

Geranium Oil Production in Suspension Cultures of *Pelargonium graveolens* L.

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ABSTRACT

Studies were conducted on the morphogenetic potential and secondary metabolite production by *P. graveolens* L. cell culture. Leaf segments excised from field grown plants, which showed the most intensive growth of callus on B₅ medium supplemented with 0.5 mg/l BA and 2.0 mg/l NAA, were used to initiate callus and cell suspension cultures. The effect of initial concentrations of two of the major medium components, sucrose and nitrate, on the growth, biosynthetic and biochemical characteristics of essential oils was examined. Callus developed most on B₅ medium containing 4% sucrose and normal level of nitrate (2500 mg/l). Maximum essential oil was produced from plants grown on M7 medium containing 8% sucrose.

Keywords: cell suspension, C/N ratio, essential oil, Geraniaceae, morphogenetic potential

Abbreviations: B₅, Gamborg medium; BA, 6-benzyladenine; Dt, doubling time; EO, essential oil; GR, growth rate; NAA, 1-naphthaleneacetic acid

INTRODUCTION

Many species of higher plants synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful raw materials for various commercial applications. Economically important plants serve as irreplaceable sources of flavours and fragrances, pharmaceuticals and many more products (Brown *et al.* 1987; Fischer *et al.* 2004). Aromatic plants occupy a prominent economic position in Egypt because of the continuous demand for their volatile products from foreign markets.

Geranium (*Pelargonium graveolens* L.), commonly known as rose-scented geranium, belongs to the family Geraniaceae and yields essential oil (EO) through distillation known commercially as geranium oil (Gomes *et al.* 2004; Lawrence 2005). *P. graveolens* with its rose-like odor is widely grown and cultivated in Egypt on a large commercial scale for oil production and is propagated by stem cuttings. Geranium plant EO is mainly concentrated in the leaves. One of the constraints is the non-availability of sufficient planting material for large-scale cultivation. Currently, South Africa, North America and Europe are the major producers and distributors of *Pelargonium* when taking the increasing demand for both local uses and international pharmaceutical and industrial market into consideration (Mithila *et al.* 2001; Lewu *et al.* 2007). A considerable amount of work has been done on ornamental geranium (Cassells and Minas 1983; Horn 1988; Aboelnil 1990), but work on *in vitro* multiplication of oil-yielding crops is limited. Tissue culture offers an effective alternative method for rapid multiplication of desirable clones that contain a high EO content. Relatively few studies have been published on mass *in vitro* clonal propagation of oil-yielding species of *Pelargonium* (Charlwood and Charlwood 1991; Agarwal and Ranu 2000; Nassour and Dorion 2002; Wojtania *et al.* 2004; Hassanein and Dorion 2005).

It is known that the ratios between sucrose and nitrate concentrations can have a profound effect on secondary metabolite production in plant cell cultures. For example, *Mucuna pruriens* cell suspension culture showed a clear

dependency of the maximum L-DOPA production and C/N ratio (Wichers *et al.* 1985). The combined effects of low nitrate and high sucrose synergistically improved anthocyanin volumetric productivity (up to 0.15 g/l per day) in grape cells cultured in liquid Gamborg's B₅ maintenance medium (Hirasuna *et al.* 1991). Kubek and Shuler (1980) studied the influence of changes in carbon/nitrogen (C/N) ratio on the production of phenolics in suspension cultures of rose and soybean. Over the C/N range tested in that study, for both species a clear maximum specific productivity was detected at 3% sucrose and normal levels of nitrate (1900 mg/l). Also the production of other secondary metabolites is influenced by initial levels of sucrose and/or nitrate, e.g. the production of anthraquinones in *Morinda citrifolia* cultures (Zenk *et al.* 1975), indole alkaloid production in *Catharanthus roseus* cultures (Zenk *et al.* 1977; Merillon *et al.* 1983) and nicotine production in *Nicotiana tabacum* cultures (Mantell *et al.* 1983). The objectives of this work were to establish the best conditions for callus induction from *P. graveolens* explants and to assess the effect of sucrose and nitrate concentrations on EO production from callus.

MATERIALS AND METHODS

Plant material, initiation and maintenance of callus cultures

Scented geranium plants were kindly provided by the Floriculture Department, Faculty of Agriculture, Cairo University. The isolated explants (i.e. leaf, petiole, stem and nodal segment) were excised from vigorously growing open-field plants (1-year-old) and briefly washed in running tap water. Sterilization was conducted by immersing the explants in a series of solutions: initially with 70% (v/v) EtOH for 1 min, then by 15% Clorox (4% available active chlorine) with a drop of Tween-20 for 30 min, and then again 70% EtOH for 1 min, and finally washed three times with sterile distilled water. During the course of the sterilization treatments, the explants were periodically agitated. Four segments (0.8-1.0 cm) from each explant were induced to form callus on solid B₅ medium (Gamborg *et al.* 1968) supplemented with 2% (w/v) sucrose, 7 g/l

Table 1 Levels of plant growth regulators (mg/l) tested for callus induction.

Plant growth regulator	Media code								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
BA	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
NAA	0.5	0.5	0.5	1.0	1.0	1.0	2.0	2.0	2.0

Table 2 Levels and ratios of sucrose and nitrate of media tested.

Media code	CN1	CN2	CN3	CN4	CN5	CN6	CN7
Sucrose (%)	0.5	2	4	8	2	2	2
Nitrate (mg/l)	2500	2500	2500	2500	625	1250	10,000

Table 3 Growth and morphogenetic responses of *P. graveolens* explants (in triplicates) during five weeks cultivation on B₅ medium addend with different combinations of BA and NAA.

BA (mg/l)	NAA (mg/l)	Leaf (g/explants)	Petiole (g/explants)	Stem (g/explants)	Nodes (g/explants)
0.5	0.5	0.72 ± 0.05 C	0.374 ± 0.02 C	0.295 ± 0.04 C	1.88 ± 0.04 C + Sh
	1.0	ND	0.338 ± 0.05 C	0.165 ± 0.02 C	ND
	2.0	1.55 ± 0.19 C	ND	ND	ND
1.0	0.5	ND	0.109 ± 0.01 C	ND	ND
	1.0	ND	0.137 ± 0.01 C	0.135 ± 0.03 C	ND
	2.0	ND	ND	ND	ND
2.0	0.5	ND	ND	ND	ND
	1.0	ND	ND	ND	ND
	2.0	ND	ND	ND	ND

Callus detection: C (detected), ND (not detected). Shoots (Sh).

agar (Fluka, Switzerland) and different concentrations and combinations of 6-benzyladenine (BA) and α -naphthalene acetic acid (NAA) as shown in **Table 1**. The medium pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. The cultures were incubated at 26 ± 2°C with 16 h daily illumination (3000 lux). Growth regulators were purchased from Sigma Aldrich, USA.

Suspension culture

A cell suspension culture was established by transferring the leaf-derived callus (grown on M7 medium) to 250 ml Erlenmeyer flasks containing 50 ml of liquid B₅ medium (at initial cell density of 50 g fw/l) to which 0.5 mg/l BA, 2.0 mg/l NAA and 2% (w/v) sucrose was added. The cultures were maintained on a gyratory shaker (100 rpm) under the same culture conditions mentioned above.

Experimental set-up

Seven different media were prepared in such a way that four different C/N ratios were obtained as shown in **Table 2**. B₅ medium is the medium used for *P. graveolens* callus maintenance is used as the standard for testing the effect of C/N ratios on callus growth and EO production in *Pelargonium* cultures by keeping ammonium sulphate concentration low or by raising it from normal levels in such the same way as the nitrate concentration.

Measurement and statistical analysis of growth

Growth data included the increase in fresh weight of a cultured tissue recorded after four weeks of cultivation by filtering under gentle suction using an air pump. Doubling time (Dt) was calculated by the equation, $Dt = 0.693/GR$, where GR is the growth rate (Shuler *et al* 1990). The data presented are the means of triplicates ± standard error. Using the F-test, statistical analysis was conducted according to Steel and Torrie (1960). Analysis of variance was determined and the value of least significant difference was calculated at 5% level to compare different treatment.

Carbohydrate, nitrate and inorganic phosphate determination

Residual reducing sugars in the cell free medium were determined according to the method of van Handel (1968); sucrose was used as the standard. Nitrate was determined according to Montgomery and Dymock (1961). Inorganic phosphate concentration in the extracellular medium was estimated according to the method of

Chen *et al.* (1956).

Extraction and determination of EO percentage

A sample of about 50 g fresh geranium leaf callus was harvested and subjected to steam-distillation for three hours according to Saxena *et al.* (2007). EO concentration in callus tissues was expressed as a percentage on a volume basis according to the following equation: $EO \% = \text{volume of EO (ml)} / \text{fresh callus weight (g)} \times 100$.

RESULTS AND DISCUSSION

Callus formation and morphogenetic responses

A preliminary screening was carried out to choose the best explants and media for callus induction. Four types of *P. graveolens* explants (i.e. leaf, petiole, stem and nodal segment) were cultured on B₅ medium supplemented with BA and NAA (each at 0.0, 0.5, 1.0 and 2.0 mg/l) in different combinations.

No callus was detected in all treatments on M3, M6 and M9 culture media while on M1 medium, callus was initiated from all tested explants (**Table 3**). Leaf explants formed a light green and hard callus in the second week of culture. Over time, it grew as a dark-green coloured callus of a compact structure (**Fig. 1A**).

Callus from petiole and stem explants appeared in the third week of culture. Initially the callus was hard and yellowish-green which turned brown by the end of the culture period (**Table 3**).

Fig. 1B indicates that morphogenesis occurred in the form of multiple shoots arising from the callus base, when nodal segments were cultured on 0.5 mg/l of both BA and NAA.

Results clarify that the medium containing 2.0 mg/l NAA + 0.5 mg/l BA shows maximum response of callus production from leaf explants (**Fig. 1**). On the other hand shoots were proliferated from nodal tissues at concentrations of 0.5 mg/l of both BA and NAA. While the other explants did not produce shoots with any tested combinations of these growth regulators.

These results support the suggestions by Pillai and Hildebrandt (1969), Hildebrandt (1970), Theiler (1977) and Chang *et al.* (1996) that the morphogenetic response of explants from various plant parts *in vitro* can be significantly different. Conversely, Brown and Charlwood (1986) re-

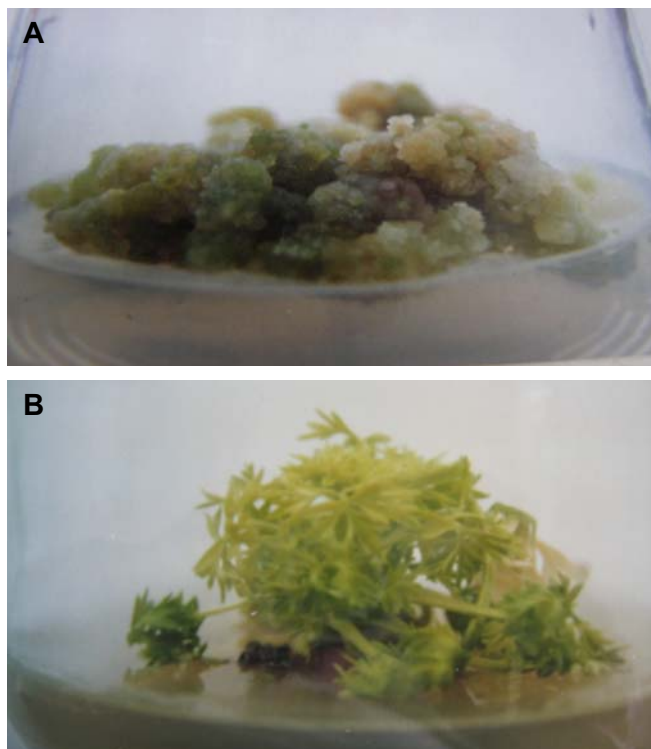


Fig. 1 Callus formation and morphogenetic responses of *P. graveolens* from leaf (A), and nodal explants (B), cultured on B5-medium containing 2 % sucrose and different concentrations and combinations of BA and NAA.

vealed that high levels of auxin and cytokinin stimulate callus initiation and production irrespective of the concentration ratios of plant hormone. Gupta *et al.* (2002) reported that MS medium (Murashige and Skoog 1962) supplemented with 4.5 mg/l BAP and 1.0 mg/l NAA resulted in the maximum induction of callus from leaf explants of *P. graveolens*. Moreover, Saxena *et al.* (2000) indicated that *in vitro* production of callus in geranium required a low auxin and high cytokinin level.

Effect of changes in C/N ratios on growth

Effect of various C/N ratios in the culture media were tested for their growth-promoting activities (as fresh weight) in leaves derived callus cultures of *P. graveolens* grown in liquid B5-medium for five weeks (Fig. 2). It can be noted that, fresh cell weight starts increasing reaching the maximum at the fourth week of cultivation and then gradually decreased. Comparing the fresh weights, the growth of callus cultured on CN3 medium supplemented with normal level of nitrate (2500 mg/l) and 4% sucrose was found to have a higher value than that grown on other C/N combinations.

It is important to mention that, when the C/N ratio was varied by changing the initial sucrose and nitrate concentrations from 0.5-8% and 0.625-10,000, g/l respectively, the growth profile was concomitantly changed. When the C/N ratio was varied by increasing the amount of potassium nitrate from 0.625-10,000 g/l in combination with 2% sucrose, a fairly constant yield of biomass was observed (Fig. 2). Only in the case of 10 g/l KNO_3 with double the culture period (i.e. 269 h), which is extremely long, more than two times the doubling time found for the other media (Fig. 3). The media with the lowest initial concentration of nitrate (0.625 g/l KNO_3), and the highest sucrose concentration (8% sucrose), resulted in a somewhat longer doubling time than the other media (Fig. 3). To some extent, this result is in contradiction to the finding of Kubek and Shuler (1980), who in their experiments with rose cell cultures found that nitrate, was already growth limiting when its initial concentration was lowered from normal levels to half the normal

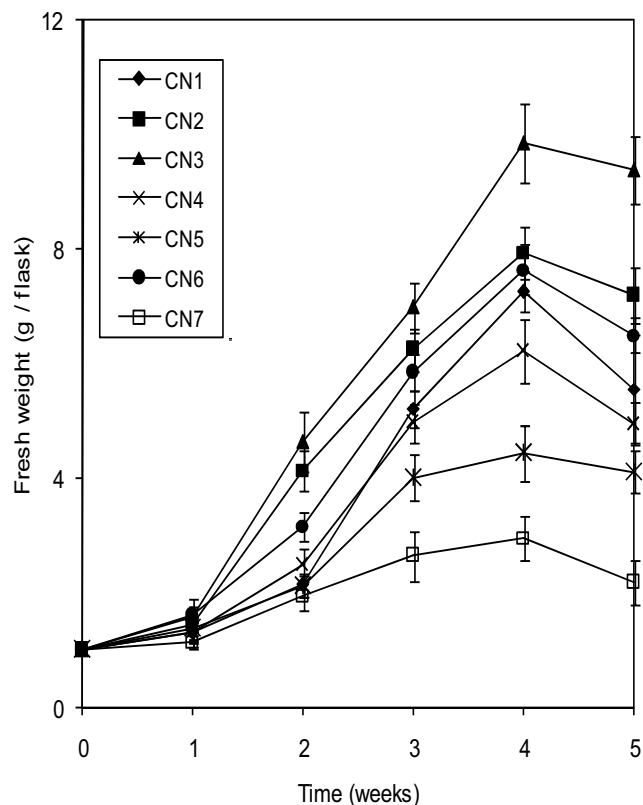


Fig. 2 Time course of change in callus fresh weight (in triplicates) of *P. graveolens* suspension culture under different C/N ratios during five weeks culture period. Error bar represents mean \pm S.E.

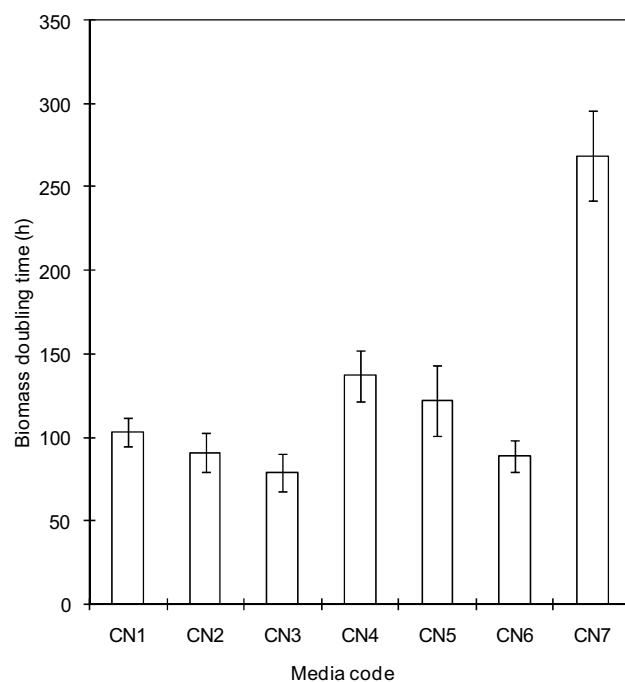


Fig. 3 Biomass doubling time of *pelargonium* suspension cultures grown on media with different C/N ratios. Error bar represents mean \pm S.E.

concentration.

Moreover, we observed that the enhancement in fresh weight of leaf-derived callus is directly proportional to the initial sucrose concentration within a range of 0.5-4% (Fig. 2). Raising the concentration of sucrose to 8% did not further increase the callus biomass. This result is in good agreement with the results of Mantell *et al.* (1983), on the effect of medium components on growth and secondary metabolite production in *N. tabacum* tissue cultures, employing media with 0.2 and 2% sucrose. Hildebrandt and

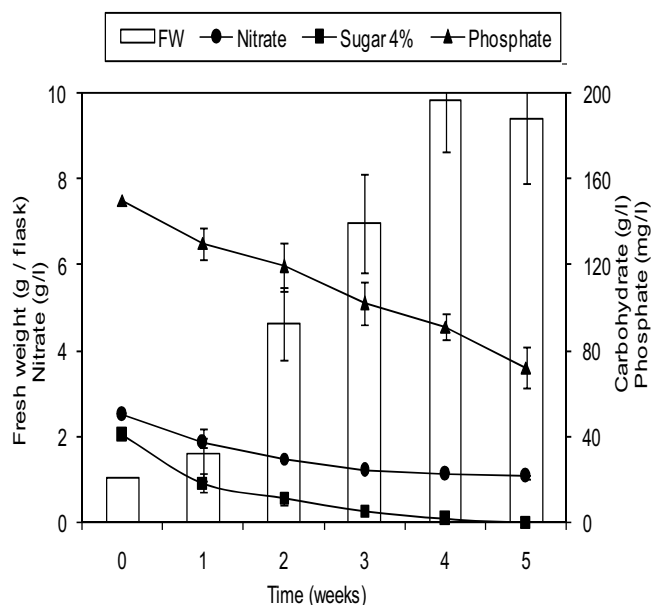


Fig. 4 Time course of change in growth, carbohydrate, nitrate and phosphate concentration of *Pelargonium graveolens* cultured in CN3 medium. Error bar represents mean \pm S.E.

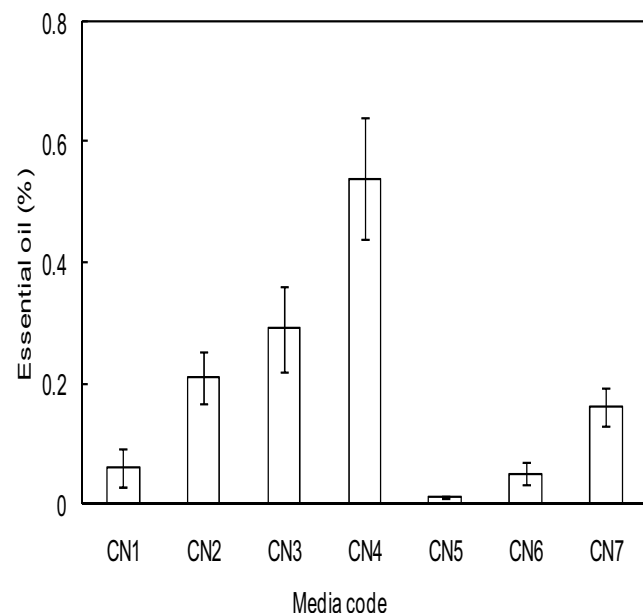


Fig. 5 Total essential oil of *pelargonium graveolens* suspension cultures grown under different C/N ratios. Error bar represents mean \pm S.E.

Riker (1949) indicated the importance of sucrose as a carbon source on the growth of marigold, Paris-daisy, periwinkle, sunflower and tobacco callus tissues.

Data presented in **Fig. 4** shows the gradual increase in *P. graveolens* callus growth during a five-week cultivation period and that the biomass doubling time during that phase was 79 h. The same figure also shows the consumption of carbohydrates and nitrate from the medium. The initial phosphate concentration of the media was the same in all the experiments; however, phosphate levels can play an important role in the biosynthesis of secondary metabolites (Poulose and Croteau 1978; Fischer *et al.* 2004). In contrast to other findings, the medium phosphate concentrations in these experiments only showed a slight gradual decrease. No sharp drop in the phosphate concentration was observed during the lag phase as was reported for *Catharanthus roseus* (Knobloch and Berlin 1983; Merillon *et al.* 1983). Regarding the nitrate concentration, it is clear however, that at the end of cultivation an adequate amount of nitrogen still exist in the culture medium, while most of sucrose

seems to be consumed during callus growth. Obviously, carbohydrates are the first to be exhausted.

Obtained data revealed that, the best medium for sustaining growth in cell suspension cultures of *P. graveolens* is a standard B₅-medium containing 40% sucrose. In this case the duration of the lag-phase is the shortest and duration of the exponential growth phase is the longest (**Fig. 4**).

Effect of changes in C/N ratios on EO formation

The effect of varying the C/N ratio on the production of EO in cell suspension cultures of *P. graveolens* could be divided into two parts, the first part is the influence of varying sucrose levels, and the second part is on the influence of varying nitrate levels. It was observed that, a clear influence on the total EO production is observed with increasing the sugar concentration (**Fig. 5**). The maximum oil production (0.54%) was detected on CN4 medium that contained 8% sucrose and normal nitrate level, followed by CN3 medium (0.29%) which contained 4% sucrose. On the other hand, by varying the initial nitrate concentrations at a constant initial level of 2% sucrose, the EO production was different. When nitrate was lowered from normal levels, the accumulation of oil was relatively similar (**Fig. 5**). However, when nitrate concentration was raised from 2500 mg/l (normal level in B₅ medium) to 10,000 mg/l, the total EO clearly declined. This could possibly be due to the stress experienced by cells at high levels of sucrose and nitrate. These results disagree with those of Brown and Charlwood (1986), who reported that the accumulation of monoterpenes in callus cultures grown under controlled culture conditions was negligible. In contrast, Charlwood and Charlwood (1983) proved that *in vitro* induction of shoots enhance the accumulation of monoterpenes. Similarly Mulder-Krieger *et al.* (1988) proved that EO production through plant cell culture is an alternative promising way that scientists should pay more attention to developing.

From these experiments it is clear that sucrose plays an important role in the stimulation of growth and EO production in *Pelargonium graveolens* cell cultures.

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