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In vitro regeneration and transformation studies on *Pelargonium graveolens* (geranium) - an important medicinal and aromatic plant

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Development of improved protocols for the regeneration of *Pelargonium graveolens* is important for their commercial utilization. Callus was induced from leaf explants on Murashige and Skoog **(MS) medium supplemented with auxins and combinations of auxins and cytokinins. Micropropagation of nodal bud explants was achieved using medium supplemented with cytokinins. The regenerated explants were acclimatized and the transformation potential of geranium was demonstrated using *Agrobacterium tumefaciens*. Maximum proliferation was obtained in medium supplemented with combinations of 20 µM indole butyric acid (IBA) + 10 µM kinetin (KIN), 20 µM IBA + 10 µM benzyl amino purine (BAP) and 20 µM indole-3-acetic acid (IAA) + 10 µM KIN. Plantlets were regenerated when callus was given a photoperiod of 16 h light and 8 h dark conditions at 25°C. After being transferred to greenhouse conditions, the plantlets were successfully acclimatized and transferred to the field condition. The study has established an efficient protocol for *in vitro* regeneration and transformation of *P. graveolens* as an alternative method for vegetative propagation.**

Key words: Callus, geranium, *in vitro* regeneration, transformation.

INTRODUCTION

Pelargonium graveolens belongs to the family Geraniaceae, and is an evergreen perennial flowering plant commonly known for its essential oil, with rose like aroma. The plant is commonly called Rose geranium or Rose scented geranium and there are about 300 geranium species. It has several medicinal and aromatic values of commercial importance (Verma et al., 2010; Brian et al., 2010). Traditionally, geranium was used to staunch bleeding, heal wounds, ulcers and skin disorders as well as treat diarrhea, dysentery and colic (Matthews, 1995). The extracts of *P. graveolens* are reported to be used as antibacterial and insecticidal agents (Jeon et al., 2008; Ooshiro et al., 2009; Tabanca et al., 2013). Geranium is now an indispensable aromatherapy oil and is considered as balancing oil for the mind and body (Dormon, 2000). Psychologically, geranium oil is evidenced by its sedative, uplifting properties, which is used to treat extreme depression, panic and anxiety. Geranium oil is considered as one of the best remedy for congested, oily and dry skin complications. It is also good for minor cuts, bruises, eczemas, burns, frostbites, chilblains, acne and broken veins. It is a vital component in the treatment of endometriosis. Application of geranium oil is very effective for menopausal problems,

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diabetes, blood disorders, and throat infections and as a nerve tonic and works well as a sedative (Bussmann et al., 2013; Karato et al., 2006; Petrie and Peck, 2000). The efficacy of the oil is exploited in many major food, alcohol and beverage industries.

Rose geranium is a native of dry rocky slopes of Cape Province in South Africa and was commercially cultivated in France, Belgium, Spain, Morocco, Madagascar, Egypt, Reunion Islands, Congo, China, India and the former USSR countries (Farooqi and Sreeram, 2001). The species was first introduced in Yercaud on the Shevroy Hills (1370 to 1525 m attitude) of Salem district of Tamil Nadu. Later, a number of scented and ornamental *Pelargonium* species were introduced into India, some of which got naturalized, escaped garden cultivation and are found growing wild on Shevroy, Kodaikanal, Nilgiri Hills of Tamil Nadu State and certain parts of Karnataka as it prefers high altitude areas with a milder climate (Rajeswara et al., 1999).

Commercially, geranium is widely used for scenting soaps and high-grade perfumes due to the presence of low molecular weight aroma compounds (Verma et al., 2010; Farooqi and Sreeram, 2001). At one time, India was producing about 20 tonnes of geranium oil, with an average of nearly 1,400 hectares (Narayana et al., 1986). However, current Indian production has decreased to less than two tonnes per year due to significant reduction in the availability of cultivated area and ever changing climatic conditions. This has necessitated the application of modern biotechnological approaches/tools to increase the productivity to compete the requirements. The present study aims at formulating a protocol for increased rate of propagation and productivity of *P. graveolens* by *in vitro* culturing and genetic transformation.

MATERIALS AND METHODS

P. graveolens saplings were obtained from Horticulture Research Institute, Udagamandalam, The Nilgiris, Tamil Nadu. Soil from Ooty hills fertilized with “panchkavyam” was used to grow the saplings. The saplings were grown in shady region of the green house and watered once a day and maintained in the department nursery.

Callus induction

The leaf explants (Figure 1A) were initially washed thoroughly with tap water to remove the dust particles and soil residues, followed by rinsing in soap water for few minutes. Further, the explants were treated with 20% teepol and 10% bleach (Ala) for 10 min. Finally, the leaves were surface sterilized by agitating them in 0.1% $HgCl_2$ for about 5 min to eliminate fungal and bacterial contaminants above and below the cell surface. The explants were rinsed thoroughly in sterile distilled water. The sterile leaf explants were cut into 5 mm long segments and plated on to Murashige and Skoog (1962) solid medium supplemented with appropriate combinations of nutrients and plant growth regulators (Table 1). The bottles were sealed and incubated in a growth chamber at 25°C under 16/8 h (light/dark) photoperiod for 25 to 30 days.

Micropropagation

Micropropagation was carried out using nodal bud explants in MS medium supplemented with auxins: cytokinins, 3% w/v sucrose and 0.7% w/v agar (Table 2). pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 30 min. The explants were incubated at 26 ± 1°C with 16/8 h light/dark cycles under white fluorescent lamps (3000 to 5000 lux). Shoots were regenerated from the callus by subjecting them in a cytokinin rich medium for 16/8 h light and dark photoperiod.

Rhizogenesis

Rhizogenesis was induced by sub culturing the *in vitro* regenerated shoots into rooting medium supplemented with higher concentrations of auxin and cytokinin and maintained under complete dark condition.

Acclimatization

The plantlets, with shoot and roots, were taken out from the culture medium and washed gently with double distilled water for removing all traces of medium from the surface. The plantlets were then transferred to small plastic cups containing sterile sand. The plastic cups were covered with sealed plastic vinyl bags to keep full humidity at 25 ± 2°C in light conditions (photon flux density at 25 µmol m^{-2} S^{-1}, 16 h). The plantlets were moistened with water. As the plants grew vigorous, the bags were poked with chopsticks to allow air into the bags until the plants were self supported. The polythene bags were removed after fifteen to twenty days. The plantlets were later transferred to larger pots containing sterile sand and soil (1:1 ratio) and kept under shade in the green house.

Transformation studies

A 35 days old callus of *P. graveolens* was used for transformation using *Agrobacterium tumifaciens* LBA4404 containing a binary vector pTOK 233. Healthy compact callus from aseptically grown *P. graveolens* explant was transferred into a sterile petridish containing Whatmann filter paper. Callus was wounded by pricking with a sterile needle all over the surface. The explants were incubated for 48 h in MS medium to increase its meristematic activity. Overnight grown culture of *A. tumefaciens* strain with pTOK 233 in yeast extract peptone (YEP) with kanamycin was directly used for co-cultivation. Approximately 5 to 10 ml of *Agrobacterium* culture were poured into a sterile petridish, 3 to 10 callus explants were taken using sterile forceps and immersed and incubated for 10 min. After incubation, the explants were transferred to a petridish with sterile Whatmann No.1 filter paper. Explants were washed with MS containing 250 mg/L carbencillin to kill the *Agrobacterium* and again transferred to sterile petridish with Whatmann No. 1 paper. The washed explant was transferred to MS and incubated for 2 days in culture room. After 2 days, explants were aseptically transferred to selection medium (MS with hygromycin and 600 µl of 250 mg/L carbenicillin). The transformed callus carrying binary vector pTOK233 from *A. tumefacie* selection medium was cut into small sections. The explant was mixed with X-gluc solution and incubated for 2 to 12 h at 37°C in the dark. The tissue sections

Table 1. Callus induction and proliferation from leaf explants of geranium.

S/No.	Treatment		Callus induction (After 16 days)	Callus proliferation (After 27 days)	Viable callus (After 45 days)
	PGR	µM			
1	2,4-D	10-60	0	0	0
2	NAA+ KIN	20 10	+ +	+ + +	Y
3	NAA+ ZEA	20 10	+	+	N
4	IAA+ KIN	20 10	+ + +	+ + + +	Y
5	IAA+ KIN	10 5	+ +	+ + +	Y
6	IAA+ BAP	20 10	+ +	+ + +	Y
7	IAA+ BAP	10 5	+ +	+ +	Y
8	IAB+ KIN	20 10	+ + + +	+ + + +	N
9	IBP+ BAP	20 10	+ + + +	+ + + +	Y

0: Explants turned brown and died, +: Only induction was there, no proliferation, ++: fair growth, callus was visible to naked eye, +++: good growth, callus covered the explants, ++++: excellent growth, prolific callus development.

were washed in ethanol. Tissues were stored in100 mM phosphate buffer and examined under dissection microscope.

RESULTS

Callus induction and regeneration

Callus was induced from the leaf explants cultured on MS medium supplemented with various combinations of indole-3-acetic acid (IAA), indole butyric acid (IBA), benzyl amino purine (BAP) and kinetin (Table 1). Curling and appearance of protuberances occurred in a time period of 4 to 6 days (Figure 1B). Callus induction was observed in 16 to 17 days of incubation in dark at 25°C. Proliferative callus was observed in 25 to 27 days of culturing (Figure 1C). Callus induction was initiated from the midrib and veins of the explants. Callus obtained in dark was white friable and compact. Callus when incubated in light turned green friable and nodular in 1 to 2 days. Pigmentation was observed in callus tissues incubated in light after about 8 to 10 days (Figure 1C). Maximum, proliferation was obtained in medium with combinations of MS with 20 µM IBA + 10 µM KIN, 20 µM IBA + 10 µM BAP and 20 µM IAA + 10 µM KIN. The callus was maintained by sub culturing in the same medium at an interval of 10 to 12 days. Proliferation of sub-cultured callus was observed within a time period of 2 to 3 days (Table 1, Figure 1C and D).

Shoots were emerged as tiny pale green even globules from 3 to 5 days old proliferative callus tissue grown under 16/8 h light/dark photoperiod at 25°C (Table 2, Figure 1D and E). The callus tissues were found responsive to higher concentrations of cytokinin. The shoot primordial developed had white petiole carrying un-incised leaf, which later showed incisions and lobes.

They were light green during the time of incubation and turned dark green as they grew, then later on acquired red pigmentation. Shoots without pigmentation were also observed in callus tissues grown in complete dark condition. Maximum proliferation and multiplication of

Table 2. *In vitro* regeneration of shoots and roots from leaf derived calli of geranium.

PGR's		Shoot regeneration (16 h light/8 h dark)			Rhizogenesis (16 h light/8 h dark)		
PGR	**µM**	**5 d**	**15 d**	**30 d**	**5 d**	**15 d**	**30d**
BAP	20	+ +	++ +	-			
KIN	20	+ +	+ +	-			
BAP+ IAA	20 5	+ + +	+ + +	+ + +			
KIN + IAA	20 25	+ +	+ + +	+ ++	← Shooting medium		
KIN + IAA	10 10	+ +	+ + +	+ +			
KIN + IBA	10 10	+ +	+ + +	+ +			
IBA + KIN	20 10				-	+	+ + +
IBA + BAP	20 10				-	+	+++
IAA+ BAP	20 5	← Rooting medium			-	+	++++
IBA + BAP	20 5				-	+	++++

++: 1-2 shootlets, +++: few shootlets, ++++: numerous shootlets, +: induction of root, visible to naked eye, +++: proliferation, +++: maximum proliferation.

shoots was observed in hormone combinations of 20 µM BAP + 5 µM IAA, 20 µM KIN + 5 µM IAA. After multiplication had started, the shoots showed elongation of petiole, which was initially white, then turned green in color. Complete proliferation was obtained after 15 to 16 days (Figure 1E and F). Different stages of shoot buds, from induction to whole bud formation was observed. It appeared as small protuberance that gave rise to nodules of globular and bent shape. These nodules on further differentiation showed dome shaped structure with apical incision, which were deepened further. The shoot buds which differentiated from the callus were meristematic in nature (Figures 1G and H).

Micropropagation

The nodal bud explants maintained in BAP and KIN supplemented medium initiated shoot development by 3 to 4 days of inoculation. Prominent shoot multiplication was observed around 15 to 20 days of culturing in shooting medium. The shoot development was rapid and covered the culture tube in 28 days. Numerous shootlets were developed when callus tissue was sub cultured in MS medium with 10 µM BAP + 5 µM IAA and 20 µM KIN + 5 µM IAA under continuous white light. All the shootlets had green petiole with lobed leaves and glandular hairs (Figure 1I).

Rhizogenesis

Roots were induced from the *in vitro* regenerated shoots grown in medium supplemented with higher concentration of auxin:cytokinin growth hormones (Table 2). Small hairy fibrous roots were developed from the shoot base after 20 to 25 days of culturing. The number of rhizome developed was observed maximum in medium supplemented with 20 µM IBA + 5 µM BAP (Figure 1J). Regenerated plantlets were acclimatized under controlled *in vivo* condition and transferred to pots and maintained (Table 2, Figure 1K).

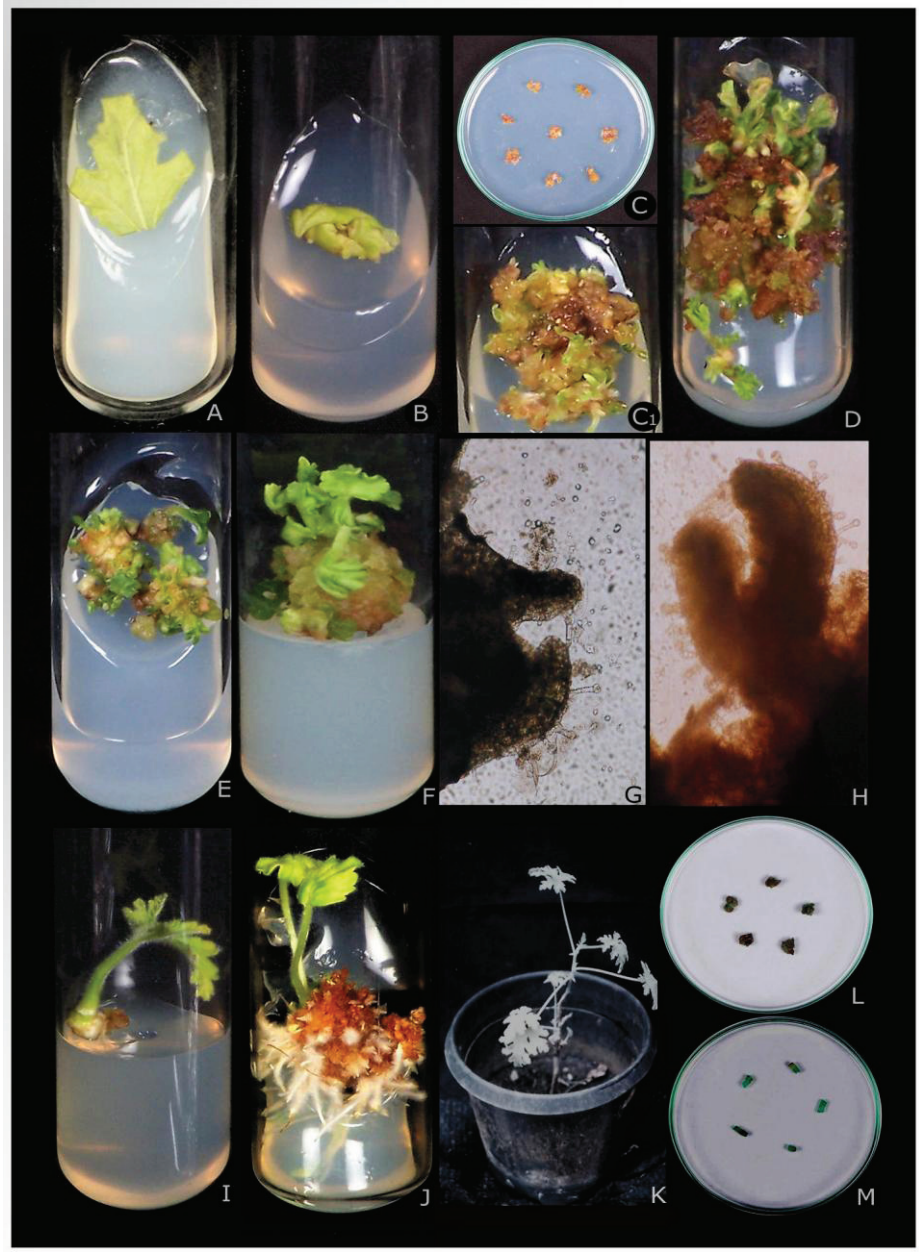

Figure 1. *In vitro* regeneration of *P. graveolens*

Transformation

An effficient protocol for transfer of genomic DNA into *P. graveolens* using *Agrobacterium* mediated transformation was developed. After 12 h incubation at 37°C in dark, transformed calli showed transient blue coloration while un-transformed calli showed no sign of colorations. Transformed calli harbored the plasmid pTOK 233 which contain the β-glucuronidase gene, which metabolized X-gluc and gave characteristic blue color (Figure 1L and M).

DISCUSSION

The present study aims at formulating a protocol for increased rate of propagation and productivity of *P. graveolens* by *in vitro* culturing and genetic transformation. The geranium oil is an indispensable essential oil in aromatherapy and perfume industry. Manipulation at molecular level to develop stable transgenic plant with increased oil production is a substitute to save the biomass. The genetic transformation attempted as a part

of this study, thus finds a significant application in increasing the production of essential oil and fragrance quality of this aromatic plant.

MS medium supplemented with various levels of 2,4-D was chosen as primary medium for callus induction but unlike the studies reported in *P. graveolens* (Sreedhar, 1999), the plant showed poor response and no sign of growth. This may be due to improper modulation of exogenous auxin and endogenous auxin content that would prove antagonistic to callus induction. This implies that the initiation of a proliferating culture from explants, involves profound change in the development state of the tissue and relates to the alterations in the basic architecture of cells or tissues resulting in the activation of quiescent cells (Irfan, 2001).

Curling of tissue and protuberances were formed that was followed by the appearance of little irregular cellular masses that were observed around the pricked end, which may be due to exogenous substances that ooze out of the injured tissue at cut end and stimulates cell division (Kumar, 1992). The callus induction was observed from the midrib as it is rich in meristamatic tissue, and meristematic tissue have high rate of growth as they are physiologically active (Razdan, 2003). Substantial callus was formed in a time period of 25 to 27 days, as usually it takes 4 weeks for complete callus formation (Kumar, 1992). The texture of callus observed was friable and compact. Texture varies according to explant and species used (Kumar, 1992).

Callus was colorless in dark and acquired green color when exposed to light, which is probably due to photo stimulation of chloroplast and development of chloroplastids in the callus tissue. Some callus tissues turned purple in color which may be due to accumulation of anthocyanins (Kumar, 1992). Maximum proliferation was obtained in medium with hormone combination MS with 20 µM IBA + 10 µM KIN, 20 µM IBA + 10 µM BAP and 20 µM IAA +10 µM KIN. This shows that a good modulation of auxin and cytokinin is required for formation of callus from geranium leaves unlike auxins alone as reported in many cases (Razdan, 2003; Batra, 2001) (Figure 1C and D). Shoot induction occurred when a photo period of 16/8 h light/dark was provided as geranium is reported to be highly sensitive to light (Sreedhar, 1999) and regeneration was delayed when exposed to light alone (Sreedhar, 1999). The differential coloration of shootlets was also observed. Albinos were formed in the dark due to lack of chloroplastids while in light, the chloroplastid differentiation was induced and procured green color (Kumar, 1992). The pink coloration in leaf was obtained, which can be the manifestation of somaclonal variation (Brown and Charlwood, 1986) or deposition of anthocyanin pigments (Razdan, 2003). Caulogenesis occurs in blue light or low intensity white light or in the dark with sudden exposure to light (Kumar, 1992). Shootlets grew into the medium and shows its sensitivity to longer period of light conditions (Sreedhar, 1999). Maximum shoot proliferation was obtained in hormone combinations MS with 20 µM BAP + 5 µM IAA and 20 µM KIN + 5 µM IAA. This shows that the plant requires large amounts of cytokinin with auxin supplement for shoot proliferation (Saxena et al., 2001) (Figure 1D to F).

Multiplication of the shoots were observed, when the callus with shoot buds was sub cultured to MS supplemented with 20 µM BAP + 5 µM IAA, 20 µM kin + 5 µM IAA. Saxena (2002) in her work on *in vitro* procedure for micropropagation had that found MS 0.5 mg/L BAP + 0.1 mg/L NAA exhibited regeneration of maximum number of shoots. Whereas, Sreedhar (1999) had reported that the maximum number of shoots for *in vitro* propagation of geranium was observed in medium containing BAP and IAA at 1 mg/L.

Different stages of shoot buds from induction to whole bud formation were observed in simple light mode of phase contrast microscope. The stages like nodular, dome shape bend shape, dome shapes with apical incision were observed. The protuberances seem to differentiate from epidermal cells and have become meristamatic. These stages are confirmed to be stages in shoot bud formation, as there are reports of histological analysis of shoot differentiation in *Chloroxylon swietenia.* Like in geranium, the microscopic examination of the *Chloroxylon* callus from hypocotyl region revealed that mitotic division actually initiated in pericyclic region, giving rise to a mass of compactly arranged cells that contained dense cytoplasm and prominent nuclei. In pericyclic region, due to continuous proliferation of cells, a pressure was developed from the radial growth of the cells, which led to the disruption and degeneration of the cortex triggering some of the newly formed cells to exhibit re-differentiation. These cells generally occurred in nests or nodules and could be appropriately referred to as ‘growth centres’ or ‘meristermoids’ that further differentiated to dome shaped structure. Oval and elongated callus cell with thick wall were also viewed in microscopical examination. This corresponds with the differentiation of callus tissues at the time of initiation and further growth shows mixed population of small, more rounded, oval and few elongated cells with dense cytoplasm.

In geranium callus, the roots formed were small, hairy, and fibrous and resembled that of field grown plant. The callus tissue developed slight yellowish brown pigmentation during rhizogenesis, (Kumar, 1992). The hormone combination MS 20 µM IBA + 10 µM BAP resulted in the highest amount of rhizogenesis. Similar observation was reported by Razdan (2003), that development of roots under light and dark suggest that the rhizogenesis in geranium is insensitive to photoperiods and high level

of auxin favours the multiplication. Regeneration occurs by complete organogenesis, usually initiation of shoot buds in calli may precede rhizogenesis or sometimes it succeeds rhizogenesis as reported by other studies (Kumar, 1992). The latter case was observed in the geranium callus. The callus for rhizogenesis was incubated in the same medium (MS + 20 µM IBA + 5 µM BAP), which gave rise to shootlets after 45 days of incubation.

Nodal buds inoculated in MS medium supplemented with 20 µM IAA + 10 µM KIN gave rise to first, a single shoot, then to multiple shoots, unlike the combination given by Saxena (2001) on geranium which was 8 mg/L KIN and 1 mg/L NAA. It took 15 to 20 days for multiplication, unlike easy breaking of dormancy of shoot bud; this may be due to usage of more auxin concentration than cytokinin. The appearance of milky white callus can also be attributed due to the auxin and cytokinin combination with more of auxin that indeed promotes callus induction (Razdan, 2003). The transformed calli pieces showed transient blue coloration. The callus was highly proliferated, and friable pre-incubation requirement was only 2 days. Pre-culture is crucial because host cell division is required for successful *Agrobacterium* transformation (Binns and Thomashow, 1988), it is not surprising that pre-culture in a high auxin medium often enhances transformation rate (Mathis and Hinchee, 1994; Sangwan et al., 1992). In addition, because there was no effect of pre-culture or transient gb-glucuronidase (GUS) expression, it is likely that plants benefit from pre-culture by increasing the competence of cells for DNA incorporation into chromosome and/or de-differentiate into callus rather than enhancing DNA transfer cells. Callus was chosen, as it was highly meristematic compared to other explants.

As per the standardized protocol for *Agrobacterium* transformation in *Nicotiana tobaccum*, in the present study, the infection time and incubation time for co-cultivation is respectively 8 to 10 min and 2 days. The same time intervals were given for geranium calli treatment and to prevent the bacterial overgrowth, selection on hygromycin and carbenicillin containing medium was carried out for 2 days, which prevented the bacterial growth. Selection was carried out in dark as these antibiotics are light sensitive (Meilan et al., 2002). The transient expression is a test for plasmid transformation into calli. The plasmid pTOK 233 harbors β-glucuronidase gene that is capable of metabolising X-gluc and gives blue color. The transient expression even though is a positive confirmatory test for transformation (Jefferson, 1987) can sometimes get mislead due to incorporation of bacteria into callus which has not been selected properly on antibiotic medium (Han et al., 2000). Thus we have developed a rapid protocol for the transformation of genomic DNA into *P. graveolens* using A. *tumefaciens*.

Conclusion

The present study aimed to establishing an efficient protocol for *in vitro* regeneration and *Agrobacterium* mediated transformation of *P. graveolens* as an alternative method for vegetative propagation. The results suggest better methods of propagation and efficient utilization of the *in vitro* protocols for the mass multiplication of this commercially important crop. Further, the transformation study provides a primary step for rapid gene transfer towards the improvement of the plant for oil content and fragrance at the molecular level.

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