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Research Article

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In Vitro Proliferation of Petunia Hybrida Vilm; Worldwide Important Ornamental Plant

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Abstrac

This project tries to artificially grow one of the most ornamentally significant Petunia hybrida when it is out of season. Murashige and Skoog Basal Medium (MS Basal Medium) was used for in vitro growth of P. hybrida, supplemented with different Plant Growth Regulators (PGRs). Various physical and chemical factors on in vitro growth of P. hybrida using different explants were analyzed. The type of auxin that proved significant for somatic embryogenesis of P. hybrida Vilm was 2,4-Dichlorophenoxyacetic acid (2.0 mg/L) using the leaf as explant under controlled environmental conditions, i.e., 92% at 23oC with a 5.8 pH and 16 hours photoperiod & 30g/l sucrose in. Friable calli were also developed from leaf, node, and internodal explants on a tissue culture medium (MS basal medium) when supplemented with other PGRs in certain combinations i.e. 2,4-Dichlorophenoxyacetic acid (2.0 mg/L), Benzyl Amino Purine (2.5mg/L), Benzyl Amino Purine + 2,4-Dichlorophenoxyacetic acid (2mg/L +2.5mg/L), Naphthalene Acetic Acid (1.5mg/L), 2,4-Dichlorophenoxyacetic acid + Naphthalene Acetic Acid (2mg/L+1.5mg/L) and Benzyl Amino Purine + Naphthalene Acetic Acid (2.5mg/L+0.5mg/L) at optimal physical factors under strict aseptic environment. These results may enable one to produce the required type of P. hybrida Vilm during off and on seasons on a commercial scale by using plant tissue culture as a tool.

Keywords: Petunia Hybrida, Plant Growth Regulator, Auxin, Explant, Tissue Culturing, Callus, MS Basal Medium

Introduction

Petunia is a genus having 35 species of the family Solanaceae [1]. Within this family, *Petunia* is exceptionally responsive to tissue culturing techniques and can be regenerated from anthers, protoplasts, seedling tips, stems, leaves, and other tissue and organs [2]. Micropropagation refers to the culture of tissues of the selected plants grown in an aseptic condition on a medium containing macro and micronutrients to produce disease-free and true-to-type plants [3, 4]. The principle behind the concept of tissue culture is totipotency which refers to the ability of cells to regenerate into a whole plant [5-7]. Apart from this, it encourages the chances of semi-to 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 [8]. It is essential to realize that hormone concentration does not necessarily reflect the level of active hormone within the explant [9]. However, *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). For *Petunia* leaf explants, the exogenous cytokinin and BA can control the commitment of leaf explants to produce shoots in tissue culture [10]. This is one of the most popular bedding plant which is being used for ornamental purpose throughout the world, thus, tissue culturing will be one of the best choice to fulfill its demand [11]. Furthermore, *Petunia* species have been widely considered as a model system due to several reasons including short cycled, feasible culture conditions, easy propagation and transformation. It's also well suited for biochemical analysis as phenyl propanoid pathway (PPP) can be obtained and induced easily through regulation of tissue and cell culture of *Petunia* species [12]. In molecular biology, various developmental stages of a plant, along with mutants, can be investigated conveniently through this plant species [13]. 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.

Materials and 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:

• Establishment of Proliferation for *P. hybrida* Vilm.
• 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 work were grown in Lahore College for Women University, Lahore. Plant 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 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 Regulator (2, 4-Dichlorophenoxyacetic acid, Benzyl Amino Purine & Naphthalene Acetic Acid) and their combinations were used in MS basal medium. Both liquid and solidifying nutrient medium was 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 using SPSS software [14].

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 intermodal 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.

Discussion

This study provides an approach to estimating the key chemical impact on the proliferation of *Petunia hybrida* Vilm. Optimizing physical factors. *In vitro* proliferation was established and effects of different PGRs on synthetic nutrient medium were recorded during this growth under controlled physical factors. 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. Callus growth and embryo differentiation eventually developed into plantlets of *P. inflata* and *P. hybrida* [15]. 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). 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 *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 figures 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 [16]. 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* [17]. 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 [18].

As for as physical factors were concerned during the present investigation, the most effective sucrose concentration for the proliferation of *P. hybrida* Vilm was 30g/L in MS basal medium. It was found that 23±2°C was the most suitable temperature and 16 hours photoperiod (2000-3000 Lux) and pH 5.8, using a leaf as explants, gave maximum proliferation i.e. 92%, on MS basal medium supplemented with 2,4-Dichlorophenoxyacetic acid (2mg/L), using the leaf explants (figure 7-9). Suggested that ≈8–9 hours was the optimal photoperiod and 20 to 24°C was the optimal temperature during the first 6 weeks after germination (figure 9) [19]. Irwin (2002) reported that 5.5-6.2 is an optimal range of pH for *P. hybrida* Vilm. However, the MS basal medium supplemented with 2, 4-Dichlorophenoxyacetic acid (2mg/L) was the best culture medium for the successful proliferation of *P. hybrida* Vilm. under controlled environmental conditions i.e., 92% containing 30g/L sucrose and 10g/L difcobacto agar under 16 hours photoperiod at 23oC.

The present study revealed that for the successful proliferation of P. hybrida Vilm on 92% of on MS basal medium, it needs to be supplemented with 2, 4-Dichlorophenoxyacetic acid (2mg/L), using a leaf as an explant. This should also be done to regenerate *P. hybrida* Vilm.

Conclusion

The *Petunia hybrida* Vilm is capable of synthetic regeneration in a controlled environment. All of the PGRs that were evaluated, whether used alone or in combination with another PGR, demonstrated the ability to start a callus when used at a certain concentration. However, 4-Dichlorophenoxyacetic acid at a concentration of 2 mg/L on an MS medium produced the greatest results for this approach.

Acknowledgments

The authors are thankful to the Department of Botany, Lahore College for Women University, for various facilities.

Data Avaialibility Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

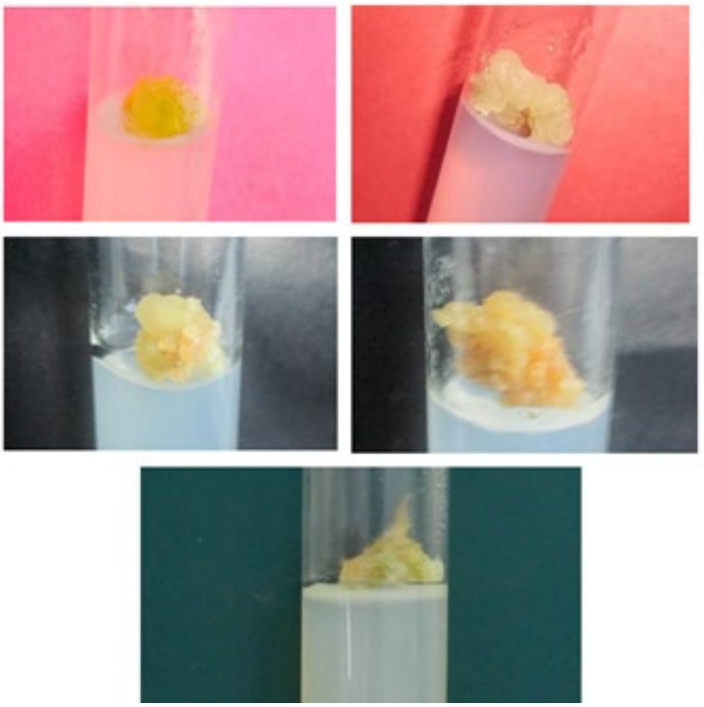

Plate 1: Proliferation from leaf explant of *Petunia hybrida* Vilm. on MS medium supplemented with 2, 4-D (2mg/L) up to 4 weeks of inoculation.

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±1.05b	1.12
ii.	Solidified medium	3	93±0.66a	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}	1.47
ii.	20	3	36±0.52^{d}	
iii.	25	3	44±0.61^{c}	
iv.	**30**	**3**	**89±0.61^{a}**	
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 hybrida*Vilm. 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}	1.42
ii.	21±2	3	55±0.51^{d}	
iii.	23±2	3	81±0.52^{a}	
iv.	**25±2**	**3**	**64±0.30^{b}**	
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}	1.26
ii.	5.6	3	42±0.23^{c}	
iii.	5.7	3	71±0.13^{b}	
iv.	**5.8**	**3**	**86±0.43^{a}**	
v.	5.9	3	26±0.42c^{d}	

± Standard error of themean
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 hybrida* 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±0.50cd	1.26
ii.	8 hours	3	42±0.55b	
iii.	**16 hours**	**3**	**76±0.44a**	
iv.	24 hours	3	33±0.32c	

± 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 No.	Explant Used	PGRs used (mg/l)	Proliferation (%Mean)	Proliferation (%Mean)	Color of Callus
1.	Leaf	2,4-Dichlorophe-noxyacetic acid (2)	92 ± 1.57[a]	Compact	Greenish
2.	Leaf	**2,4-Dichlorophe-noxyacetic acid + Benzyl Amino Pu-rine (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 are significantly different according to Duncan's multiple range tests (0.05p value) ± = Standard mean of error

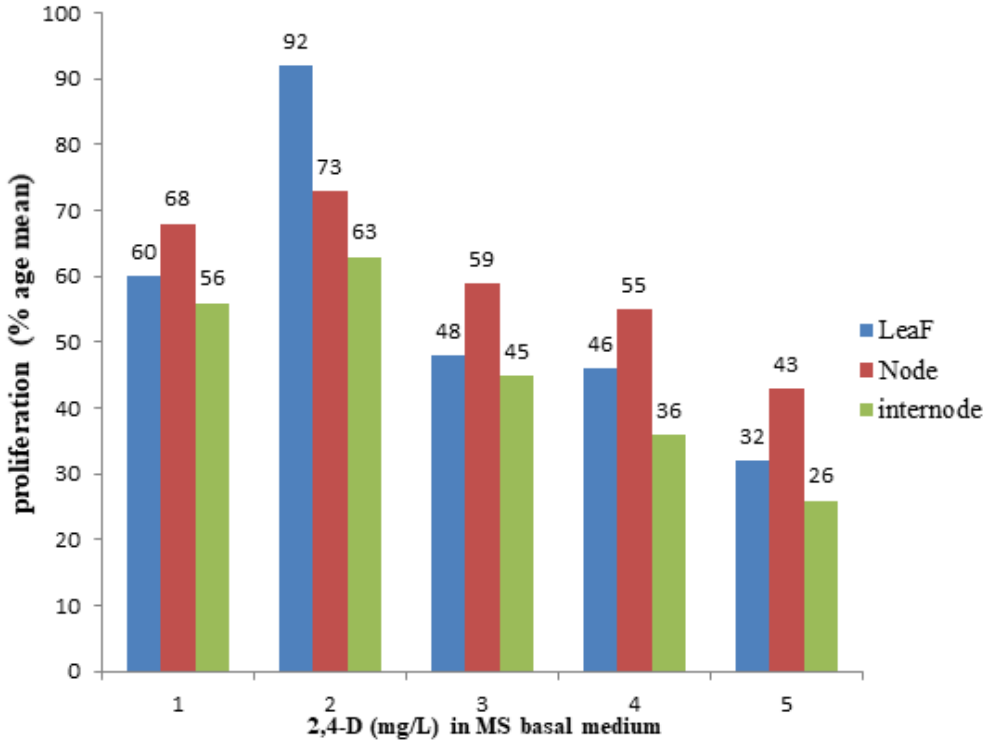

Figure 1: Effect of different concentrations of 2,4-D in MS basal medium on proliferation of *Petunia hybrida* Vilm

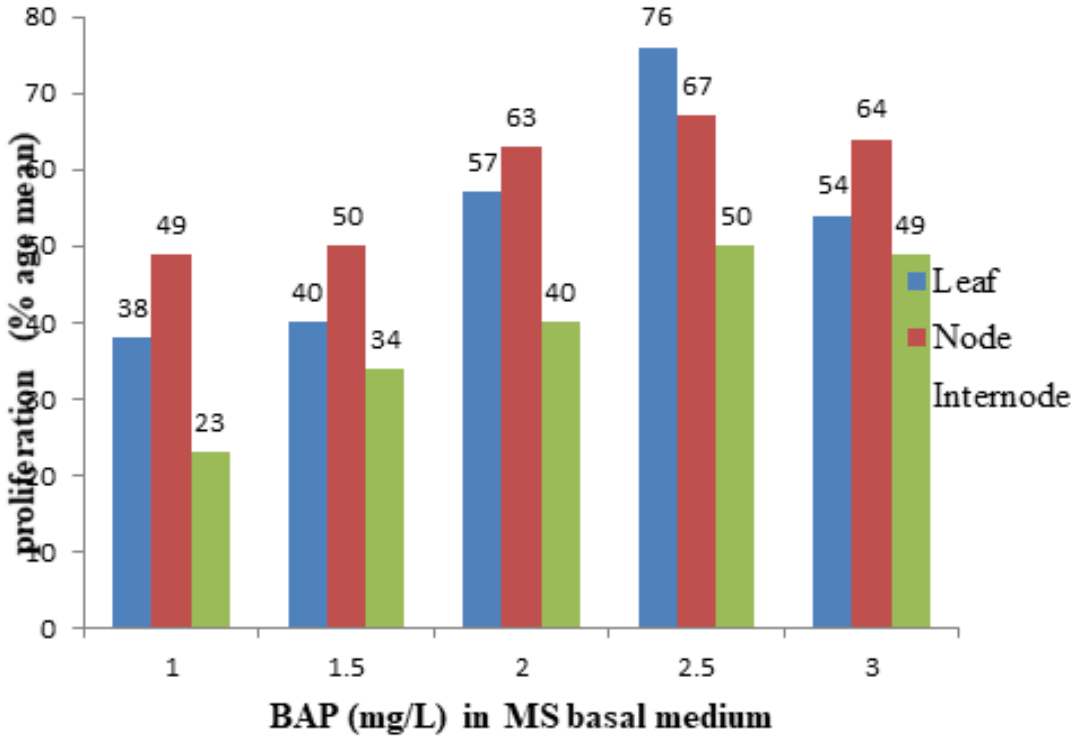

Figure 2: Effect of Different concentrations of BAP in MS basal medium on proliferation of Petunia hybridaVilm. using different explants

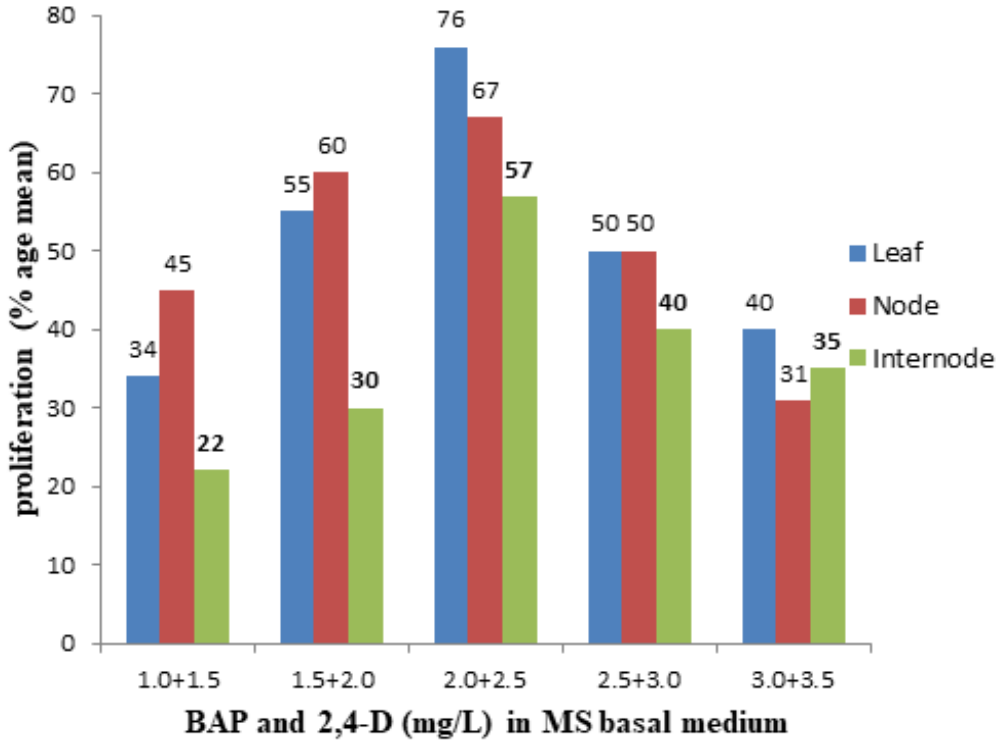

Figure 3: Effect of different concentations of BAP+2,4-D in MS basal medium on proliferation of Petunia hybridaVilm. using different explants

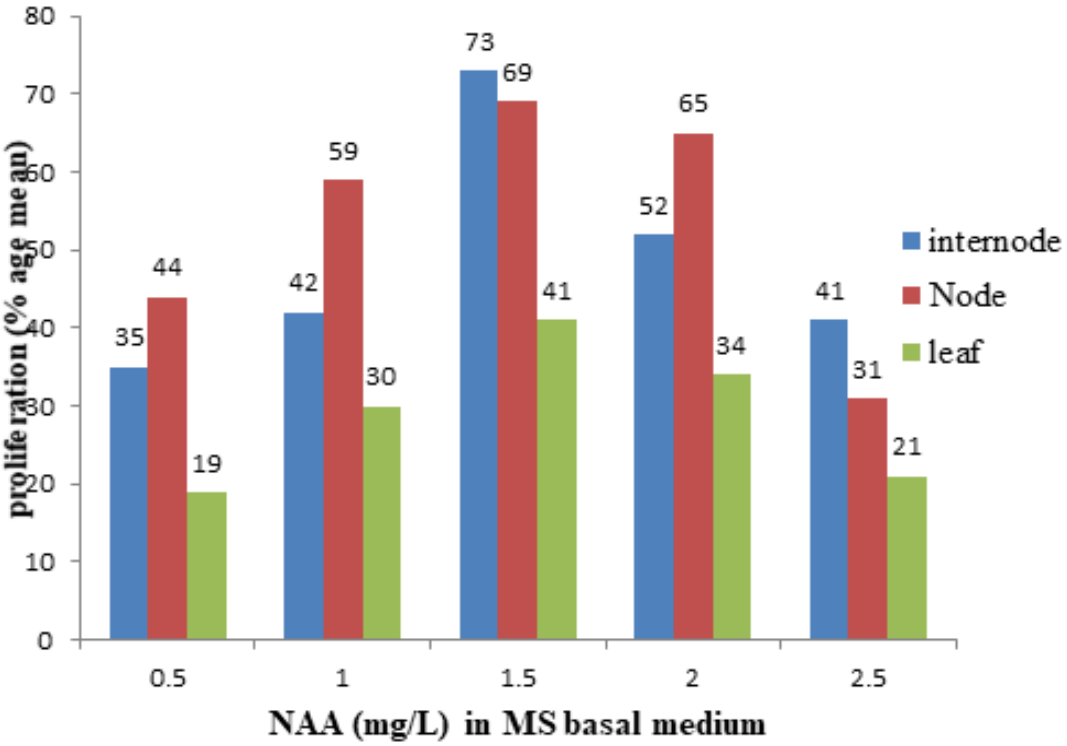

Figure 4: Effect of different concentrations of NAA in MS basal medium on proliferation of Petunia hybridaVilm. using different explants

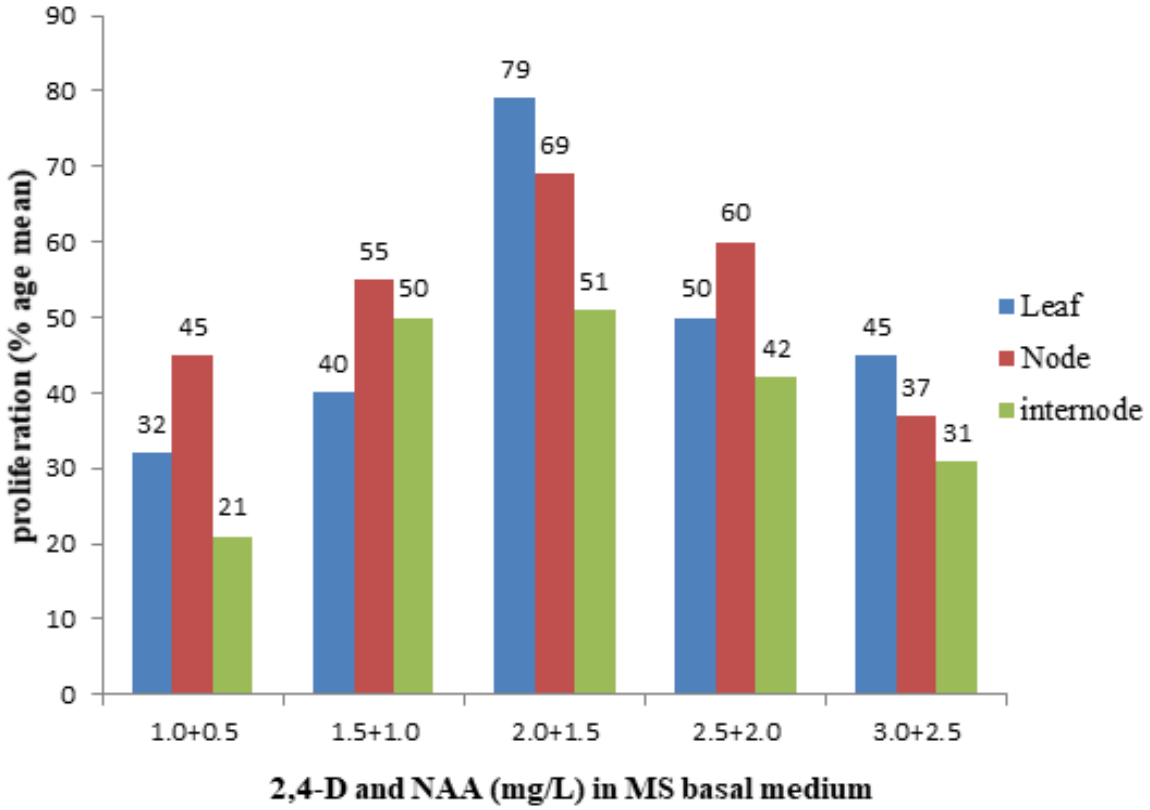

Figure 5: Effect of different concentrations of 2,4-D and NAA in MS basal medium on proliferation of Petunia hybrid Vilm. Using different explants

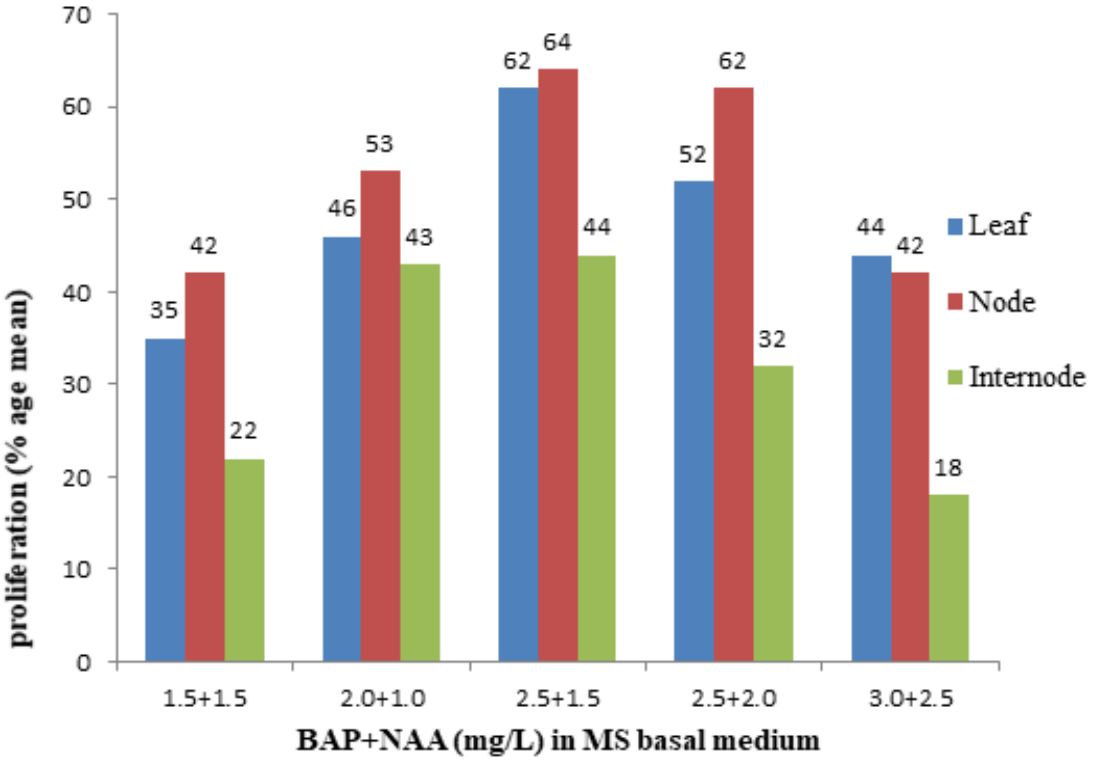

Figure 6: Effect of different concentrations of BAP and NAA on Petunia hybridaVilm. using different explants

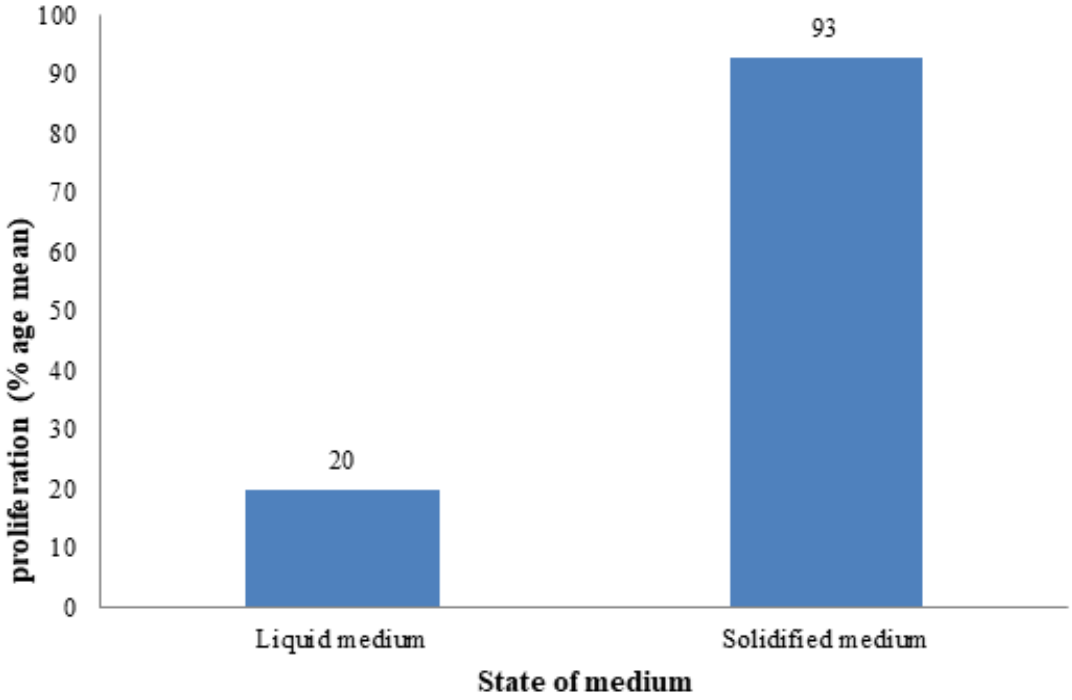

Figure 7: Effect of Liquid and solidified medium with 2.0 mg/L 2,4-D in MS basal medium on proliferation of *Petunia hybrida*Vilm. using leaf as a explants

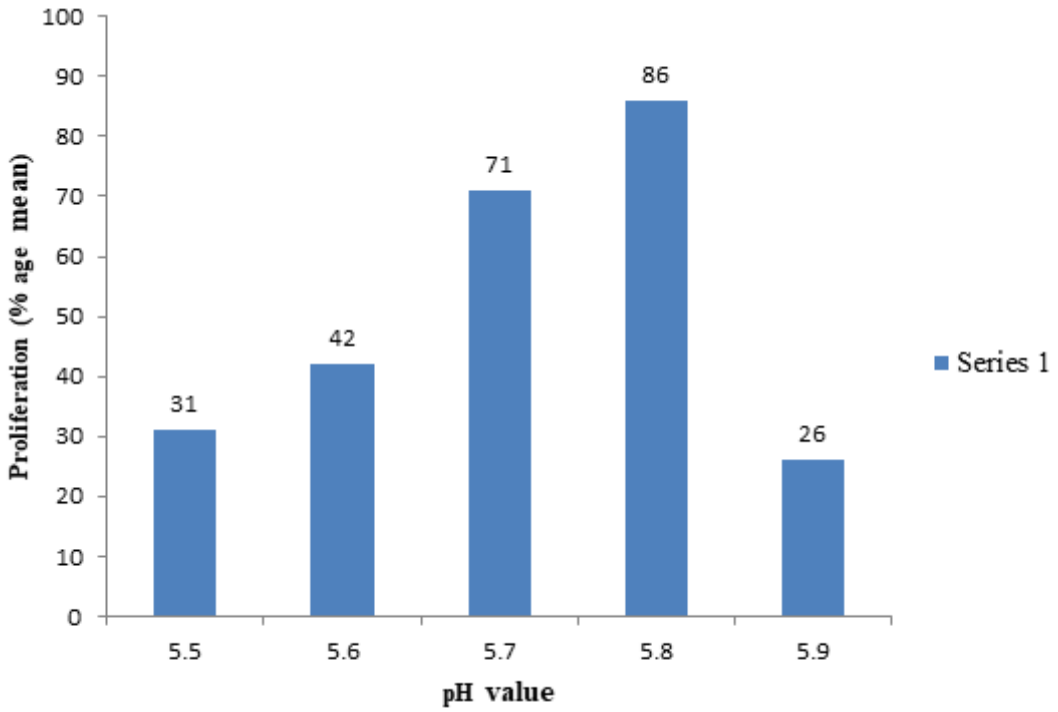

Figure 8: Effect of different pH on proliferation of leaf explants of Petunia hybridaVilm. in MS basal medium using 2,4-D (2.0mg/L)

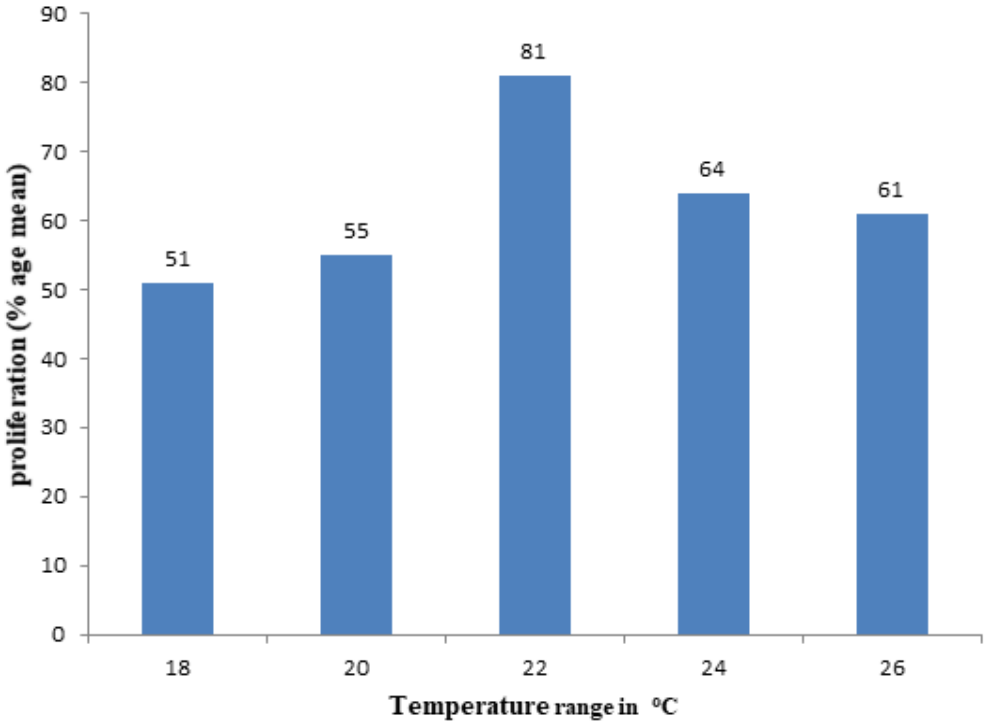

Figure 9: Effect of different temperatures proliferation of leaf explants of Petunia hybridaVilm. in MS basal medium using 2,4-D (2.0mg/L)

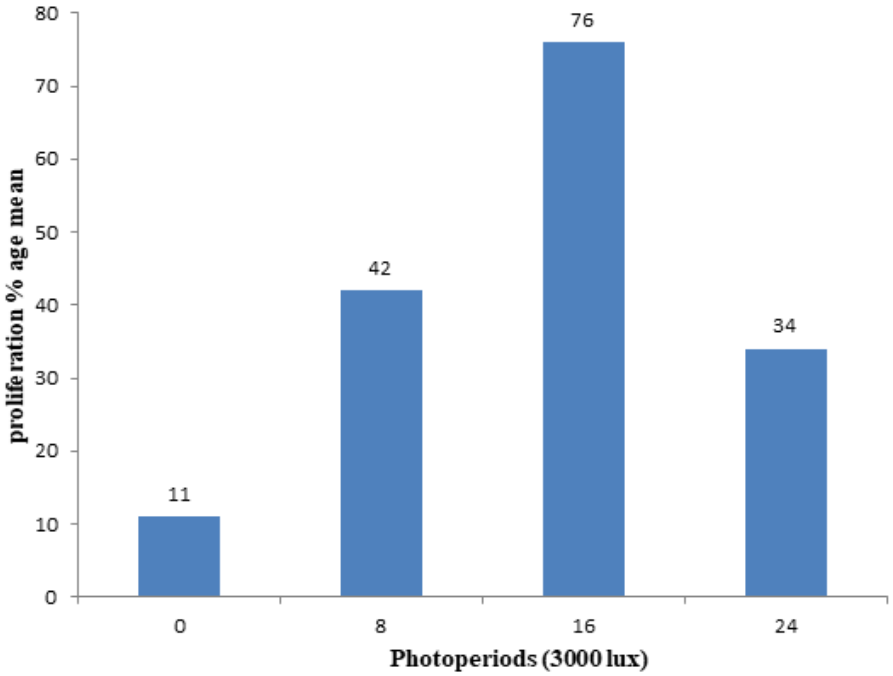

Figure 10: Effect of different photoperiods on proliferation of Petunia hybridaVilm. in MS basal medium using 2,4-D (2.0mg/L).

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