sup> and the sample size was 1 µL (injector temperature 210°C; split ratio 1:15). The ion source

temperature was 200°C and the ionizing potential was 70 eV with an accelerating voltage of 3 kV; the sensitivity was 1.45 and the scan speed 1 sec: spectra were scanned in the range 35-400 µ. Quantitative analysis of monoterpene of the essential oils of regenerated tissues from different treatments were carried out after 4 passage using GC (Monoterpene were identified by comparing retention time with authentic standard).

**Plant regeneration and re-establishment in soil:** Callus material of *P. nervosum* was transferred from maintenance medium to solidified MS medium with various concentrations of BAP and NAA, when significant morphological differentiation has occurred in the subsequent passages. Plantlets were grown to minimum height of 4 cm before attempting root induction. Such induction was brought about by reculturing plantlets onto MS medium containing 3% sucrose and omitting hormones<sup>[8]</sup>, then regenerated plantlets were transferred to solidified MS medium without sucrose or hormones and left for further 4 weeks. In order to re-establish new plants, after root induction, plantlets were removed from the growth vessel, washed with sterile water to remove traces of agar, transferred to a mixture of sterile mold and sand moister with water and maintained in the growth chamber under continuous light for 15 days before moved to the experimental pots.

## RESULTS

**Effect of growth regulators on callus growth:** The mean results from experiments carried out in triplicate showed the response of *P. nervosum* callus to treatment with varying plant growth regulators, a general development pattern can be observed where callus was initiated on all concentrations but the growth rate and shoot differentiation varied during 5 subcultures.

The average doubling times for fresh and dry weights of *P. nervosum* callus maintained on solidified MS medium containing 5.0 mg L<sup>-1</sup> BAP: 1.0 mg L<sup>-1</sup> NAA (Fig. 1) were 5.4 and 5.7 days, respectively. When samples from these calli were cultured on similar medium supplemented with 0.5 mg L<sup>-1</sup> BAP and NAA at 0.1 or 0.2 mg L<sup>-1</sup>, a maximum frequency of shoot induction (97%) was attained where, small shoots of *P. nervosum* were observed after only one passage (14 day growth cycle) under this regime and a stable shoot-proliferation culture (Fig. 2 plate c) was obtained after a further 3 passages. Such shoot cultures grow faster, showing t<sub>d</sub> values of 4.5 and 4.2 days for dry weight of cultures, respectively after 3 passage. On the other hand, high levels (2 or 5 mg L<sup>-1</sup>) of BAP resulted in continuous production of callus with very little shoots (Fig. 2 plate a) irrespective of the ratio of

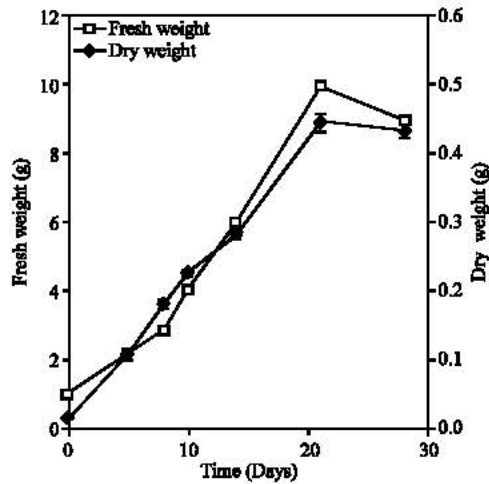


Fig. 1: Growth curve of unorganized callus cultures of *P. nervosum* cultivated in ( $5 \text{ mg L}^{-1}$  BAP :  $1 \text{ mg L}^{-1}$  NAA) MS medium (When absent, standard deviation are smaller than symbols)

NAA ( $0.1$  or  $0.2 \text{ mg L}^{-1}$ ). However, the morphology of the callus mass did not alter with the ratio of BAP to NAA. When cytokinin (BAP) was maintained at  $1.0 \text{ mg L}^{-1}$  and auxin at ( $0.2$  or  $0.1 \text{ mg L}^{-1}$  NAA) the growth rate accelerated ( $t_D$  values being  $4.9$ ;  $4.6$  days for fresh and  $4.6$ ;  $4.6$  for dry weights of cultures, respectively) and accompanied with an increase in shoot production being ( $80\%$ ) after 4 subculture (Fig. 2 plate b).

With exceptions of high levels (i.e  $5.0$  and  $2.0 \text{ mg L}^{-1}$ ) of BAP, the maximum increase in fresh and dry weight of culture were obtained after 3 subcultures in all concentrations of BAP and NAA, then they decreased in 4th subculture compared to 3rd one. However the reverse was obtained with high level of BAP at 4th and 5th subcultures (Fig. 3 and 4).

**Effect of growth regulators on essential oil content:**

Qualitative analysis by GC-MS revealed the major components of the essential oils of the parent plant, regenerated plant and partially differentiated callus (Fig. 5), whilst partially differentiated callus grown on  $2.0 \text{ mg L}^{-1}$  BAP and  $0.1 \text{ mg L}^{-1}$  NAA (Fig. 5A), accumulated negligible amounts of mono- and sesquiterpenes, namely, 3-carene, limonene, citral,  $\alpha$ -terpinyl acetate and  $\gamma$ -muurolene, there was a gradual restoration of mono- and sesquiterpenes accumulation as morphological differentiation was induced. Thus, the callus grown on  $0.5 \text{ mg L}^{-1}$  BAP and  $0.2 \text{ mg L}^{-1}$  NAA (Fig. 5B), exhibited the development of shoots with glandular hairs after 4 passages. Such cultures accumulated menthone, myrtenol, citral, neryl acetate,  $\alpha$ -muurolene, allo-aromadenderene,  $\beta$ -farnesene,

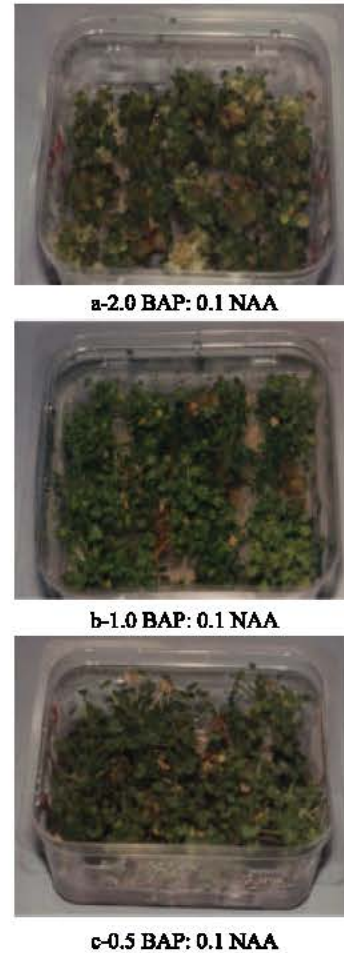


Fig. 2: The response of *P. nervosum* callus to treatment with varying plant growth regulator concentrations ( $\text{mg L}^{-1}$ ) after 4 subcultures

$\gamma$ -muurolene and  $\delta$ -cadiene. On the other hand, the parent plant produced oil with different profile to that present in the regenerated shoots (Fig. 5C and B, respectively).

Staining with Nile blue sulphate and oil red O revealed that the epidermal hairs are the sites of accumulation of the essential oil in the intact plant as well as in the shoot cultures.

Quantitative analysis (GC) of monoterpene products of the essential oils of various treatments after 4 passage showed that, in *P. nervosum* cyto-differentiation may be controlled by manipulation of the concentration ratios of the plant growth regulators within the range  $5.0 \text{ mg L}^{-1}$  BAP:  $0.2 \text{ mg L}^{-1}$  NAA to  $0.5 \text{ mg L}^{-1}$  BAP:  $0.1 \text{ mg L}^{-1}$  NAA. Thus, in callus culture system, supplementation of the medium with  $2.0 \text{ mg L}^{-1}$  BAP:  $0.1 \text{ mg L}^{-1}$  NAA gave rise to a low level of shoot initiation but an increase in oil accumulation (equivalent to  $1.22 \text{ mg oil g}^{-1}$  fresh weight) compared with the unorganized culture grown on

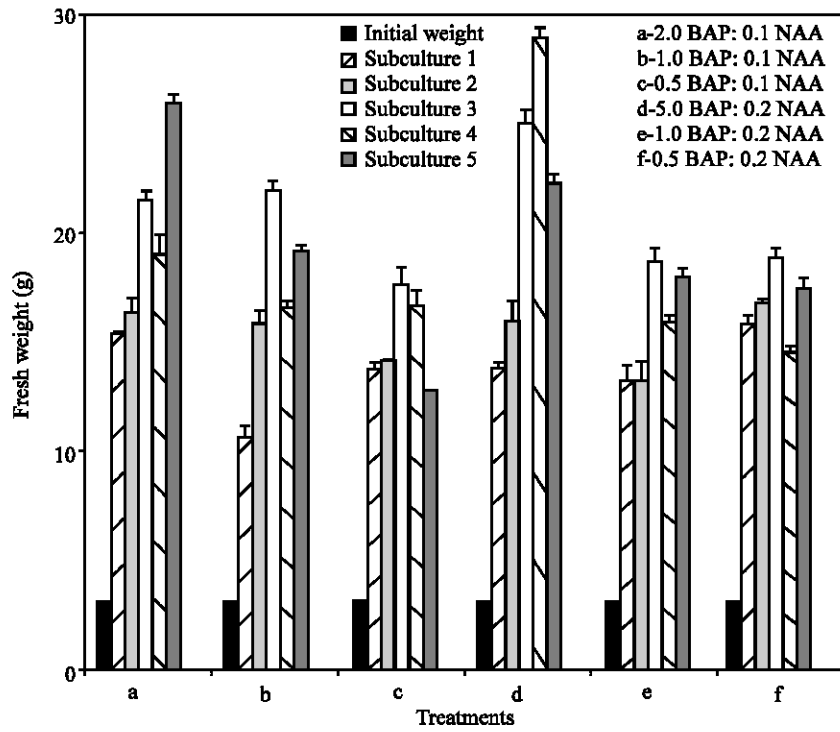


Fig. 3: The effect of varying plant growth regulator concentrations ( $\text{mg L}^{-1}$ ) on fresh weight of *P. nervosum* (subcultures 1-5)

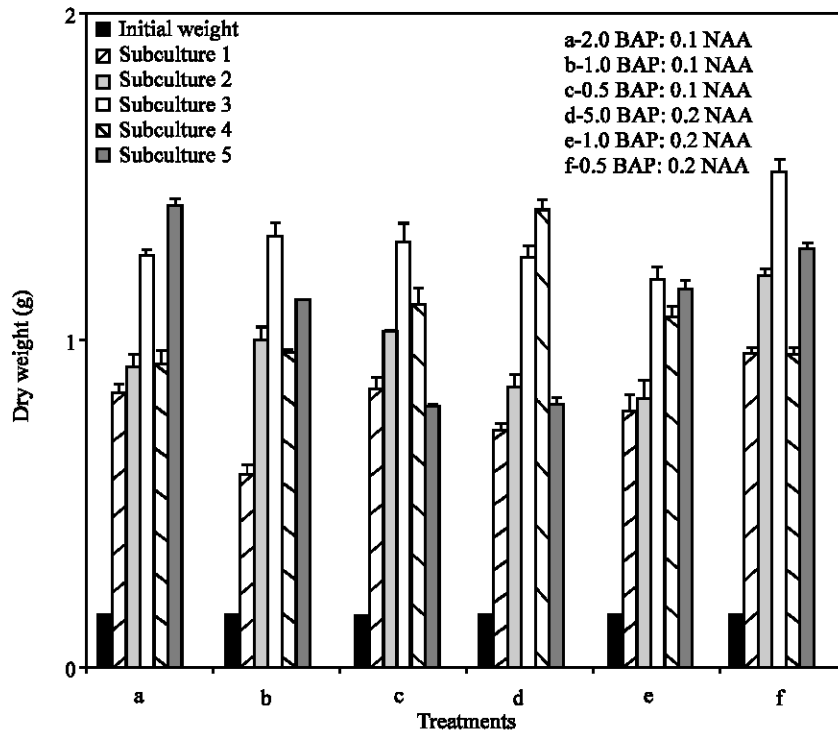


Fig. 4: The effect of varying plant growth regulator concentrations ( $\text{mg L}^{-1}$ ) on dry weight of *P. nervosum* (subcultures 1-5)

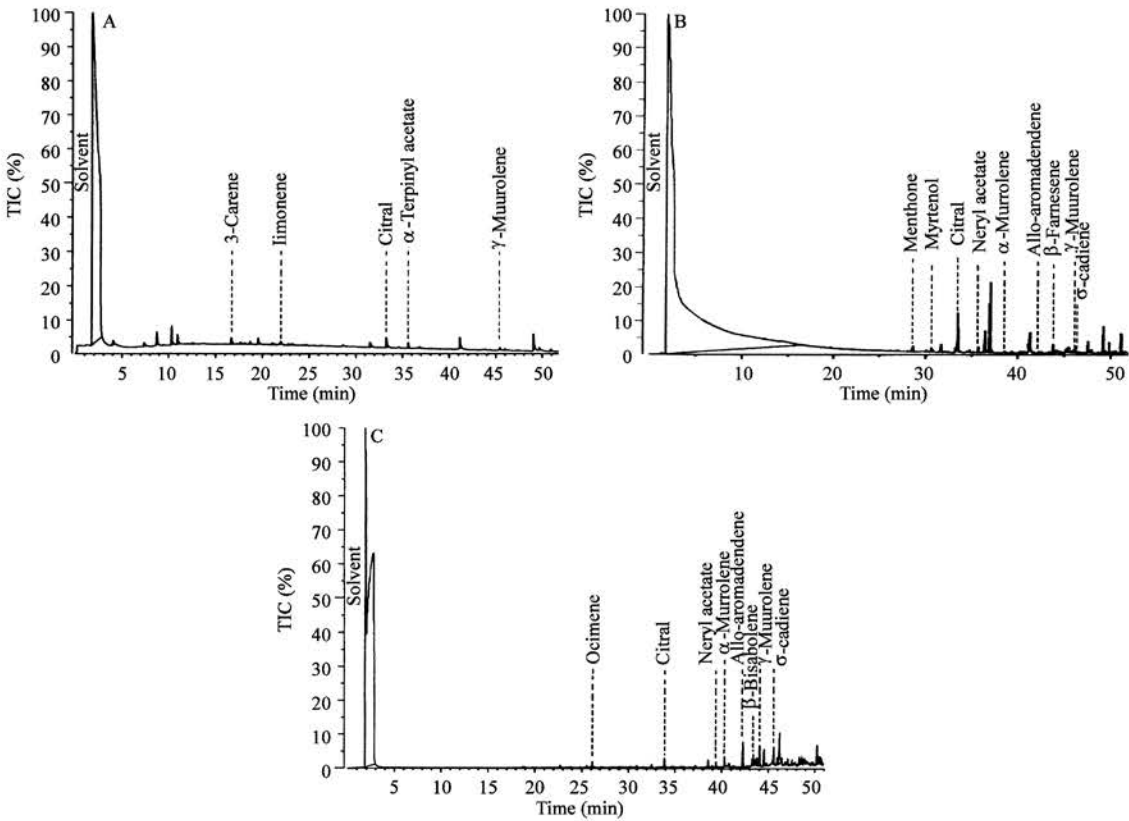


Fig. 5: Gas chromatograms of extracts of tissues of *P. nervosum* (A) partially differentiated callus, (B) regenerated shoots, (C) leaves of intact plant

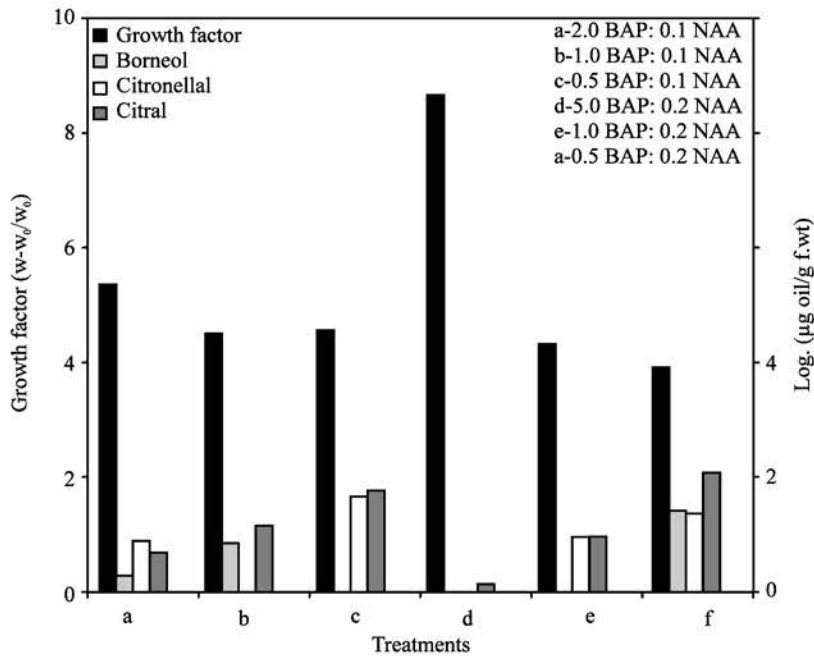


Fig. 6: The effect of varying plant growth regulator concentrations ( $\text{mg L}^{-1}$ ) on growth and essential oil profiles in shoot cultures of *P. nervosum* (subculture 4)



Fig. 7: Many well developed plantlets of *P. nervosum* on rooting MS medium omitting hormones under continuous light



Fig. 8: Regenerated plantlets of *P. nervosum* successfully reestablished in soil

maintenance medium (maintained on solidified MS medium containing  $5.0 \text{ mg L}^{-1}$  BAP;  $1 \text{ mg L}^{-1}$  NAA). However, applications of shoot-proliferation cultures by reduction of the growth regulator levels a dramatic effect in product accumulation by culture was observed. After 4 passages, levels of oil production increased to a quantity similar to that of the parent plants (equivalent to  $4.55 \text{ mg oil/g}$  fresh weight). This increase in oil accumulation due to  $0.5 \text{ mg L}^{-1}$  BAP +  $0.2 \text{ mg L}^{-1}$  NAA was accompanied by a reduction in growth rate and a shift in oil profile to from oxygenated derivatives oil (they encourage the biosynthesis of monoterpene) with

maximum value of borneol, citronellal and citral (Fig. 6). Moreover, the callus growth and shoot differentiation capacity was enhanced at  $1.0 \text{ mg L}^{-1}$  BAP + NAA at  $0.1$  or  $0.2 \text{ mg L}^{-1}$  showing the same growth rate  $4.5$ ,  $4.3$ , respectively and similar value of citral but citronellal and borneol disappeared, respectively. Again, high level of cytokinin (i.e  $2$  or  $5 \text{ mg L}^{-1}$ ) was effective for callus induction till 4th subculture with highest growth factor being ( $5.38$  and  $8.68$ ) at the same time, it increased oil accumulation and is accompanied by a shift in oil profile from the oxygenated derivatives to sesquiterpenes; borneol and citronellal formation were inhibited as cytokinins concentration increased to  $5 \text{ mg L}^{-1}$  more strongly than citral biosynthesis.

**Plant regeneration and re-establishment in soil:** Explants of *P. nervosum* formed a white friable callus after 7 days on maintenance medium which grew as a friable green colored callus by continuous subculturing. Regenerated shoots of *P. nervosum* could be obtained after 3 passages incubation of the calli on MS medium containing  $0.5$  or  $1.0 \text{ mg L}^{-1}$  BAP and NAA at  $0.1$  or  $0.2 \text{ mg L}^{-1}$ . Root is differentiated in regenerated plantlets (Fig. 7) only after two weeks on MS medium omitting hormones. Such regenerated shoots showed high photosynthetic capacity, behaving as autotrophic plants on MS medium without sucrose or hormones. The complete plantlets with well-developed roots (Fig. 8), were maintained in the growth chamber under continuous light for acclimatization before being moved to the field conditions where they survived; visual examination showed no signs of morphological variation.

## DISCUSSION

Although it is well established that monoterpene and sesquiterpene production in the intact plant is affected by growth regulators, few investigations on this aspect have been carried out on *P. nervosum* *in vitro*. In general, callus was initiated accompanied by shoot regeneration and an increase in fresh and dry weight of cultures in different concentrations of BAP and NAA was observed. The doubling time also decreased by various concentrations of growth regulators and the faster growth of culture was due to  $5.0 \text{ mg L}^{-1}$  BAP and NAA at  $0.2 \text{ mg L}^{-1}$  showing ( $t_d$  values of  $4.3$  and  $4.4$  days after 4 passages) in contrast with ( $5.4$  and  $5.7$  days, for fresh and dry weights, respectively) for callus tissue on maintenance medium. Furthermore, such cultures grow faster due to the accelerating effect of high cytokinin/auxin ratio on producing shooty cultures,

whereas intermediate ratios produced normal callus growth in *pelargonium* variants as reported by Brown and Charlwood<sup>[6]</sup>.

The yield of essential oil in shoot cultures of *P. nervosum* could be significantly improved after 4 passages, compared with the unorganized callus culture grown under maintenance conditions where no essential oil could be detected. However for maximum activity, the growth regulators had to be applied at 0.5 mg L<sup>-1</sup> BAP : 0.2 mg L<sup>-1</sup> NAA this gave rise to a maximum frequency of shoot induction and coupled with an increase in essential oil accumulation (up to 4.55 mg oil/g fresh) which was similar to that found in the parent plant, but with a vastly altered profile. Such increase of accumulation of secondary compounds is accompanied by a reduction in growth rate with respect to other treatments and a shift in oil profile to form oxygenated derivatives and sesquiterpenes; similar results in *P. fragrans* were obtained by Charlwood *et al.*<sup>[2]</sup>.

On the other hand, the regenerated shoots produced oil with different profile to that present in the parent plant, in this respect, Charlwood *et al.*<sup>[19]</sup> has found that the profiles of secondary compounds accumulated by shoot cultures, do not necessarily match those of the parent plant. Moreover, in the oil of the regenerated shoot of *P. nervosum* 9 components were unambiguously identified namely, menthone, myrtenol, citral, neryl acetate,  $\alpha$ -muurolene, allo-aromadenderene,  $\beta$ -farnesene,  $\gamma$ -muurolene and  $\delta$ -cadiene, both citral and  $\gamma$ -muurolene have been identified in partially differentiated callus cultures and intact tissue of this plant. Similarly quantitative changes in the major monoterpene and sesquiterpene content of *Lavandula dentate* plantlet oil were observed in response to the effect of varying growth regulator concentration in the culture medium<sup>[9]</sup>. Again, whereas the essential oils component profile of shoot cultures of *P. nervosum* was markedly different from that of the corresponding leaf oil, a similar situation appears to pertain in case of *P. fragrans*<sup>[10]</sup>, on *Pimpinella anisum*<sup>[11]</sup> and on *P. graveolens*<sup>[12]</sup>. However, the essential oils component of leaf oil and shoot culture of *P. tomentosum*<sup>[10]</sup>, of *Mentha tricata*<sup>[13]</sup> and of *P. robertianum*<sup>[14]</sup> were very similar.

It has been shown that it is possible to produce regenerated shoot cultures from petioles and leaf blades of *P. nervosum*. The regenerated shoots of *P. nervosum* were rooted, acclimatized and transferred to soil where they showed a normal phenotype. Similar results were obtained by Dumber and Stephens<sup>[15]</sup> on *P. domesticum*; Qureshi and Saxena<sup>[16]</sup> on *P. hortorum*; Robichon *et al.*<sup>[17]</sup> on *P. peltatum*. In contrast, significantly large variance for some morphological traits, herb yield, essential oil content and essential oil composition were observed among

*in vitro* regenerated *P. graveolens* plants of the first generation than among parental plants<sup>[18]</sup>.

It is clear that both the growth rate of *P. nervosum* culture, level and composition of the accumulated oil are modified by regulating the concentrations of auxin and cytokinin of the growth medium. In general, there is a marked improvement in overall accumulation of secondary compounds within shoot tissue and the developing leaflets bear glandular hairs on their surface, such shoot cultures accumulate oil in large amounts in contrast with the situation pertaining to callus cultures grown under maintenance conditions and parent plant: it has been suggested by Charlwood and Moustou<sup>[10]</sup> on *P. tomentosum*; Sudria *et al.*<sup>[9]</sup> on *Lavandula dentate*) that, all cultured showed a positive correlation between oil accumulation and the percentage of glandular hairs in secretary stage although the composition of the oil so formed differ significantly in *Lavandula dentate* plantlets. Also, it is demonstrated that morphological differentiation is not necessary for product synthesis. However, without such differentiation accumulation levels were low. This level of differentiation may therefore be necessary to obtain accumulation levels comparable to that of the parent plant. From the results, the most efficient regeneration medium is MS supplemented with 0.5 mg L<sup>-1</sup> BAP and NAA at 0.2 mg L<sup>-1</sup>, this medium induced shoot regeneration in callus after one passage, after root induction of such regenerated shoots on M.S medium, plantlets were acclimatized and transferred to soil.

Finally, the development of regeneration system for *P. nervosum* may facilitate conventional crop improvement programs, thereby providing a valuable resource to the horticultural industry.

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