RAPID *IN VITRO* PLANT REGENERATION OF GERANIUM (*Pelargonium graveolens* L.)

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Abstract:

Rose-scented geranium (Pelargonium graveolens L.) is a highly sought-after plant known for its rose-like fragrance. To meet the increasing demand for geranium oil, efficient propagation methods are essential. This study aimed to optimize in vitro techniques for the propagation of rose-scented geranium.Successful surface sterilization was achieved using a 0.1% $HgCl_2$ treatment, resulting in a survival rate exceeding 95%. Shoot initiation was accomplished using Murashige and Skoog (MS) medium supplemented with 1.5 mg/L BAP and 0.1 mg/L IAA, resulting in a 90% shoot induction rate. Shoot multiplication was achieved on MS medium supplemented with 1.5 mg/L BAP and 0.1 mg/L NAA. The rooting of micro-shoots was successful using a rooting medium with 0.5 mg/L IBA, resulting in 5.6 cm root length per shoot. In vitro-raised plantlets were successfully acclimatized to a mixture of cocopeat, compost, and soil, with a 100% survival rate after primary acclimatization and 75% after secondary acclimatization. These optimized protocols for surface sterilization, shoot initiation, multiplication, and rooting provide a foundation for large-scale production and commercial cultivation of rose-scented geranium. Implementing these techniques can help meet the demand for geranium oil while ensuring its sustainable supply and conservation.

Keywords*: In vitro propagation, Geranium, Shoot multiplication, Sustainable supply Micropropagation*

Introduction:

A rose-scented geranium is a species of the Geraniaceae family, whose genus is *Pelargonium graveolens* L. This perennial flowering plant is primarily known for its essential oil, which has a rose-like fragrance. There are over 200 known species of Pelargonium, according to estimates. A variety of cultivated geranium can be found in South Africa. There are more than 700 varieties. The scented geranium was introduced to India in the early 20th century, but cultivation and oil production was limited to the hilly regions of Southern India.(Pandey *et al*., 2020).

Due to its versatile applications in perfumery, pharmaceuticals, cosmetics, and the flavour industry, Geranium oil is considered to be one of the top 20 essential oils in the world (Douglas G, 1969; Aggarwal *et al.*, 2000; Cristea *et al*., 2009; Gupta *et al*., 2002). The rose-scented geranium essential oil is renowned for its effectiveness in aromatherapy and various health-related treatments, owing to its antimicrobial properties (Narnoliya *et al*., 2019, 2018; Jadaun *et al*., 2017). Rose like smell of geranium oil is due to major constituents citronellol and Geraniol. Which may varying proporsions upon the origin of oil (Mahindru, 1992). Despite the estimated global production of Geranium oil being around 250-300 tons per year, its demand reportedly exceeds 800 tons annually. The limited supply of Geranium oil has resulted in its high value as a sought-after commodity in the global market. The primary countries producing Geranium oil are Egypt, China, South Africa, and Morocco. Given the high demand and limited supply, the prices of Geranium oil can be relatively high (Guthar, 1950)

Traditionally, Geranium is propagated through stem cuttings and` seeds, but both methods have limitations. Cuttings have a relatively low multiplication rate, a risk of propagating systemic pathogens, a limited propagation period, and a high mortality rate. Seed germination can be challenging due to cross-pollination, lengthy germination time, and risk of changes in the plant's genetic composition, which can adversely affect the oil composition or yield, making the plant unsuitable for use in perfumery, cosmetic, or pharmaceutical industry (Rabuma, 2015). Additionally, Geranium is susceptible to various diseases, including bacterial blight, Verticillium wilt, Botrytis blight, root rot, bacterial fasciation, rust, and Pelargonium flower break virus, etc. (Bi *et al.*, 1999; Nameth *et al.*, 1999; Swanson *et al.*, 2005) which can significantly impact crop production. To overcome these challenges, researchers have proposed using *in vitro* multiplication techniques, such as nodal culture, to obtain a variety of Geranium with desirable agricultural traits, such as large leaves with high essential oil yield and disease resistance (Narnoliya *et al*., 2019).

Material And Methods

Experimental site

The study was conducted at the Plant Tissue Culture Laboratory of the Department of Plant Biotechnology at K. K. Wagh College of Agricultural Biotechnology in Nashik, Maharashtra, India.

Plant Materials

Geranium plants were taken from a field and planted in the greenhouse at the K. K. Wagh College of Agriculture farm. The plants were maintained by watering them on an alternate day, and they were treated with 2% (w/v) fungicide solution (Bavistin) every two weeks for four months. This fungicide treatment likely helped to prevent the growth of fungal pathogens that could have damaged the explants.**Preparation of Stock and Working Solutions of MS Media and Different Hormone Concentrations**

The study used MS (Murashige and Skoog, 1962) media with macro, micro and vitamin compositions. These stock solutions were prepared by dissolving the appropriate amount of macro and micronutrients and organic supplements in distilled water. Similarly, growth regulator stock solutions of BAP and IBA were prepared by dissolving them in a 1:1 ratio (1 mg/mL) using 1 N NaOH or ethanol and then adjusting the total volume with distilled water. The stock solutions were then stored in the deep freezer at -4 °C for one month before they were used in the experiment. Standard MS medium was prepared by using stock solutions and supplemented with 30 g/L sucrose and different concentrations of 6-BAP and IAA with a combination.

The pH of the medium used in the experiment was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl as required. 0.8% (w/v) agar was then added to the medium and it was boiled to mix the agar with the media. The media was then autoclaved at a temperature of 121 °C for 45 minutes with a pressure of 15psi. There were four treatment combinations, and a control (hormone-free) treatment for shoot initiation as listed in Table 1, and each treatment combination was replicated six times. These treatment combinations likely involve the use of different concentrations of growth regulators to promote shoot initiation.

Explant Collection and Sterilization

The vigorously growing nodal parts of the plant including the internodes were cut at about 3 cm length, and taken into the laboratory for sterilization treatment. The explants were thoroughly washed under tap water for 20 min. Then treated with 0.5% Bavistin for 10 min followed by rinsing 4-5 times in sterile distilled water. In the laminar air flow cabinet treatment was continued by dipping explants in a 0.1%-0.3% Mercuric chloride solution for time intervals of 3 and 5 minutes. The disinfection process was done with 70% ethanol for 5 seconds. Finally, explants were then washed with autoclaved distilled water 3-4 times to ensure that they were completely sterile. The surface sterilized explants were then trimmed at the cut ends with the help of a surgical blade.

Shoot Initiation

Surface sterilized explants were cut into 2-3 cm with the help of autoclaved blade, and then inoculated on MS media supplemented with different concentrations as mentioned in Table 1. Each treatment is replicated six times One node was inoculated in each culture bottle and therefore a total of 30 bottles were inoculated. Culture bottles were incubated inside a growth room where the temperature is about 25 ± 2 °C with 16 hours of profuse light and 8 hours of dark cycles under the lamp (1000-2000 Lux). After 2 weeks subculturing was done. Responses were recorded after 3 weeks of culture.

Shoot Multiplication

One-month shoot-initiated cultures were inoculated on MS-medium (Murashige and Skoog medium) with different concentrations. Each treatment is replicated three times, with one node being inoculated per culture bottle. In total, 12 nodes are being inoculated across all treatment combinations for shoot multiplication as shown in Table no. 2 After inoculation the vessels of the bottles were tightly sealed with paraffin paper and incubated inside a growth room where the temperature is about 25 ± 2 °C with 16 hours of profuse light and 8 hours of dark cycles under a lamp (1000-2000 Lux). Data on the number of shoots induced per explant was recorded after three week

Rooting

Multiplied shoots were transferred on half-strength MS-medium with different concentrations and hormone-free full-strength MS-medium was prepared as shown in table 3. Each treatment had one shoot per culture bottle and was replicated three times. Culture bottles were tightly sealed and kept in a growth room where the temperature is about 25 ± 2 °C with 16 hours of profuse light and 8 hours of dark cycles under the lamp (1000-2000 Lux). Data for the number of roots and length of roots were recorded after 3 weeks.

Acclimatization

For primary hardening, the nutrient medium was gently removed and washed from rooted shoots thoroughly in running tap water ensuring that all agar particles werecompletely removed without damaging the roots. Different media like compost, coco peat and soil were mixed in a combination of 1:1:1 and filled in plastic cups. The rooted plantlets were then, dipped in 0.1% Bavistin and planted in plastic cups. They were covered with a transparent plastic bag continuously for 2 weeks and kept at 28°C. The cover was gradually removed after seven days, initially for 3 h in an external environment followed by 6 h and 12 h in the next three days. Primary hardened plantlets were gently removed from the plastic cups. For secondary hardening plantlets were transferred to the plastic pots containing a mixture such as garden soil and coco-peat in a ratio of 1:1 and kept in a shade house.

Result And Discussion

Surface sterilization

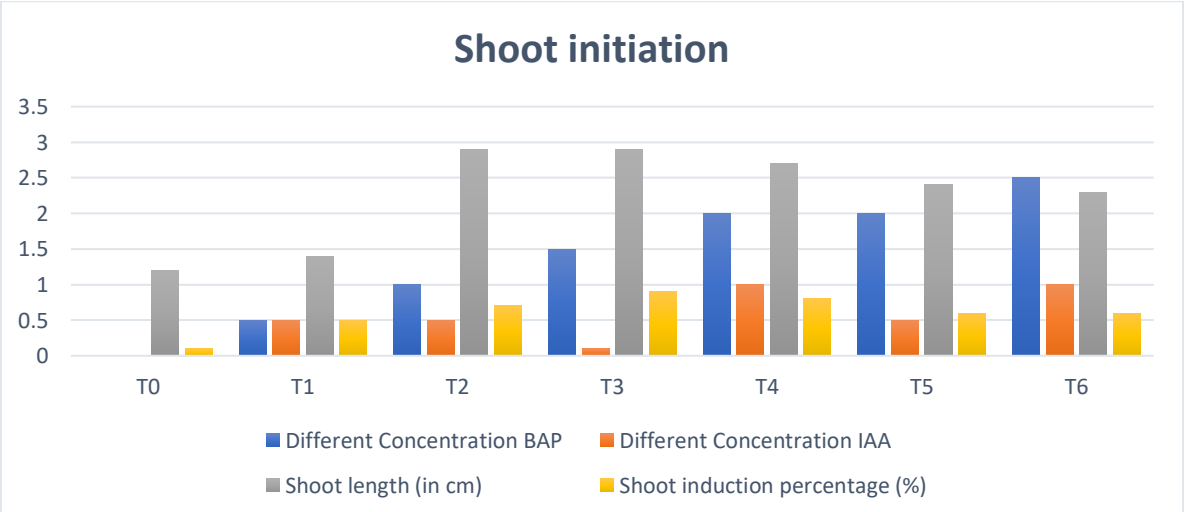
Collected explants were thoroughly washed under running tap water for 30 min, then treated with Bavistin solution (0.5 w/v) for 10 minutes. The surface sterilization was done by using three different concentrations of $HgCl_2$, and 0.1% for three minutes was found to be the most effective, resulting in a survival rate exceeding 95%. Although the established explants in 0.5% showed an improved response, the survival rate was low, only 35% of the cultures were established aseptically. Hence, 0.1% was deemed the optimal concentration for the successful establishment of the nodal segments.

Initiation of shoots

Explants were inoculated on nine different concentrations of media, with the most successful being a medium supplemented with 1.5 BAP + 0.1 IAA mg/L. This concentration resulted in the highest shoot induction percentage (90%) as Ten out of ten bottles showed shoot induction. This medium was therefore considered the best for shoot initiation production in terms of both quality and quantity.

Table 1. Effect of different concentrations of BAP and NAA on shoot induction

Sr. No.	Different Concentration		Shoot length (in cm)	Shoot induction percentage (%)
	BAP	IAA		
T0	Control		1.2	10%
T1	0.5	0.5	1.4	50%
T2	1	0.5	2.9	70%
T3	1.5	0.1	2.9	90%
T4	2	1.0	2.7	80%
T5	2	0.5	2.4	60%
T6	2.5	1.0	2.3	60%

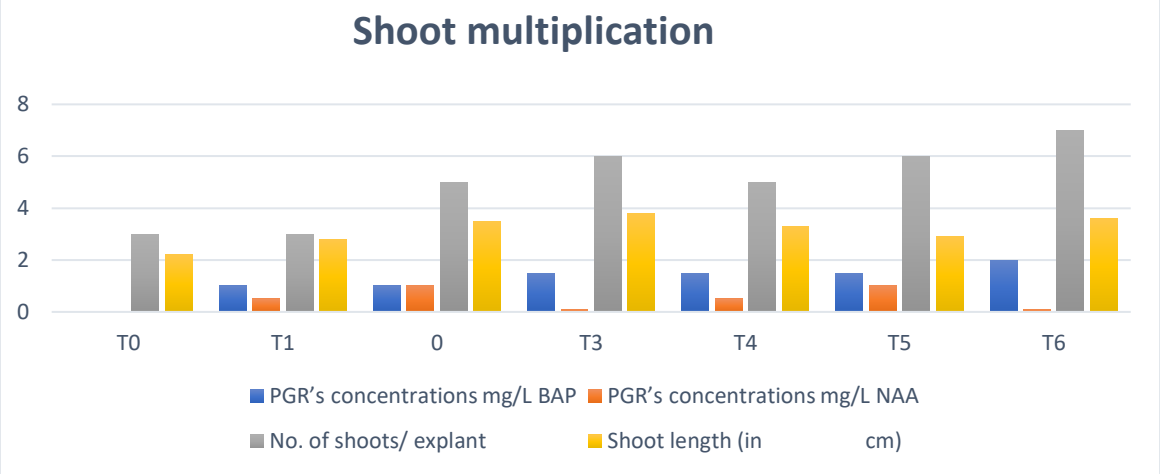


Multiplication of shoots

After successful shoot induction, the cultures with newly formed multiple shoots were carefully separated and then subcultured onto MS basal media with different concentrations of cytokinin to achieve rapid multiplication in subsequent cycles. From the media tested, the medium containing 1.5 BAP + 0.1 NAA mg was found to be the most advantageous for the quick multiplication of high-quality micro shoots, yielding the maximum number of shoots.

Table 2. Effect Of Different Concentrations Of BAP And NAA On Shoot Multiplication

Sr. No.	PGR's concentrations mg/L		No. of shoots/explant	Shoot length (incm)
	BAP	NAA		
T0	Control		3	2.2
T1	1	0.5	3	2.8
-T2	1	1	5	3.5
T3	1.5	0.1	6	3.8
T4	1.5	0.5	5	3.3
T5	1.5	1	6	2.9
T6	2	0.1	7	3.6

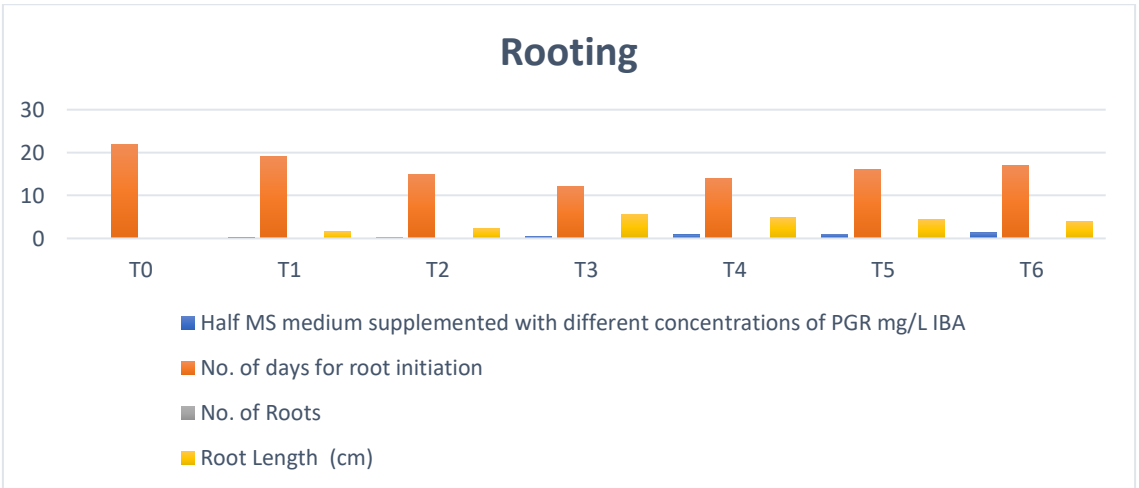


Rooting

Shoot cuttings of 4-5cm length that were developed in vitro were transferred to a rooting medium. The medium was supplemented with both full-strength and half-strength MS basal medium, with varying amounts of the plant growth regulator IBA. Results indicated that root induction occurred in all concentrations of the medium, with variations based on the concentration of IBA. The best response was seen with the half-strength MS medium containing 0.5 mg/L of IBA, with an average of 16.66 roots per shoot and a length of 5.6 cm. This study found that the half-strength MS basal medium combined with three different concentrations of IBA was more effective for root induction compared to the full-strength basal medium

Table 2. Effect of different concentrations of IBA on shoot multiplication

No.	Half MS medium supplemented with different concentrations of PGR mg/L	No. of days for root initiation	No. of Roots	Root Length (cm)
	IBA			
T0	Control	22	Not observed	0
T1	0.1	19	Observed	1.6
T2	0.3	15	Observed	2.3
T3	0.5	12	Observed	5.6
T4	0.8	14	Observed	4.8
T5	1	16	Observed	4.3
T6	1.3	17	Observed	3.9



Acclimatization

The *in vitro* raised plantlets with well-developed roots and shoots were transferred to small plastic cups containing a mixture of cocopeat: compost: soil (1:1:1) for initial acclimatization. They were covered with a plastic bag and after two weeks, showed good survival and acclimatization, with a survival rate of 100%. For secondary acclimatization plantlets mixture of cocopeat: soil (1:1) was used and kept in a shade house, the plants exhibited vigorous growth with a survival rate of 75% after two weeks.

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Plate: (A) Inoculation on MS media, (B) Shoot induction on MS media supplemented with BAP and IAA, (C) Shoot induction after 13 days, (D) Shoot multiplication on MS media supplemented with BAP and NAA, (E) Shoots with well-developed roots on the MS medium with IBA, (F) Primary hardening of regenerated plants in a medium containing Cocopeat: Soil (1:1)