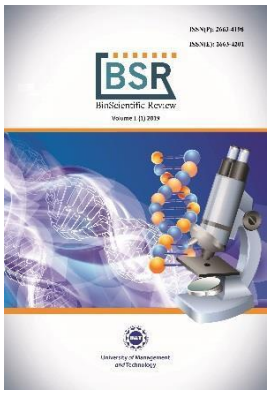

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Development and Optimization of Micro-Propagation, *In Vitro* Methodology for Gladiolus

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Abstract

Gladiolus is a non-native, costly but an important flowering plant in South Asia. Considering its lower production rate and economic importance, micro-propagation technology was optimized in this study to establish a protocol for pathogen free clonal genotypes. Taken the results together, the best response was observed at 27°C as growing temperature, table sugar (sucrose) as a carbon source, apical meristem of 3mm for shoot formation as an explant. The best plant regeneration was observed for shoot apical meristem (within 7.8 days after inoculation) by using Murashige and Skoog MS basal medium with supplementing 1.0 mg/L BAP and 0.5 mg/L of Kinetin. The best shoot multiplication was 98% in 18.2 days of inoculation using MS media with 1.0 mg/L BAP only. For root induction, MS media which was supplemented with 1.0 mg/L NAA and 2.0 mg/L IBA gave the best results (100% in 6.8 days). The best supporting agent was found to be cotton swab while the best media for hardening and acclimatization of well-developed plants was a combination of sand, soil, and peat in an equal ratio (1:1:1), leading to
80% survival rate of plants. The results obtained in this study may better help grow *Gladiolus* plants on an industrial scale with a lower cost of production

1. Introduction

Floriculture industry is one of the oldest and most profitable industries in the world. Due to the recent advances in agricultural technologies, it is expected that per capita consumption and production of cut flowers will rise throughout the world. *Gladiolus* is a group [Genus *Gladiolous*] of cut flowers that belongs to Iridaceae family. *Gladiolous* are herbaceous plants, two to three feet in height, with sturdy sword-shaped leaves, producing flower spikes with trumpet shaped forests.

Gladiolus plant is principally native to South Africa, with some species explored in the wild in Southern Europe. For the past two thousand years, Greeks and Romans have been using *Gladiolus* to decorate their ceremonies and events (1) There are approximately 180 species and 10,000 cultivars of *Gladiolus* (2). *Gladiolus* cultivation at commercial level is limited due to infection by pathogens and the low rate of multiplication of corms (3). Furthermore, the physiological dormancy of cornels, corns and corm rot during storage also contributes to its low yield and production. Plant biotechnology has paved ways of sustainable development in agriculture. Among various biotechnological tools, Plant Tissue Culture (PTC), also known as in *vitro* propagation or micro-propagation, is the most successful and widely used discipline. Advances in *in vitro* technology have made the floriculture much more productive and cost effective, thereby utilizing comparatively less land, labor resources and time. *In vitro* propagation offers a tool for large scale propagation of high yield and premium quality of the starting material for plantation and provides novel procedures for improving plants. The best commercial application of micro-propagation has been found in ornamental plants (4). Micro-propagated plants are raised under sterile conditions that avoid pathogens and induce rapid multiplication. They further maintain clonal and phenotype uniformity among the offspring, round the year production and the disease free plants make the technology much more acceptable (5). Tissue culture techniques are used as routine procedures to obtain large amounts of good quality planting material (6). The clonal propagation technique has generated a good deal of interest among nursery growers and has made a tremendous impact on floriculture industry worldwide. An entire crop can be produced with the premium qualities of a selected individual plant by employing micro-propagation technology. Considering the shortcomings with *Gladiolus* production, the research work was designed to optimize protocol for micro-propagation, while manipulating different growth factors like plant hormones (auxins and cytokinins), temperature, carbon source and finally, the hardening media.

2. Material and methods

2.1. Procurement and sterilization of explant

Gladiolus explants used in this study were collected from the seed center at Punjab University, Lahore, Pakistan. The apical portion was sterilized, initially washed with distilled water, then rinsed with 10% sodium hypochlorite, and at the end again washed with distilled water. The culture media was also sterilized by autoclaving culture tubes while all other instruments to be used were also autoclaved. Before starting micro-propagation, hands were also sanitized using cotton dipped in 70% ethanol.

2.2. Stock solution preparation

Preparation of stock solutions was carried out by the use of Murashige and Skoog MS basal media which was supplemented with micronutrients, macronutrients, Iron EDTA, vitamins and growth regulators Auxins [α-naphthylacetic] acid (NAA), Indole butyric acid (IBA) and Cytokinins [6-Benzyl amino purine (BAP) and Kinetin]. The media was stored in brown bottles at around 4-10ºC until further use (7).

2.3. Explant inoculation and meristem culture

The explant inoculation and meristem excision was performed in laminar flow. The culture was prepared carefully and placed at a temperature of 27 ± 2ºC, under the illumination of 40-watt fluorescent tubes (40-watt), which were fixed at a distance of 40 cm. The light intensity varied from 2,000-3,000 flux with 16 hour photoperiod. For the growth of meristem, the media culture was prepared from the stock solutions supplemented with 0.8% DifcoBacto agar.

2.4. Micro-propagation

Micro-propagation procedure was used to induce shooting and rooting of the explants.

2.5. Parameters and statistical treatments

The different stock solutions with a consistent change in the concentration of different categorical ingredients may alter the effect on growth and micro-propagation of *Gladiolus*. Different concentration of the supplements including cytokinins, auxins, carbon source as well as supporting agents may undergo different rates of root induction and multiple shoot formation. Different supporting items/agents like cotton swab, agar and phytagel were used to analyze shoot multiplication with respect to the time period. The effect of different temperature levels ranging from 23ºC ±1ºC to 30ºC ±1ºC was studied for shoot initiation while using explants of apical meristem. Five different BAP concentrations ranging from 0.5 to 2.5 mg/L BAP were supplemented in MS basal medium for analyzing the optimum shoot formation. Moreover, different combinations of peat, soil and sand were tested for their effects on the hardening of plants. All the parameters including the rate of micro-propagation, growth and multiple shoot formation were analyzed carefully and tested by Duncan's multiple range tests, statistically (8).

3. Results

Different factors were observed in order to analyze the growth and shoot initiation along with shoot multiplication using *Gladiolus* micro-propagation.

3.1. Effect of temperature on shoot formation

With the rise in temperature from 23ºC±1ºC to 27ºC±1ºC, the formation of shoot also increased. However, with further increase in temperature from 27ºC±1ºC to 30ºC±1ºC, there was a gradual decline observed in shoot initiation (Table 1).

Table 1. Effect of Temperature on Shooting from using Shoot Apical Meristem.

Temperature (°C)							
23±1	24±1	25±1	26±1	27±1	28±1	29±1	30±1
++	++	+++	+++	++++	+++	+++	++

+Poor, ++Fair, +++Good, ++++Excellent

Figure1. *In vitro* shoot formation using 3 mm long shoot apical meristem after 12 days of culturing.

3.2. Effect of different sizes of shoot apical meristem on formation of shoots

The results revealed that the rate of shoot formation is increased by increasing the size of apical meristem. Meristem of size 0.5 mm took an average of 27.4 days for shoot initiation with a rate of survival estimated at 70% and the regeneration potential estimated to be 40%. The maximum survival rate of 100% and regeneration potential of 90% within 12 days of inoculation were achieved using 3 mm of meristem (Table 2, Figure 1).

Table 2. Effect of Size of Shoot Apical Meristem on *In Vitro* Shoot Formation**.**

Size of Meristem (mm)	No. of days taken for shoot formation	Meristems survived	Rate of survival (%)	Meristems showing formation of shoots	Shoot formation rate (%)	Growth
0.5	27.4 ± 0.456^{a}	7 ± 0.282^{d}	70	4 ± 0.282^{d}	40	+
1.0	22.4 ± 0.456^{b}	8 ± 0.282^{c}	80	6 ± 0.282^{c}	60	++
2.0	17.8 ± 0.334^{c}	9 ± 0.282^{b}	90	8 ± 0.632^{ab}	80	+++
3.0	12 ± 0.282^{d}	10 ± 0.000^{a}	100	9 ± 0.400^{a}	90	++++
4.0	12 ± 0.400^{d}	10 ± 0.000^{a}	100	7 ± 0.282^{bc}	70	+++
5.0	12 ± 0.282^{d}	9 ± 0.282^{b}	90	9 ± 0.282^{a}	90	++++
LSD	**1.240**	**0.753**		**1.249**		

+Poor, ++Fair, +++Good, ++++Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.

3.3. Effect of cytokinins (BAP and Kinetin) on shoot formation and multiplication

The rate of shoot formation along with the time taken was analyzed (Figure 2).

To conclude, as BAP concentration increases from 0.5 mg/L to 1.0 mg/L, shooting increases to 96% within 8.4 culturing days. However, a further increase leads to low shoot formation rate in a longer time span (Table 3).

Figure2. Shoot formation from shoot apical meristem after 8 days of inoculation on MS medium containing 1.0 mg/l BAP.

Table 3. Effect of Various Quantities of BAP on Formation of Shoots Using Shoot Apical Meristem

Media	Composition (mg/l)	Days for shoot formation	Rate of shoot formation (%)	Growth
MS_1	MS+BAP 0.5	13.2 ± 0.178^c	78	+++
MS_2	MS+BAP 1.0	8.4 ± 0.219^e	96	++++
MS_3	MS+BAP 1.5	9.4 ± 0.219^d	94	++++
MS_4	MS+BAP 2.0	15.4 ± 0.219^b	84	+++
MS_5	MS+BAP 2.5	20.4 ± 0.219^a	64	++
LSD	-	**0.698**	-	

+Poor, ++Fair, +++Good, ++++ Excellent

*Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests. MS: Murashige and Skooge media

Figure 3. Formation of shoot by using shoot apical meristem after 15 days of inoculation on MS medium which was supplemented with 1.0 mg/l BAP + 0.5 mg/l of Kinetin.

Shoot formation rate with the amount of consumed time was observed (Figure 3). It was revealed that Kinetin also enhances the shoot formation rate with decreasing time period. The maximum shoot initiation was observed as 96% in 7.8 days on media with 1.0mg/L BAP and 0.5mg/L Kinetin (Table 4).

Table 4. Effect of Various Concentrations of BAP with Kinetin on Shoot Formation**.**

Media	Composition (mg/l)	Days for shoot formation	Shoot formation rate (%)	Growth
MB_1	MS+ BAP0.5+Kinetin0.5	8.2 ± 0.178^a	82	+++
MB_2	MS+ BAP1.0+Kinetin0.5	7.8 ± 0.178^b	96	++++
MB_3	MS+ BAP1.5+Kinetin0.5	8.0 ± 0.282^b	88	+++
MB_4	MS+ BAP2.0+Kinetin0.5	9.2 ± 0.178^b	54	++
MB_5	MS+ BAP2.0+Kinetin1.0	9.6 ± 0.219^a	48	+
LSD	-	**0.698**	-	

+Poor, ++Fair, +++Good, ++++Excellent

*Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests. MS:

Figure 4. Growth and proliferation of multiplied shoots of Gladiolus on MS medium supplemented with 1.0 mg/l BAP within 18 days of culturing.

Surprisingly, the maximum multiplication of shoots was observed with 1.0mg/L of BAP as shown in Figure 4 (98% in 18.2 days), while the second maximum was at 1.5mg/L of BAP (98% shoot multiplication in 21.4 days). This was followed by the media having 1.0mg/L of BAP with 0.25mg/L of Kinetin (92% shoot multiplication in 22.4 days) (Table 5).

Table 5. Effect of Different Cytokinins on *In Vitro* Shoot Multiplication**.**

Media	Composition (mg/l)	Days taken for shoot multiplication	No. of shoots formed per culture vial	Rate of Shoot Multiplication (%)	Growth
MH_1	MS+BAP 0.5	23 ± 0.282^{ab}	13.4 ± 0.219^{b}	86	+++
MH_2	MS+ BAP1.0	18.2 ± 0.178^{d}	18.8 ± 0.178^{a}	98	++++
MH_3	MS+ BAP1.5	21.4 ± 0.219^{c}	18.6 ± 0.219^{a}	98	++++
MH_4	MS+ BAP1.0+Kinetin0.25	22.4 ± 0.219^{c}	18.2 ± 0.178^{a}	92	++++
MH_5	MS+ BAP1.0+Kinetin0.5	23.4 ± 0.357^{a}	13.2 ± 0.178^{b}	84	+++
MH_6	M.S+ BAP1.5+Kinetin0.25	22.2 ± 0.178^{b}	13.4 ± 0.219^{b}	86	+++
MH_7	MS+ BAP1.5+Kinetin 0.5	23.8 ± 0.178^{a}	11.2 ± 0.334^{c}	66	++
LSD	-	**0.774**	**0.726**	-	-

+Poor, ++Fair, +++Good, ++++ Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.

Figure 5. *In vitro* developed plants having well developed roots on MS medium containing 1.0 mg/l of NAA with 2 mg/l IBA after 8 days of culturing.

3.4. Effect of auxins on root induction

Our results revealed that maximum root induction in minimum time was with 1.0 mg/L of NAA with MS. The rate of root induction was 98% in a time period of 7.6 days. If a greater concentration of NAA was used, it resulted in decline in root induction rate (Table 6).

Table 6. Effect of Various Concentrations of NAA on Rooting of Well-Developed Plants.

Media	Composition (mg/l)	Days taken for root induction	Average roots produced per plant	Rate of root induction (%)	Growth
RN_1	M.S+NAA0.5	11.4 ± 0.219^{a}	2.4 ± 0.279^{b}	46	+
RN_2	M.S+NAA1.0	7.6 ± 0.357^{c}	3.6 ± 0.219^{a}	98	++++
RN_3	M.S+NAA1.5	9.4 ± 0.219^{b}	3 ± 0.282^{ab}	86	+++
RN_4	M.S+NAA2.0	9.4 ± 0.219^{b}	2.8 ± 0.178^{b}	76	++
RN_5	M.S+NAA2.5	11.6 ± 0.357^{a}	2.4 ± 0.219^{b}	30	+
LSD	-	**0.932**	**0.746**	-	-

+Poor, ++Fair, +++Good, ++++ Excellent
* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests

To find out the rate of root induction using other auxins like IBA, the same procedure was followed with five different amounts (in a range of 0.5 to 2.5 mg/L). The maximum root induction (96%) with the minimum time of induction (7.2 days) was observed on media supplemented with 1.0 mg/L of IBA (Table 7)

Figure 6. Growth and development of *in vitro* shoot formed from shoot apical meristem on MS medium using table sugar as carbon source.

Table 7. Effect of Different Concentration of IBA on Rooting of Well-Developed Plants.

Media	Composition (mg/l)	Days for root induction	Average roots per plant	Rate of root induction (%)	Growth
RB_1	M.S + IBA 0.5	12.6 ± 0.219^{a}	2.4 ± 0.219^{bc}	40	+
RB_2	M.S + IBA 1.0	7.2 ± 0.334^{c}	3.2 ± 0.178^{a}	96	++++
RB_3	M.S + IBA 1.5	9.8 ± 0.178^{b}	3 ± 0.282^{ab}	90	++++
RB_4	M.S + IBA 2.0	10 ± 0.282^{b}	2.4 ± 0.219^{bc}	74	+++
RB_5	M.S + IBA 2.5	12.8 ± 0.334^{a}	2.2 ± 0.178^{c}	32	+
LSD	**-**	**0.914**	**0.722**	**-**	

+Poor, ++Fair, +++Good, ++++ Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.

Since the results with both auxins were promising, the mixture of both auxins using different concentrations were tested for root induction with respect to time. Maximum root induction (100% in 6.8 days) was observed with 1.0mg/L NAA and 2.0 mg/L of IBA (Figure 5; Table 8).

Figure 7. Well proliferated *in vitro* plants of Gladiolus on MS medium which was supplemented with cotton as supporting agent within 18 days of inoculation.

Table 8. Effect of NAA in Combination with IBA on Rooting of *In Vitro* Grown Plants.

Media	Composition (mg/l)	Days taken for inducing rooting	Average roots per plant	Rate of root induction (%)	Growth
NB1	M.S+NAA1.0+IBA 1.0	12.4 ± 0.219^{a}	2.4 ± 0.219^{abc}	44	+
NB2	M.S+NAA1.0+IBA 2.0	6.8 ± 0.178^{c}	3.6 ± 0.219^{a}	100	++++
NB3	M.S+NAA1.0+ IBA 3.0	9.4 ± 0.219^{b}	3 ± 0.282^{ab}	88	+++
NB4	M.S+NAA1.5+ IBA 2.0	9.6 ± 0.219^{b}	2.8 ± 0.178^{bc}	74	++
NB5	M.S+NAA2.0+ IBA 2.5	12.6 ± 0.219^{a}	2.4 ± 0.219^{c}	40	+
LSD	-	**0.698**	**0.746**	-	-

+Poor, ++Fair, +++Good, ++++ Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.

3.5. Effect of carbon sources on shoot regeneration

There may be a number of different sources which can be used. The laboratory grade sucrose and table sugar (sucrose) were tested as carbon sources. As a result, it was found that table sugar was a comparable but a cheaper carbon source to

the laboratory grade sucrose (Figure 6). The MS media containing pure sucrose with 1.0mg/L BAP showed the rate of shoot formation as 92% in 7.6 days. Whereas table sugar produced 94% of shoot induction in 7.4 days (Table 9).

3.6. Effect of different supporting agents on shoot multiplication

Phytagel provided 84% shoot multiplication response within 21.2 days of culturing having 15.6 shoots per culture vial. Whereas 90% shoot multiplication response was obtained within 19.2 days of culturing, with 17.6 shoots per culture vial using agar. As far as cotton is concerned, it provided the best results with 94% shoot multiplication within 18 days of inoculation which resulted in an average of approximately 19.8 shoots per culture vial (Figure 7) (Table 10).

Table 9. Effect of Different Sources of Carbon on In Vitro Shoot Formation

Media	Composition (mg/l)	Source of Carbon	No. of test tubes inoculated	Days for shoot formation	No. of test tubes showing shoot formation	Rate of shoot formation (%)	Growth
MC1	M.S+ AP1.0	Sucrose	10	7.6 ±0.219a	9.2 ± 0.178*a*	92	++++
MC2	M.S+BAP1.0	Table Sugar	10	7.4 ±0.219a	9.4 ± 0.219*a*	94	++++
LSD	**-**	**-**	**-**	**0.798**	**0.729**	**-**	-

*Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.
+Poor, ++Fair, +++Good, ++++ Excellent

3.7. Effect of different media on hardening of well-developed plants

The effect of media was also analyzed for the hardening of plantlets which were well-developed and raised in *vitro*. The autoclaved sand resulted in the survival of 60% plants taking 40 days for hardening, while the mixture of soil and sand (1:1) resulted in the same. In case of the mixture of sand, soil and peat (1:1:1), 80% plants survived within 31.8 days of hardening (Figure 8) (Table 11).

Table 10. Effect of Various Supporting Agents on *In Vitro* Shoot Multiplication**.**

Composition (mg/l)	Type of media	Supporting Agent	Days for shoot multiplication	Number of multiple shoots formed per culture vial	Growth
MS + BAP 1.0	Solid	Agar	19.2 ±0.521^{c}	17.6 ± 0.219^{b}	+++
MS + BAP 1.0	Solid	Phyta gel	21.2 ±0.334^{b}	15.6 ± 0.456^{c}	++
MS + BAP 1.0	Liquid	---	23.8 ±0.334^{a}	13.8 ± 0.521^{d}	+
MS + BAP 1.0	Liquid	Cotton	18.8 ±0.334^{c}	19.8 ± 0.334^{a}	++++
LSD	-	-	**1.306**	**1.340**	

+Poor, ++Fair, +++Good, ++++ Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range test

Table 11. Hardening of *In Vitro* Developed Plants

Sr. No	Medium composition	Days for hardening	% age of plants survived	Growth
1	Autoclaved sand	40 ± 0.632^{a}	60	++
2	Sand + Soil (1:1)	40 ± 0.456^{a}	60	++
3	Sand + Soil+ Peat (1:1:1)	31.8 ± 0.334^{b}	80	+++
LSD	-	**1.68**		

+Poor, ++Fair, +++Good, ++++ Excellent

* Means which are being followed by different letters in the same column significantly differ at P=0.05, as per Duncan's new multiple range tests.

Figure 8. Hardening and acclimatization of well-developed *in vitro* plants of Gladiolus.

4. Discussion

In vitro culture technique offers new possibilities for plant species that are recalcitrant to grow in normal agronomic conditions. Micro-propagation technique has introduced a new era for the multiplication of plants and for the efficient production of disease free stock (9). It also results in multiple shoot formation without any alteration from parent stock in a short time span. The applicability of this technology to the propagation of trees and shrubs has previously been documented (10). Thus, the current work is an effort of *Gladiolus* micro-propagation to optimize conditions for in *vitro* initiation of root, shoot proliferation, and root induction by using shoot apical meristem tissues.

It was concluded that only 27°C ± 1°C was the optimal temperature for shoot formation and any further increase or decrease in temperature would lead only to decrease in shoot formation process. The effect of shoot apical meristem size and proliferation was a determining factor for this technique, since the increased size of meristem resulted in the increased rate of shoot formation (11, 12). When meristem of 5.0 mm was used, efficient results were obtained; however, the use of 3.0 mm meristem gave the best results due to its maximum shoot response along with a high rate of shoot multiplication.

Maximum shoot formation (96% within 7.8 days of culturing) was observed at 1.0mg/L of BAP in MS basal medium. Previously, many researchers reported in *vitro* shoot regeneration by using explant such as shoot apical meristem when MS basal medium was supplemented with 1.0mg/L BAP (10, 13).

At 1.0mg/L of BAP with 0.5mg/L of Kinetin, shoot formation response obtained was 96% after approximately seven days of inoculation (Table 4 and Fig.

4). Many researchers have also observed that the response of in vitro shoot formation was found in MS basal medium which contained both BAP and Kinetin in it. Since both of these growth factors individually help cell division, it is proposed that when used in combination, they can pronounce the effect of receptors to bind growth regulators and better conduct signaling cascade inside cell,

which ultimately leads to effective shoot formation (14-16).

Basal medium which contained 1.0 mg/L BAP without Kinetin showed maximum shoot rate (98%) within 18.2 days of culturing. These results confirm some previous studies (17-19).

NAA of 1.0 mg/L provided the most promising results (98% root induction rate in 7.6 days), thereby confirming the previous studies (20, 21). To evaluate the full strength, another auxin IBA was supplemented in a different concentration with NAA. The best rooting response of the developed shoots was obtained in MS medium which contained 1.0 mg/L NAA

and 2.0 mg/L IBA. The same results were reported previously by many researchers (22-24).

The carbon from sucrose resulted in 92% shoot formation. Whereas table sugar provided 94% shoot formation which proved it to be more efficient and a comparatively cheap carbon source for shoot formation.

All three supporting agents showed nearly similar rates of *in vitro* multiplication with cotton as the most suitable supporting agent for shoot multiplication, up to 94% within 18.8 days. These results are in agreement with Safiullah & Ahmed (2001) (25).

The best hardening response (80% of plantlets survived) of *in vitro* micro-propagated *Gladiolus* plants was observed on media containing a combination of soil, sand and peat (1:1:1). These results are in agreement with Pospisilova *et al.*, 1999 (26).

5. Conclusion

In conclusion, the current study describes optimized conditions for obtaining multiple, pure and disease free *Gladiolous*. Using explants such as shoot apical meristem, best shoot induction and shoot multiplication response was found on a medium which contained 1.0 mg/L BAP. For root induction, best results were found on NAA at 1.0 mg/L with IBA 2.0 mg/L. Among different supporting agents and sources of carbon used, table sugar as carbon source with cotton swab as supporting agent was found to be the best. For hardening of well-developed plants, a combination of soil, sand and peat (1:1:1) provided best hardening and acclimatization response.

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