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Optimized in vitro propagation of *petunia hybrida*: influence of plant growth regulators and physical factors on callus induction and proliferation

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Abstract

This study focuses on the optimization of in vitro culture techniques for the commercial, off-season propagation of *Petunia hybrida*, an important ornamental bedding plant. Explants (leaf, node, and internode) were cultured on Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs)—2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP), and naphthaleneacetic acid (NAA)—either individually or in combination. The effects of sucrose concentration, temperature, pH, and photoperiod were also evaluated. Among explants tested, leaf explants demonstrated the highest regenerative capacity. Optimal proliferation (92%) was obtained with 2,4-D (2.0 mg/L) under controlled conditions of 23 °C, pH 5.8, 30 g/L sucrose, and a 16-hour photoperiod. Callus induction was also achieved with BAP (2.5 mg/L), NAA (1.5 mg/L), and their combinations. Solid MS medium proved more effective than liquid medium for callus development. The findings highlight the importance of precisely tailored PGR concentrations and environmental conditions for maximizing regeneration efficiency. This protocol provides a reliable framework for large-scale, year-round production of *P. hybrida,* thereby supporting the floriculture industry during non-growing seasons.

Keywords: *Petunia hybrida*, Auxin, Explant, tissue culturing, Callus, MS basal medium.

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1. Introduction

Petunia is a genus having 35 species of the family Solanaceae (Bombarely et al. 2016). Within this family, *Petunia* is exceptionally responsive to tissue culturing techniques and can be regenerated from anthers, protoplasts, seedling tips, stems, leaves, and other tissues and organs (Martín-Pizarro, 2020). The principle behind the concept of tissue culture is totipotency which refers to the ability of cells to regenerate into a whole plant (Fehér, 2019; Kaya and Huyop 2020). Apart from this, it encourages the chances of a complete perennial nature of any specific variety that is grown as an annual under temperate conditions. Applying PGRs at the incorrect rate or time can result in stunted plants, delayed flowering, or market unsuitability (Asgher et al. 2015; Reddy, 2024). It is essential to realize that hormone concentration does not necessarily reflect the level of active hormone within the explant (Seale et al. 2017; Ibrahim, 2022). However, the *in vitro* organogenesis of *Petunia* plants was affected by different environmental factors as leaf discs are affected by explant size,

configuration, and duration of exposure to benzyladenine (BA) (Druege and Franken, 2018; Loyola-Vargas and Ochoa-Alejo 2024). For *Petuni*a leaf explants, the exogenous cytokinin and BA can control the commitment of leaf explants to produce shoots in tissue culture (Kaviani and Kazemi, 2017; Kamal et al., 2024). This is one of the most popular bedding plants that is being used for ornamental purposes throughout the world; thus, tissue culturing will be one of the best choices to fulfill its demand (Van der Krol and Immink, 2016). Furthermore, *Petunia* species have been widely considered as a model system due to several reasons, including short cycle, feasible culture conditions, easy propagation, and transformation (Zhang et al., 2024). It's also well suited for biochemical analysis as the phenylpropanoid pathway (PPP) can be obtained and induced easily through the regulation of tissue and cell culture of *Petunia* species (Zerche et al. 2016; Lv et al., 2024). In molecular biology, various developmental stages of a plant, along with mutants, can be investigated conveniently through this plant species (Subburaj et al. 2016). *P. hybrid* has been the interest of different studies (El-Hawaz et al., 2019; Farooq et al., 2021; Borovaya et al., 2022). In this study, we focused on the involvement of the Plant Growth Regulator's concentration in distinct combinations that induces rapid callus formation of *P. hybrida*. These calli induced from leaf explants of *P. hybrida* were maintained to regenerate the plantlet.

2. Materials & Methods

The present work was carried out in "Plant Biotechnology and Molecular Genetics Lab, Department of Botany, Lahore College for Women University, Lahore", Pakistan. Seeds were purchased from a seed center in Lahore and grown in pots. Explants were taken from these grown *P. hybrida* plants. In the laboratory, the experimental procedure was divided into the following two steps:

i. Establishment of Proliferation for *P. hybrida* Vilm.

ii: Evaluation of PGRs that induce callus of *P. hybrida* Vilm. on MS basal medium.

Establishment of Proliferation for *P. hybrida* Vilm.

Leaf explants used for this study were grown in Lahore College for Women University, Lahore. Plants were grown on regular soil to get different explants, i.e., leaf, internode, and node. The explants were surface sterilized by washing with tap water using a few drops of liquid detergent, then dipping (for 20 minutes) in 5% commercial sodium hypochlorite or ordinary bleach so as to minimize the risks of contamination. For the proliferation of *Petunia*, different Plant Growth Regulators (2, 4-Dichlorophenoxyacetic acid, Benzyl Amino Purine & Naphthalene Acetic Acid) and their combinations were used in MS basal medium. Both liquid and solidifying nutrient media were tested to achieve the best organogenesis of *P. hybrida* explants. Five different ranges of sucrose, temperature, pH, and photoperiods were analyzed to standardize the best proliferation.

Evaluation of Plant Growth Regulators that induced Proliferation of *P. hybrida* Vilm. on MS basal medium

MS medium was supplemented with three PGRs alone and in concentrations of two, to observe their effect on formation of *P. hybrida* callus cultures. Each treatment was performed in triplicates. Proliferation of all the cultured test tubes were snapped at different days, maximum time period was 30-35 days. The results obtained were statistically analyzed.

Statistical Analysis

The results obtained were statistically analyzed. The means were separated by Duncan's new multiple range test at 1% level if significance as described by Steel et al., (1997) using SPSS software (Leveseqe, 2007).

3. Results

Response of different explants i.e. leaf, internode, and node of *P. hybrida* on MS medium supplemented with different PGRs with different concentrations, were recorded (Figure 1-6). All the physical elements that were used in various ranges were noted, however we are simply presenting the Dichlorophenoxyacetic acid (2.0mg/L), in Table 1-5 here. Out of 3 inoculated cultures for each explant, the best response was observed with 2,4-Dichlorophenoxyacetic acid (2.0mg/L), whereas the minimum percentage i.e. 32% was found in 2,4-Dichlorophenoxyacetic acid (5.0mg/L). Node explants gave a maximum rate of proliferation i.e. 73% in 2,4-Dichlorophenoxyacetic acid (2.0mg/L), while in the medium containing 2,4-Dichlorophenoxyacetic acid (5.0mg/L) showed a minimum proliferation rate i.e. 43%. Internode explants gave the maximum growth i.e. 63% in the medium containing 2,4-Dichlorophenoxyacetic acid (2.0mg/L), while the minimum growth i.e. 26% was found in 2,4-Dichlorophenoxyacetic acid (5.0mg/L). It was observed that leaf explant proved to be the best for the proliferation of *P. hybrida* Vilm. (Plate 1). The maximum proliferation of the leaf explant on MS medium was obtained at 2 mg/L with 2,4-Dichlorophenoxyacetic acid. Temperature, sucrose, pH and photoperiod were adjusted at 23±2°C, 30%, 5.8 and 16 hours respectively. Although comparatively lesser callus (79%) was formed with a combination of Naphthalene Acetic Acid (1.5mg/L) & 76% with Benzyl Amino Purine (2.5 mg/l) in MS medium using leaf explants under the same above mentioned physical conditions. Furthermore, Benzyl Amino Purine (2.5mg/L) alone gave 76% proliferation rate with leaf explant; Naphthalene Acetic Acid (1.5mg/L) alone gave 73% proliferation rate with internodal explant, while their combination (2.5mg/L+1.5mg/L) gave 64% proliferation rate with nodal explant. The present study may help to find the way to proliferate *P. hybrida* Vilm. from various explants. It was observed that 2, 4-Dichlorophenoxyacetic acid (2mg/L) (Figure 1), Benzyl Amino Purine (2.5mg/L) (Figure 2), Benzyl Amino Purine and 2,4-Dichlorophenoxyacetic acid (2mg/L+2.5mg/L) (Figure 3), Naphthalene Acetic Acid (1.5mg/L) (Figure 4), 2,4-Dichlorophenoxyacetic acid and Naphthalene Acetic Acid (2mg/L+1.5mg/L) (Figure 5) and Benzyl Amino Purine and Naphthalene Acetic Acid (2.5mg/L+0.5mg/L) (Figure 6) are the combinations at which calli for excised explants of *P. hybrida* Vilm were obtained.

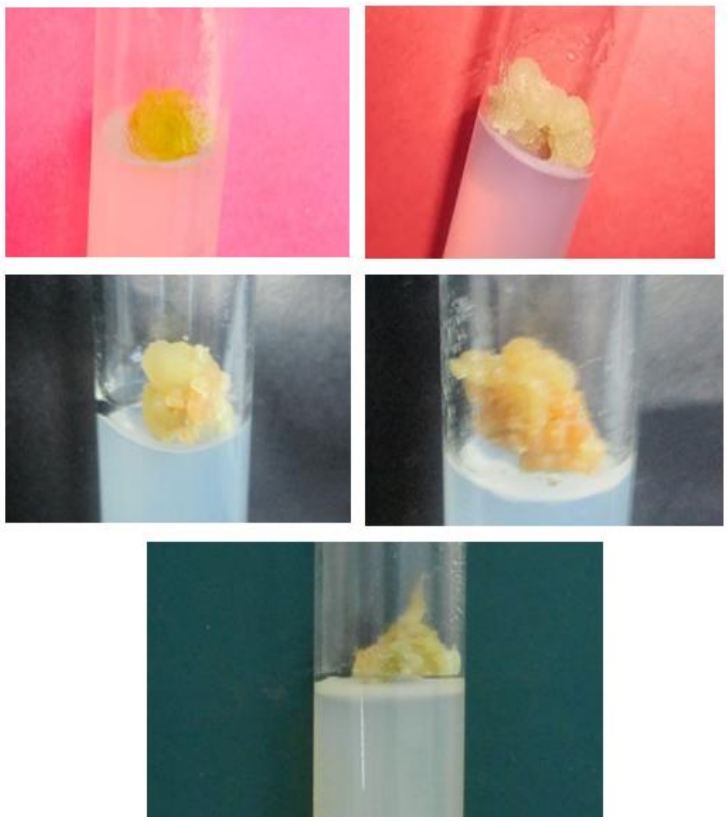

Plate 1: Proliferation from leaf explant of *Petunia hybrida* Vilm. on MS medium supplemented with 2, 4-D (2mg/L) up to 4 weeks of inoculation.

Table 1. Effect of liquid and solid medium on proliferation of *Petunia hybrida*Vilm. using leaf explants with 2,4-D (2.0mg/L) in MS basal medium.

Sr. no.	State of medium	Number of cultures inoculated	Proliferation (%) age mean	LSD value
i.	Liquid medium	3	20±1.05[b]	1.12
ii.	Solidified medium	3	93±0.66[a]	1.32

± Standard error of the mean; The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value).

Table 2. Effect of different concentrations of sucrose on proliferation of *Petunia hybrida*Vilm. using the leaf explants with 2,4-D (2.0mg/L) in MS basal medium.

Sr no.	Sucrose concentration (g/L) used	Number of cultures inoculated	Proliferation(%age mean).	LSD value
i.	15	3	14±0.32[cd]	
ii.	20	3	36±0.52[d]	
iii.	25	3	44±0.61[c]	
iv.	**30**	**3**	**89±0.61[a]**	1.47
v.	35	3	61±0.13[b]	

± Standard error of the mean; The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value).

Table 3. Effect of temperature on proliferation of Petunia hybridaVilm. Using leaf explants with 2,4-D (2.0mg/L) in MS medium.

Sr. no.	Temperature ranges (ºC)	Number of cultures inoculated	Proliferation %age mean	LSD value
i.	19±2	3	51±0.21[cd]	
ii.	21±2	3	55±0.51[d]	
iii.	23±2	3	81±0.52[a]	
iv.	25±2	3	64±0.30[b]	1.42
v.	27±2	3	61±0.1[c]	

± Standard error of the mean; The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value)

Table 4. Effect of pH on proliferation of *Petunia hybrida*Vilm. using leaf explants with 2,4-D (2.0mg/L) in MS basal medium.

Sr. no.	pH ranges	Number of cultures inoculated	proliferation mean (%) age	LSD value
i.	5.5	3	31±0.2[d]	
ii.	5.6	3	42±0.23[c]	

iii.	5.7	3	71±0.13[b]	
iv.	5.8	3	86±0.43[a]	1.26
v.	5.9	3	26±0.42c[d]	

± Standard error of the mean; The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value)

Table 5. Effect of photoperiod on proliferation of *Petunia hybrid* Vilm using leaf explants with 2,4-D (2.0mg/L) in MS basal medium.

Sr. no.	Photoperiods 2000-3000 Lux	Number of cultures inoculated	Proliferation % age mean	LSD value
i.	0 hours	3	11±0.50[cd]	
ii.	8 hours	3	42±0.55[b]	
iii.	**16 hours**	**3**	**76±0.44[a]**	1.66
iv.	24 hours	3	33±0.32[c]	

± Standard error of the mean; The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value).

Table 1: Effect of 2,4-Dichlorophenoxyacetic acid in MS basal medium on the maximum proliferation of *Petunia hybrida* Vilm. using different explants:

Serial	Explant	PGRs (mg/l)	Proliferation %	Texture of Callus	Color of Callus
1.	Leaf	2,4-Dichlorophenoxyacetic acid (2)	92 ± 1.57[a]	Compact	Greenish
2.	Leaf	2,4-Dichlorophenoxyacetic acid + Benzyl Amino Purine (2+2.5)	76± 1.08[a]	Compact	Brown
3.	Node	Benzyl Amino Purine +Naphthalene Acetic Acid (2.5+1.5)	80 ± 0.58[a]	Compact	Brown

The mean with different letter in each column is significantly different according to Duncan's multiple range tests (0.05p value) ± = Standard mean of error

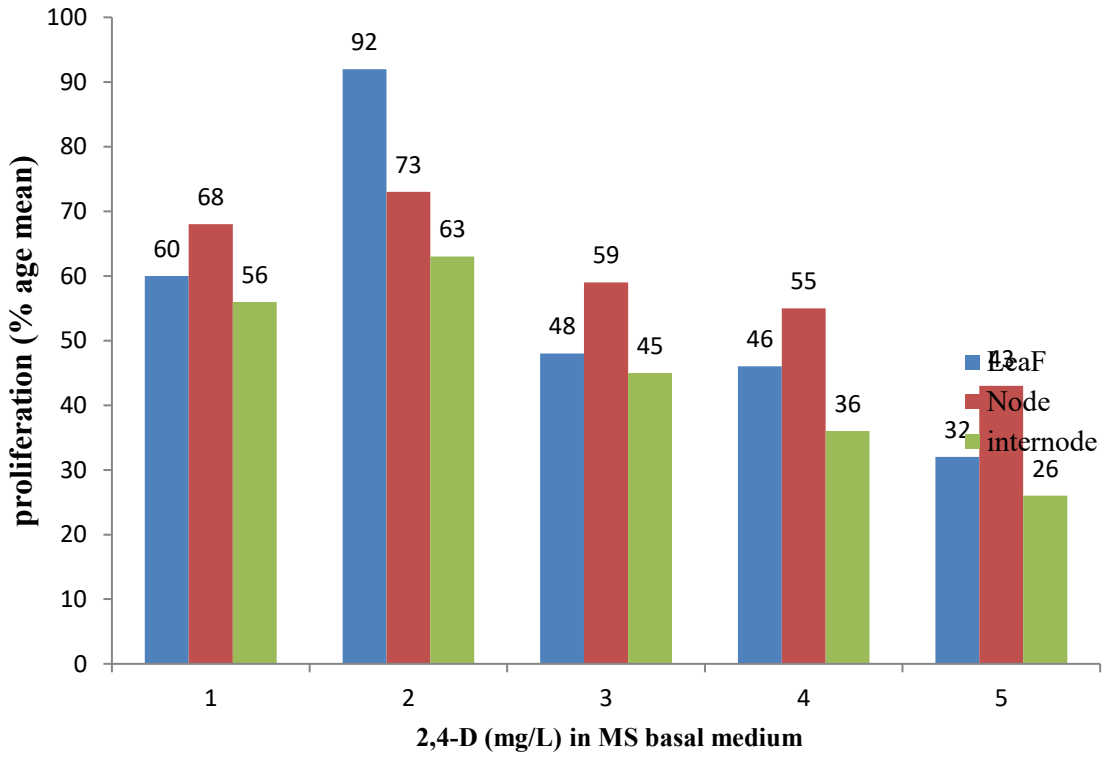

Figure 1. Effect of different concentrations of 2,4-D in MS basal medium on proliferation of *Petunia hybrida* Vilm.

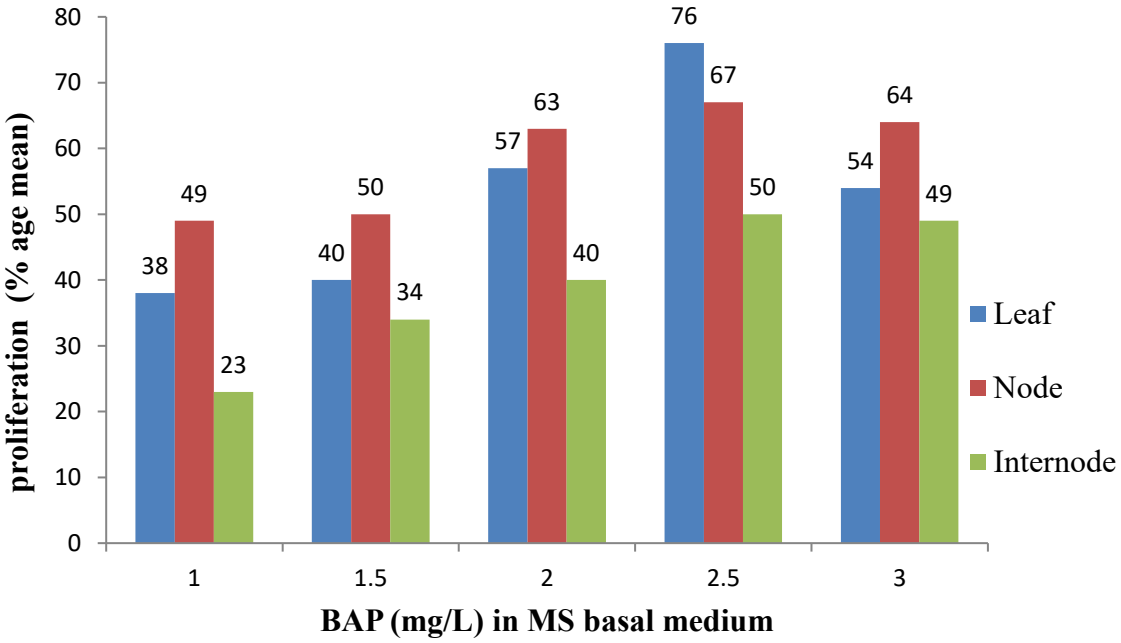

Figure 2. Effect of Different concentrations of BAP in MS basal medium on proliferation of *Petunia hybrida*- Vilm. using different explants

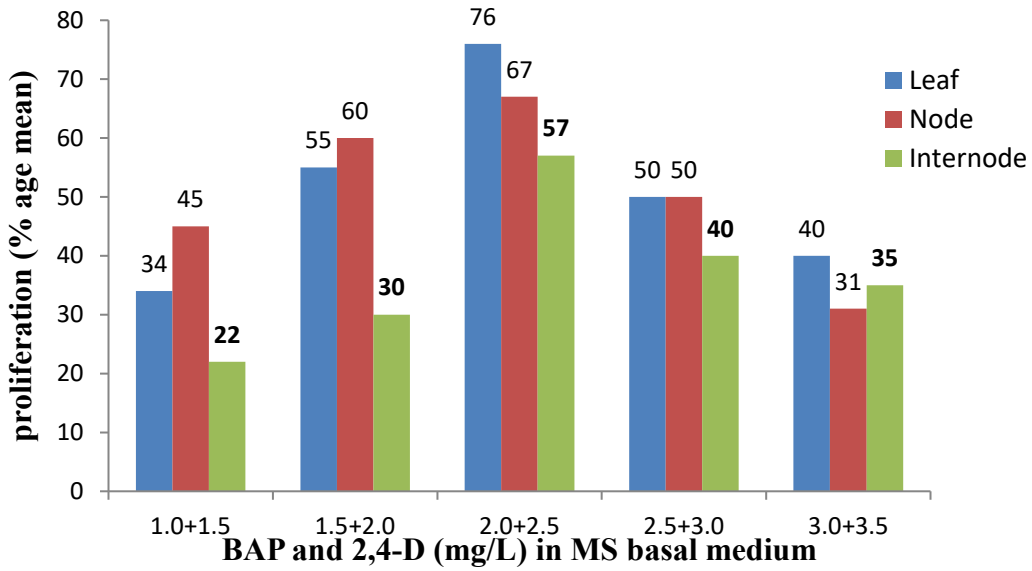

Figure 3. Effect of different concentrations of BAP+2,4-D in MS basal medium on proliferation of *Petunia hybrida*Vilm. using different explants

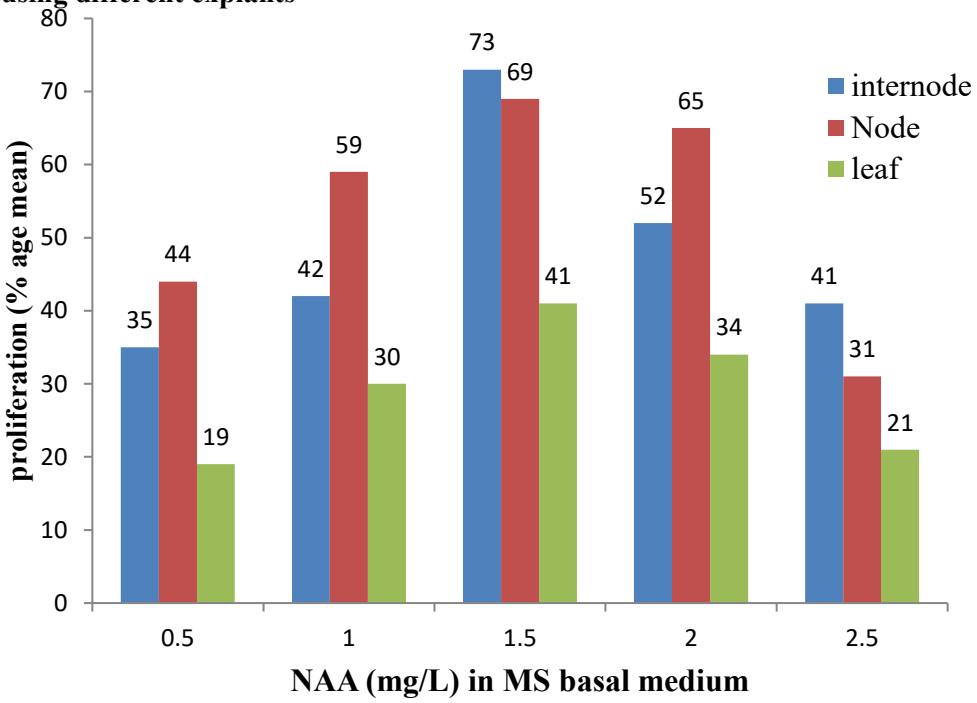

Figure 4. Effect of different concentrations of NAA in MS basal medium on proliferation of *Petunia hybrida*Vilm. using different explants.

Figure 6. Effect of different concentrations of BAP and NAA on *Petunia hybrida*Vilm. using different explants.

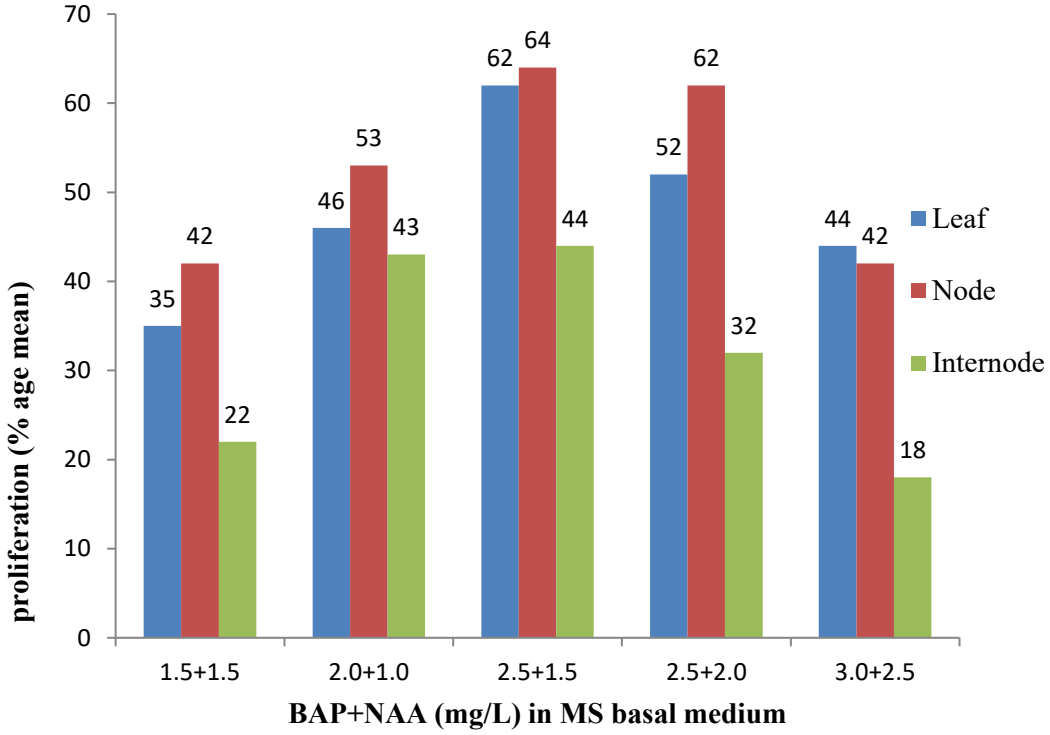

Figure 5. Effect of different concentrations of 2,4-D and NAA in MS basal medium on proliferation of *Petunia hybrid* Vilm. Using different explants

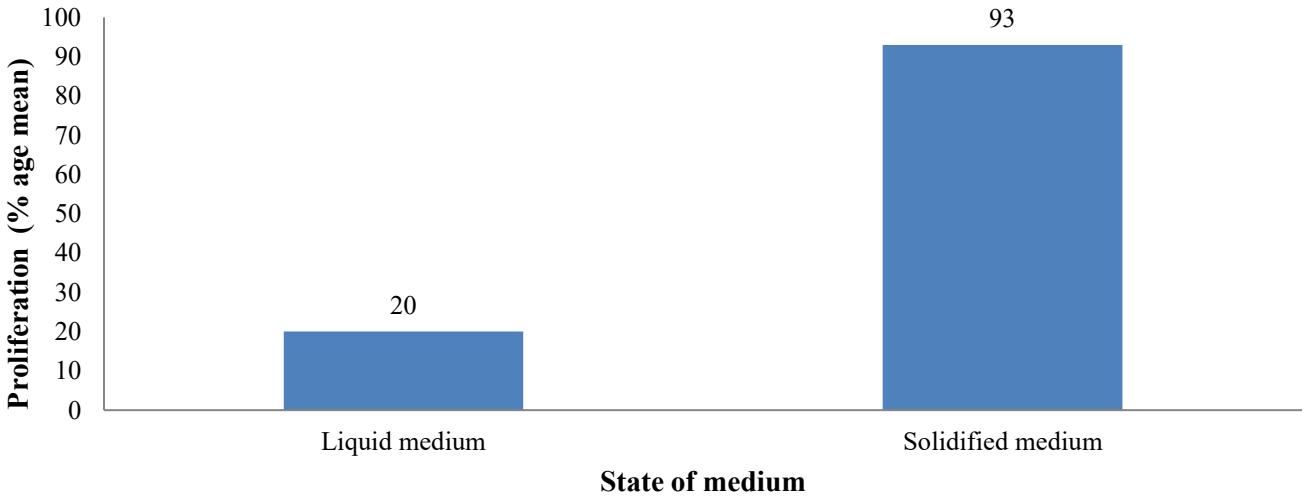

Figure 7. Effect of Liquid and solidified medium with 2.0 mg/L 2,4-D in MS basal medium on proliferation of Petunia hbri-daVilm. using leaf as a explant.

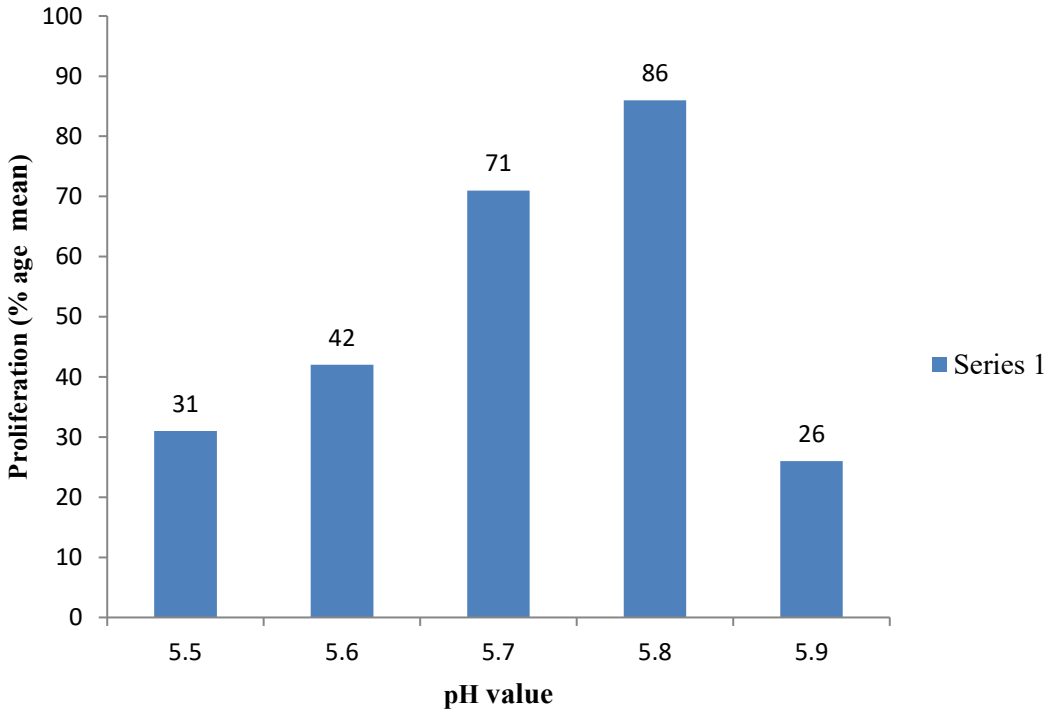

Figure 8. Effect of different pH on proliferation of leaf explants of *Petunia hybrida* Vilm. in MS basal medium using 2,4-D (2.0mg/L)

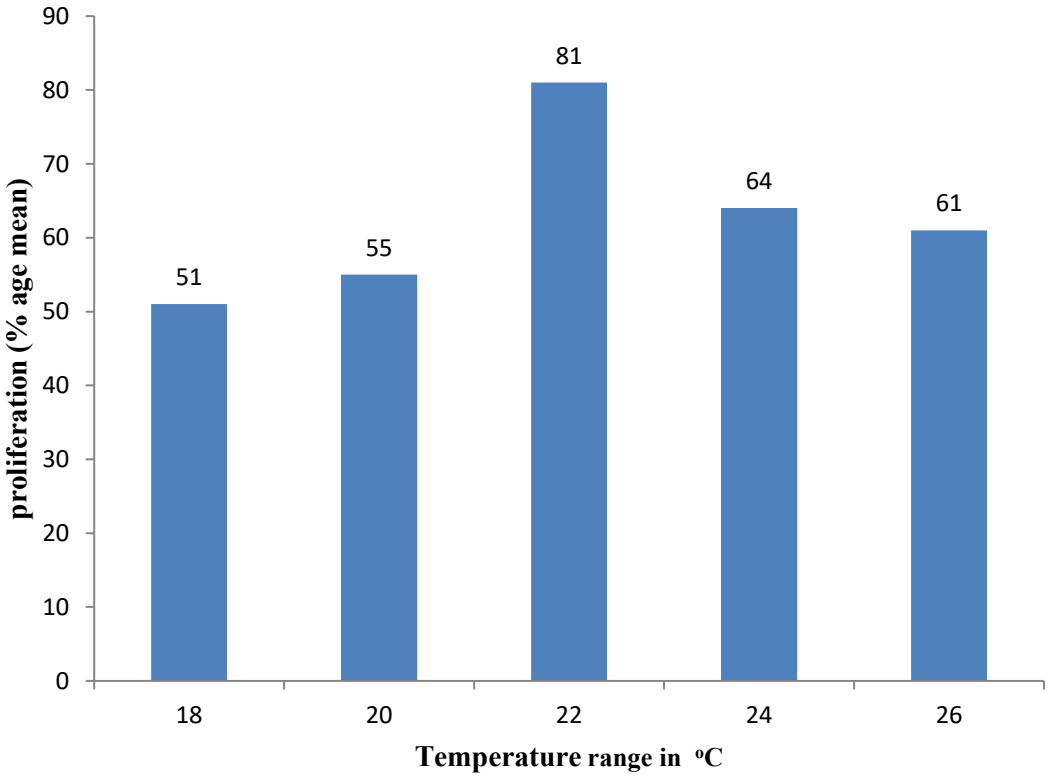

Figure 9. Effect of different temperatures proliferation of leaf explants of Petunia hybridaVilm. in MS basal medium using 2,4-D (2.0mg/L)

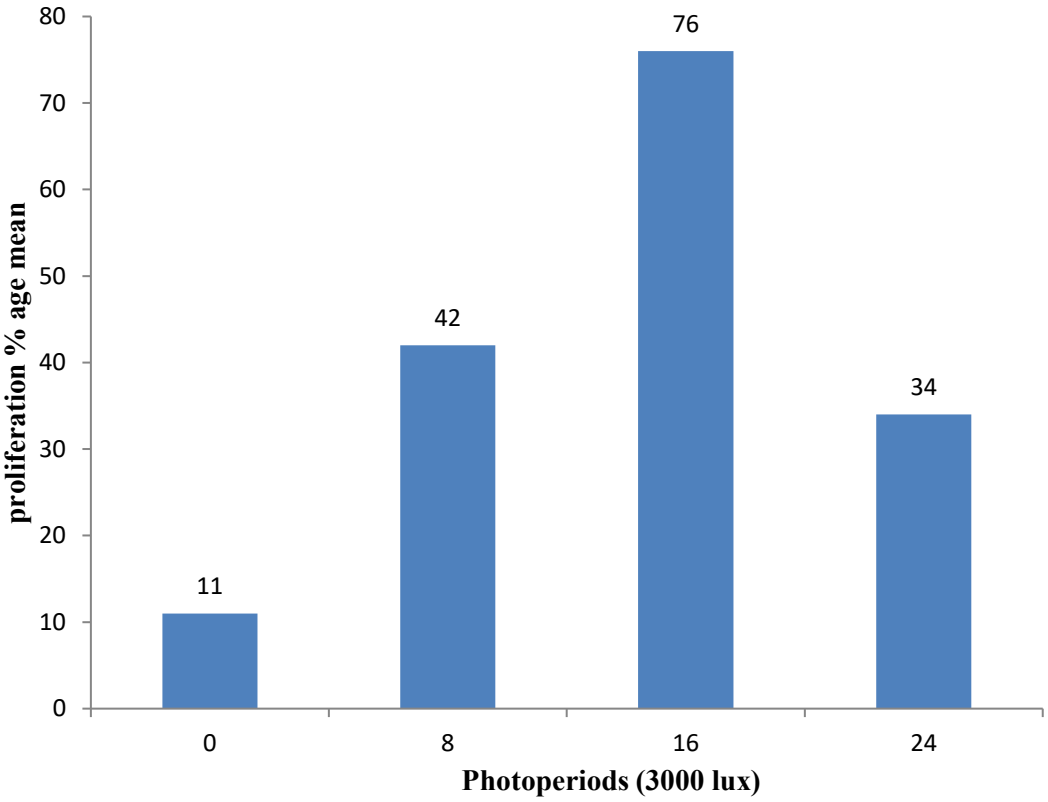

Figure 10. Effect of different photoperiods on proliferation of *Petunia hybrida Vilm.* in MS basal medium using 2,4-D (2.0mg/L).

4. Discussion

This study provides an approach to estimating the key chemical impact on the proliferation of *Petunia hybrida* Vilm. optimizing physical factors. For this study, *In vitro* proliferation was established and effects of different PGRs on the synthetic nutrient medium were recorded during this growth under controlled physical factors. Fujishima et al. (2000) studied factors influencing adventitious bud and root development and reported callus induction and embryogenesis was reported in stem and leaf cultures of *Petunia inflata* and *Petunia hybrida* on MS basal medium supplemented with 2,4-Dichlorophenoxyacetic acid. The same species has been selected to optimize for an improved, efficient, and rapid in vitro micropropagation, where they recorded maximum proliferation percent of shoots (97.90%), the highest number of shoots (20.50 explant−1), and maximum length of shoot (2.70 cm) was recorded in PGR combination of IBA and BAP both at 0.5 mg L−1 concentration level (Farooq et al., 2021). In 2022, Borovaya and Boginskaya demonstrated a high regeneration rate of *P. hybrida* on the hormone-free MS medium – it had a fast growth and development rate and good rhizogenesis; the reproductive rate was 8.77. In another study, El-Hawaz et al. (2019) demonstrated the feasibility of keeping *P. hybrida* for at least 32 weeks without subculture when stored in a low-temperature environment. They found from their two experiments that the longest storage period required low temperature (12 C), low sucrose, and a low light level for increased T0 micro-cutting production and more flexibility in variable storage periods alternatively, without a cool room, storage at 23°C for 16 weeks, with high sucrose and high light intensity maximized T0 micro-cutting production.Callus growth and embryo differentiation eventually developed into plantlets of *P. inflata* and *P. hybrida* (Rao et al. 1973). The effect of another combination of Benzyl Amino Purine & 2,4-Dichlorophenoxyacetic acid (2.5mg/L + 2mg/L) on MS basal medium was also checked and it was observed that leaf explants of *P. hybrida* Vilm showed maximum %age of calli i.e. 76% and gave minimum %age of proliferation i.e. 57% with internodal explants during present study (figure 3). Li et al. (2013)

optimized *in vitro* conditions for *P. hybrida* on 8p-KM medium supplemented with different PGRs; glucose (0.4M) along with mannitol (0.1M), 2, 4-D (0.3 mg/L) and Benzyl Amino Purine (0.3 mg) /L) were found to be best in this concern. During the present work, the effect of a combination of Benzyl Amino Purine & Naphthalene Acetic Acid was also observed on different explants in vitro growth (figure 6). The best proliferation rate i.e. 64% was achieved on the MS basal medium containing Benzyl Amino Purine & Naphthalene Acetic Acid (2.5 mg/L+0.5 mg/L) nodal explants whereas the lowest % age (44) was achieved from internodal explants while Kaviani and Kazemi (2017) proved this combination best with different concentrations of N6-benzyladenine (BA) (0.25, 0.50 and 1.00 mg l−1) and α-naphthalene acetic acid (NAA) (0.10, 0.20 and 0.30 mg l−1) when cultured on same basal Murashige and Skoog (MS) medium supplemented. Similarly, the same combinations were observed by Abu-Qaoud et al. (2010), who studied the effect of four different concentrations of BA and two different concentrations of Naphthalene Acetic Acid on the proliferation rate of *P. hybrida* Vilm. He found that the highest shoot number of *P. hybrida* was obtained with MS basal medium supplemented with BA and Naphthalene Acetic Acid (0.4mg/L + 0.1 mg/L). Other than these combinations, we also tried single PGRs in distinct concentrations as shown in Figure 1 (2,4, D), Figure 2 (BAP), and Figure 4 (NAA). During this study, amounts of 1-2 mg/L of PGRs were found to be significant in the *P. hybridra* calli's proliferation. A few plant biotechnologists have approached adequate concentrations of plant growth regulators in different combinations for *P. hybrida* on *in vitro* growth from alternate explants. For the long-term subculture of the callus of *P. hybrida,* BA media was used (Liskova et al. 2016). Plant regeneration was successfully implemented when transferred onto shoot induction media supplemented with a low concentration of plant growth regulators (Gupta et al. 2017). The culture system presented was effective in obtaining somatic variants for *P. hybrida* (Guo-gui et al. 2007). Literature also revealed that Benzyl Amino Purine and Naphthalene Acetic Acid have been the most effective combination to induce callus for *P. hybrida* Vilm within 30 days at optimized physical and chemical conditions. When shoot apex was used as explant, cultured on modified MS media (MS salts, B5 vitamins) with different concentrations of Naphthalene Acetic Acid (0, 0.2, 0.5 mg/L) and Benzyl Amino Purine (0, 0.1, 0.5, 1 mg/L). The highest callus induction percentage was obtained in the medium containing Benzyl Amino Purine (0.5 mg/L) and (Naphthalene Acetic Acid 0.5 mg/L), and it was noticed that callus induction was reduced significantly when cytokinin was used without Naphthalene Acetic Acid (Natalija et al. 2015).

Conclusion and Recommendations

The present study demonstrates that *Petunia hybrida* can be successfully propagated in vitro through optimized culture conditions and carefully adjusted combinations of plant growth regulators. Among all explants tested, leaf explants proved to be the most responsive, particularly when cultured on MS medium supplemented with 2,4-D (2.0 mg/L). This treatment consistently produced the highest proliferation rates and compact, greenish callus, indicating its strong potential for reliable regeneration. In addition, sucrose concentration (30 g/L), temperature (23 °C), pH (5.8), and photoperiod (16 h light) were identified as key physical factors that significantly enhanced proliferation efficiency. While other PGRs such as BAP and NAA also supported callus induction, their effects were most beneficial in combination with auxins.

Overall, the study establishes a reproducible and efficient protocol for off-season and large-scale propagation of *P. hybrida*. By integrating optimal hormonal and physical conditions, this method can meet the increasing demand for ornamental plants in the commercial floriculture sector. The standardized protocol developed here provides a foundation for further research on somatic embryogenesis, genetic transformation, and conservation of Petunia species, and may be adapted to other ornamentals with similar propagation challenges. Future research should focus on refining regeneration efficiency by testing additional growth regulators such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) in different concentrations and combinations with cytokinins. Molecular-level studies investigating gene expression during callus induction and somatic embryogenesis would provide deeper insights into the underlying regulatory mechanisms. Moreover, scaling up the developed protocol to bioreactor-based systems could help achieve industrial-level propagation. The use of this protocol for genetic transformation, secondary metabolite production, and conservation of rare or endangered Petunia varieties also represents a promising avenue. Finally, adapting this standardized protocol to related ornamental plants may expand its commercial applicability and further benefit the floriculture industry.

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