# Proliferation in vitro of *Petunia hybrida* Vilm, a globally significant ornamental plant

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**ABSTRACT**

As part of this endeavor, one of the most visually striking Petunia hybrida is being cultivated during the off-season. Murashige and Skoog Basal Medium (MS Basal Medium) was used for in vitro growth of *P. hybrida*, which was supplemented with several plant growth regulators (PGRs). In this study, we used a variety of explants to examine how different physical and chemical variables affected the in vitro development of *P. hybrida*. Using the leaf as an explant and subjected to controlled environmental conditions (i.e., 92% at 23oC, 5.8 pH, 16 hours photoperiod, and 30g/L sucrose), the auxin 2,4-Dichlorophenoxyacetic acid (2.0 mg/L) was shown to be significant for the somatic embryogenesis of *P. hybrida* Vilm. Utilizing plant tissue culture, it may be feasible to commercially produce the desired *P. hybrida* Vilm strain during non-growing seasons by supplementing leaf, node, and internodal explants with specific combinations of PGRs, such as 2,4-Dichlorophenoxyacetic acid (2.0 mg/L), Benzyl Amino Purine (2.5 mg/L), or Benzyl Amino Purine + 2,4-Dichlorophenoxyacetic acid (2 mg/L + 2.5 mg/L), or Naphthalene Acetic.

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

## 1. INTRODUCTION

The Solanaceae family counts 35 species under the genus *Petunia* (Bombarely et al. 2016). *Petunia* is one of the most adaptable plant families when it comes to tissue culture; it is possible to grow new plants from a variety of plant parts, including protoplasts, seedling tips, stems, leaves, and anthers (Zerche et al. 2016). According to Ahmad et al. (2006), Ahmad et al. (2007), and Kaya and Huyop (2020), the capacity of cells to regenerate into a whole plant is known as totipotency, and it is the fundamental premise behind the idea of tissue culture. Beyond this, it promotes the possibility of a fully perennial character in any particular variety when cultivated as an annual in temperate regions. Plant growth regulators (PGRs) might cause stunted growth, delayed blooming, or unsuitability for sale if applied at the wrong rate or timing (Asgher et al. 2015). Understanding that hormone concentration does not always correspond to the amount of active hormone in the explant is crucial (Seale et al. 2017). Various environmental conditions

influenced the in vitro organogenesis of Petunia plants, as the size, shape, and length of exposure to benzyladenine (BA) all had an effect on the leaf discs (Druege and Franken, 2018). In tissue culture, the ability of *Petunia* leaf explants to generate shoots may be modulated by the addition of exogenous cytokinin and BA (Kaviani and Kazemi, 2017). According to Van der Krol and Immink (2016), tissue culture is a great way to meet the demand for this bedding plant, which is widely utilized for decorative reasons across the globe. In addition, *Petunia* species have been extensively regarded as a model system for a number of reasons, such as their short cycle, simple propagation, transformation, and practical culture conditions. Biochemical studies may benefit from it as well since the phenylpropanoid pathway (PPP) can be readily produced and generated in Petunia species via the control of cell and tissue cultures (Zerche et al. 2016). Subburaj et al. (2016) noted that this plant variety is useful in molecular biology for studying mutations and different phases of plant growth. Various studies have shown interest in *P. hybrid* (El-Hawaz et al., 2019; Farooq et al., 2021; Borovaya et al., 2022). Here, we zeroed down on the specific combinations of Plant Growth Regulator concentrations that cause *P. hybrida* to develop calluses quickly. In order to restore the plantlet, these calli were kept alive from *P. hybrida* leaf explants.

## 2. MATERIALS AND METHODS

A lab at Pakistan's Lahore College for Women University (Department of Botany) called the "Plant Biotechnology and Molecular Genetics Lab" conducted the current study. We bought the seeds from a seed bank in Lahore and nurtured them in containers. These *P. hybrida* plants were cultivated and then harvested for their explants. The experimental technique was split into two phases in the laboratory:

i. Proliferation Establishment for *P. hybrida* Vilm.
ii. Assessment of PGRs that cause *P. hybrida* Vilm. to develop a callus on MS basal medium.

### 2.1. Establishment of Proliferation for *P. hybrida* Vilm.

The research team from Lahore College for Women University grew the leaf explants that were used in this investigation. The plants were cultivated in ordinary soil in order to harvest several explants, including leaves, internodes, and nodes. To limit the risks of contamination, the explants were surface sterilized by washing them with tap water and a few drops of liquid detergent. Then, they were submerged in either ordinary bleach or 5% commercial sodium hypochlorite for 20 minutes. Various Plant Growth Regulators (2, 4-Dichlorophenoxyacetic acid, Benzyl Amino Purine & Naphthalene Acetic Acid) and mixtures of these were introduced into MS basal medium in order to facilitate the Petunia's proliferation. In order to produce the optimal organogenesis of *P. hybrida* explants, both liquid and solidifying nutritional media were

investigated. In order to determine the optimal growth conditions, five distinct ranges of sucrose, temperature, pH, and photoperiods were examined.

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

To determine how three PGRs, either alone or in concentrations of two, affected the callus production of *P. hybrida* cells, MS media was added with these chemicals. Triplicates of each treatment were conducted. The maximum time duration for the proliferation of all the cultured test tubes was 30-35 days, and they were snapped at various days. Statistical analysis was performed on the collected data.

### 2.3. Statistical Analysis:

Statistical analysis was performed on the collected data. Using SPSS software, the means were separated using Duncan's new multiple range test at a 1% level of significance, following the method outlined by Steel et al., (1997) (Leveseqe, 2007).

## 3. RESULTS

The experiment included recording the response of several *P. hybrida* explants, including leaves, internodes, and nodes, on MS media that had been added with various PGRs at varying doses (Figure 1-6). Tables 1–5 show all the physical components utilized in different concentrations, however we're only showing the Dichlorophenoxyacetic acid (2.0 mg/L) here. From the three inoculated cultures per explant, 2,4-Dichlorophenoxyacetic acid (2.0mg/L) had the greatest response, while 2,4-Dichlorophenoxyacetic acid (5.0mg/L) yielded the lowest percentage, at 32%. In the presence of 2,4-Dichlorophenoxyacetic acid (2.0mg/L), node explants exhibited a maximum proliferation rate of 73%, but in the presence of 5.0mg/L, the proliferation rate was 43%. The medium containing 2,4-Dichlorophenoxyacetic acid (2.0mg/L) resulted in the greatest growth rate of 63% for internode explants, while the media containing 5.0mg/L showed the lowest growth rate of 26%. The most effective medium for *P. hybrida* Vilm. proliferation was found to be leaf explant (Plate 1). The leaf explant on MS media grew the most when treated with 2,4-Dichlorophenoxyacetic acid at a concentration of 2 mg/L. The variables that were modified were temperature (23□2□C), sucrose (30%), pH (5.8), and photoperiod (16 hours). Even though the aforementioned physical parameters were maintained, a somewhat lower callus (79% with 1.5 mg/L Naphthalene Acetic Acid and 76% with 2.5 mg/L Benzyl Amino Purine) was generated in MS media employing leaf explants. In addition, when tested individually, Benzyl Amino Purine (2.5 mg/L) resulted in a 76% proliferation rate with leaf explants, Naphthalene Acetic Acid (1.5 mg/L) produced a 73% rate with internodal explants, and when

tested together, the two compounds produced a 64% rate with nodal explants. The current research has the potential to provide light on how to cultivate *P. hybrida* Vilm. from different types of explants. An investigation revealed the following: 2,4-Dichlorophenoxyacetic acid (2mg/L) (Figure 1), Benzyl Amino Purine (2.5mg/L) (Figure 2), Benzyl Amino Purine and Naphthalene Acetic Acid (1.5mg/L) (Figure 3), Benzyl Amino Purine and 2,4-Dichlorophenoxyacetic acid (2mg/L+1.5mg/L) (Figure 5),

## 4. DISCUSSION

In order to optimize physical parameters, this research offers a method for assessing the critical chemical effect on Petunia hybrida Vilm. proliferation. In this work, we used a controlled physical environment to set up in vitro proliferation and record the effects of several PGRs on a synthetic nutritional medium. Callus induction and embryogenesis were seen in stem and leaf cultures of Petunia inflata and Petunia hybrida on MS basal medium supplemented with 2,4-Dichlorophenoxyacetic acid, according to Fujishima et al. (2000), who investigated parameters impacting adventitious bud and root formation. The same species was chosen for in vitro micropropagation because it is efficient, quick, and produces the highest percentage of shoot proliferation (97.90%), the most number of shoots (20.50 explant−1), and the longest shoots (2.70 cm) in a PGR combination of IBA and BAP at a concentration level of 0.5 mg L−1 (Farooq et al., 2021). Borovaya and Boginskaya showed in 2022 that *P. hybrida* regenerated rapidly on hormone-free MS media; the plant also grew quickly, had strong rhizogenesis, and produced 8.77 offspring per 100 plants. El-Hawaz et al. (2019) showed that *P. hybrida* can be kept in a low-temperature environment for 32 weeks without subcultivation in another investigation. Their two experiments showed that increased T0 micro-cutting production and more flexibility in variable storage periods were achieved with low temperature (12 C), low sucrose, and low light level for the longest period of storage. On the other hand, without a cool room, maximum T0 micro-cutting production was achieved with storage at 23°C for 16 weeks, high sucrose, and high light intensity.

Plantlets of *P. inflata* and *P. hybrida* were finally produced by the process of embryo differentiation and callus expansion (Rao et al. 1973). Internodal explants from *P. hybrida* Vilm showed the lowest proliferation rate of 57% and the highest calli percentage of 76% when treated with a different combination of Benzyl Amino Purine and 2,4-Dichlorophenoxyacetic acid (2.5 mg/L + 2 mg/L) in MS basal medium (figure 3). The optimal in vitro conditions for *P. hybrida* were determined by Li et al. (2013) to be 8p-KM medium supplemented with several PGRs; specifically, glucose (0.4M) and mannitol (0.1M), 2, 4-D (0.3 mg/L), and Benzyl Amino Purine (0.3 mg/L). The current study additionally examined the impact of a combination of Benzyl Amino Purine and Naphthalene Acetic Acid on the in vitro development of various explants

(figure 6). The nodal explants cultured on the MS basal medium supplemented with Benzyl Amino Purine and Naphthalene Acetic Acid (2.5 mg/L+0.5 mg/L) had the highest proliferation rate at 64%, while the internodal explants cultured on the same medium had the lowest rate at 44%. Kaviani and Kazemi (2017) demonstrated that this combination worked best with varying 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 the same MS basal medium. While investigating the impact of four distinct BA concentrations and two distinct Naphthalene Acetic Acid concentrations on the proliferation rate of *P. hybrida* Vilm, Abu-Qaoud et al. (2010) also found the same combinations. When he added BA and Naphthalene Acetic Acid to MS basal medium (0.4 mg/L + 0.1 mg/L), he saw the most shoots of *P. hybrida*. Figures 1 (2,4, D), 2 (BAP), and 4 (NAA) demonstrate the results of our experiments with different combinations of PGRs. We also tested individual PGRs at different dosages. This research discovered that PGRs at concentrations of 1-2 mg/L significantly impacted the growth of *P. hybridra* calli. In vitro growth from diverse explants has been successfully achieved by a small group of plant biotechnologists using various combinations of plant growth regulators at doses that are considered sufficient for *P. hybrida*. According to Liskova et al. (2016), BA media was used for the long-term culturing of the *P. hybrida* callus. After being moved to shoot induction medium that included a small amount of plant growth regulators, plant regeneration could be effectively achieved (Gupta et al. 2017). In a 2007 study by Guo-gui et al., the offered culture method successfully produced somatic variations of *P. hybrida*. According to the literature, the best physical and chemical conditions for inducing callus in *P. hybrida* Vilm within 30 days include Benzyl Amino Purine and Naphthalene Acetic Acid. A variety of quantities of Naphthalene Acetic Acid (0.0, 0.2, 0.5 mg/L) and Benzyl Amino Purine (0.0, 0.1, 0.5, 1 mg/L) were introduced to modified MS medium (MS salts, B5 vitamins) when shoot apex was used as an explant. The medium containing Benzyl Amino Purine and Naphthalene Acetic Acid had the maximum callus induction % at 0.5 mg/L. Using cytokinin without Naphthalene Acetic Acid resulted in a considerable decrease in callus induction, as seen in a study by Natalija et al. (2015).

Regarding the physical parameters that were considered in this study, the optimal concentration of sucrose for the growth of *P. hybrida* Vilm in MS basal medium was 30g/L. The most optimal conditions for maximal proliferation, measuring 92%, were determined to be a temperature of 23±2□C, a photoperiod of 16 hours (2000-3000 Lux), and a pH of 5.8 when utilizing leaf explants in MS baseline medium supplemented with 2,4-Dichlorophenoxyacetic acid (2mg/L) (figure 7-9). Figure 9 from Erwin et al. (1997) shows that during the first six weeks following germination, the ideal temperature was 20 to 24°C and the ideal photoperiod was around 8 to 9 hours. According to Irwin (2002), *P. hybrida* Vilm thrives at a pH range of 5.5-6.2. The most effective culture medium for the successful proliferation of *P. hybrida* Vilm. under controlled environmental conditions, including 92% sucrose and 10% difcobacto agar, was the

MS basal medium supplemented with 2,4-Dichlorophenoxyacetic acid (2 mg/L). The experiment was conducted under a 16-hour photoperiod at 23oC.

Using a leaf explant, the current research found that 2,4-Dichlorophenoxyacetic acid (2mg/L) is necessary for the effective proliferation of *P. hybrida* Vilm on 92% of MS basal medium. Regenerating *P. hybrida* Vilm also requires this action.

## 5. CONCLUSION

In a lab setting, the *Petunia hybrida* Vilm can regenerate itself synthetically. At a certain concentration, every one of the PGRs that were tested—solo or in conjunction with another—showed the capacity to initiate callus formation. The method was most effective, however, when used to an MS medium with a concentration of 2 mg/L of 4-dichlorophenoxyacetic acid.

## 6. ACKNOWLEDGMENTS

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

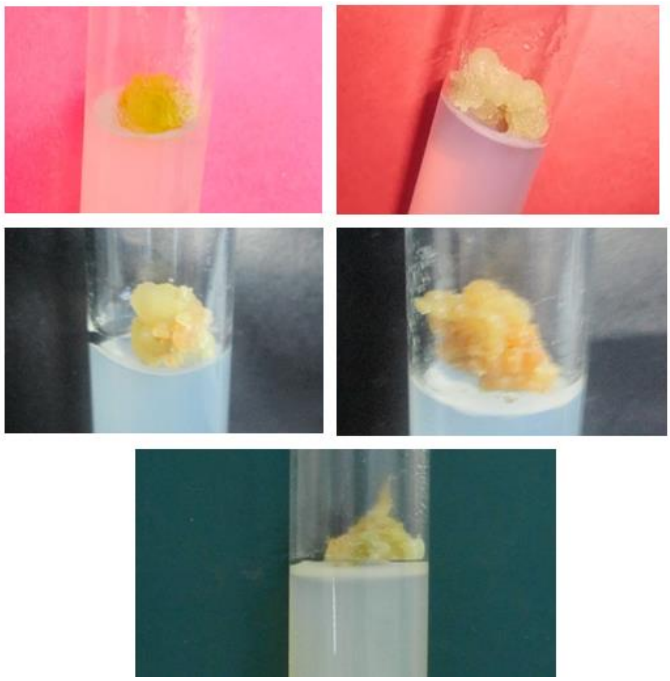


**Plate 1: Proliferation from leaf explant of *Petunia hybrida* Vilm. on MS medium supplemented with 2, 4-D (2mg/L) up to 4 weeks of inoculation.**

**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 rate (%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] | |
| 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 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±0.50[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 No. | Explant Used | PGRs used (mg/l) | Proliferation (%Mean) | 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 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.**

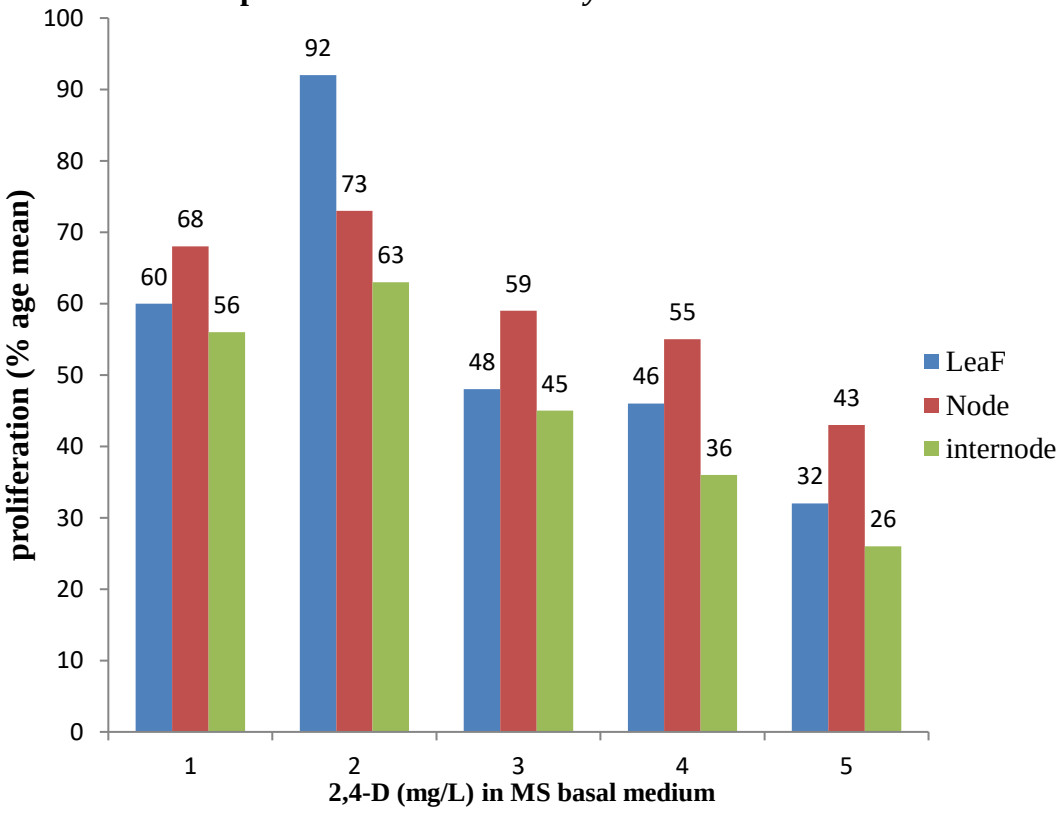


**Figure 2. Effect of Different concentrations of BAP in MS basal medium on proliferation of Petunia hybridaVilm. using different explants**

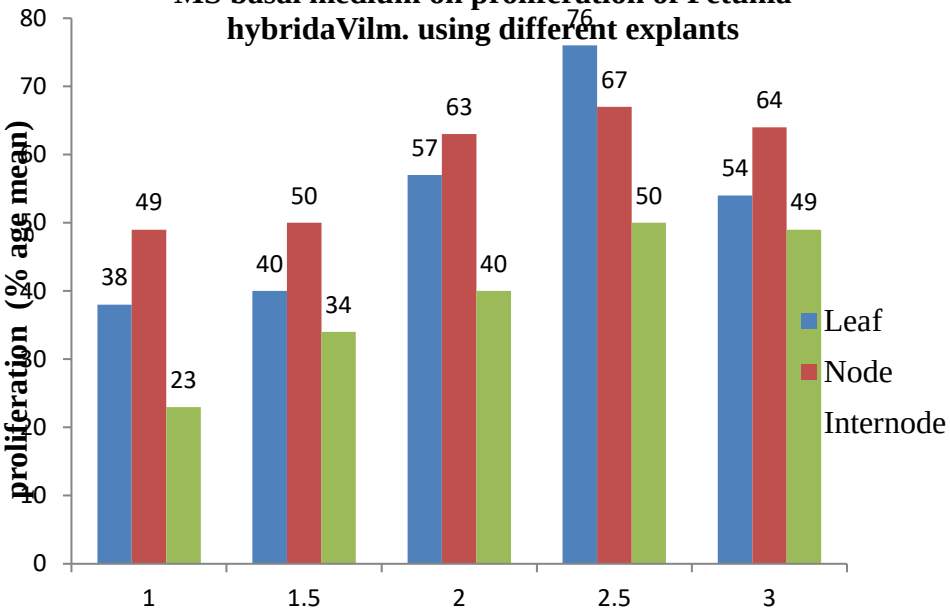



**Figure 3. Effect of different concentations of BAP+2,4-D in MS basal medium on proliferation of Petunia hybridaVilm. using different explants**

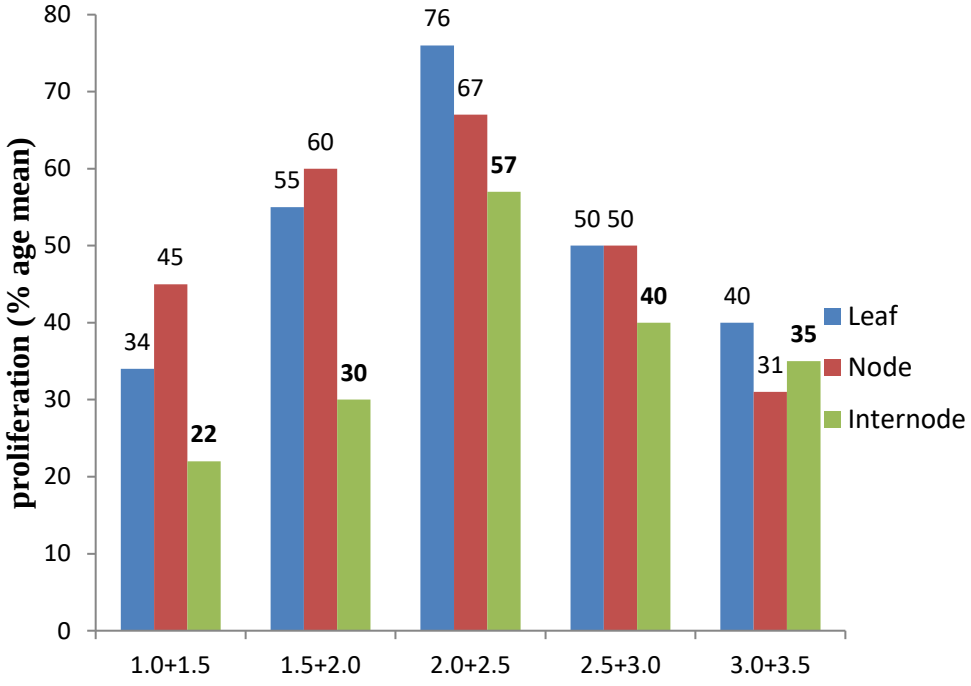


**Figure 4. Effect of different concentrations of NAA in MS basal medium on proliferation of Petunia hybridaVilm. using different explants.**

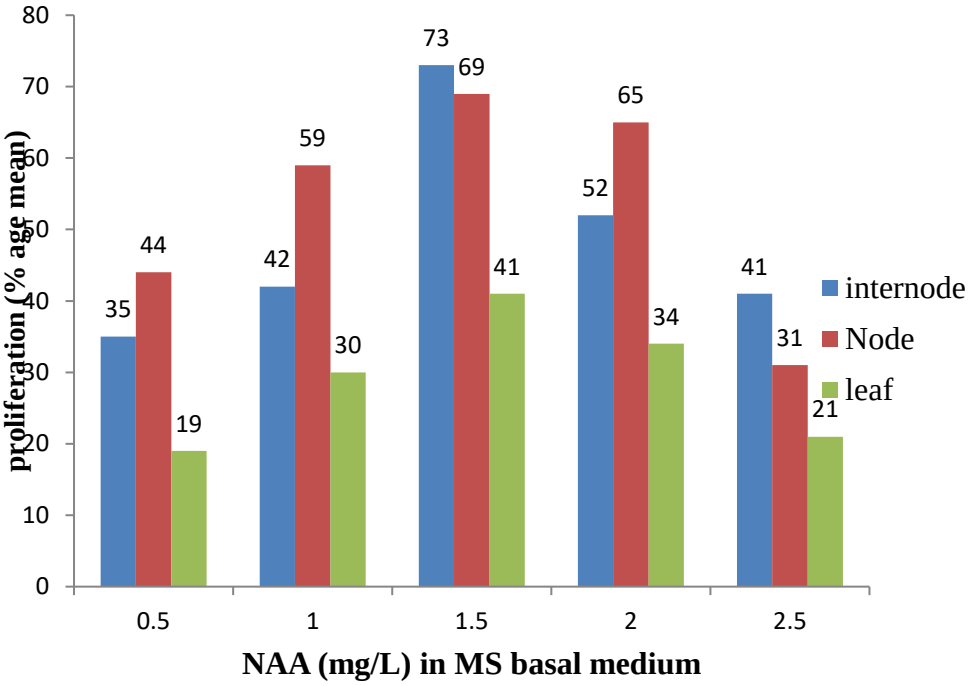



**Figure 5. Effect of different concentrations of 2,4-D and NAA in MS basal medium on proliferation of Petunia hybrid Vilm. Using**

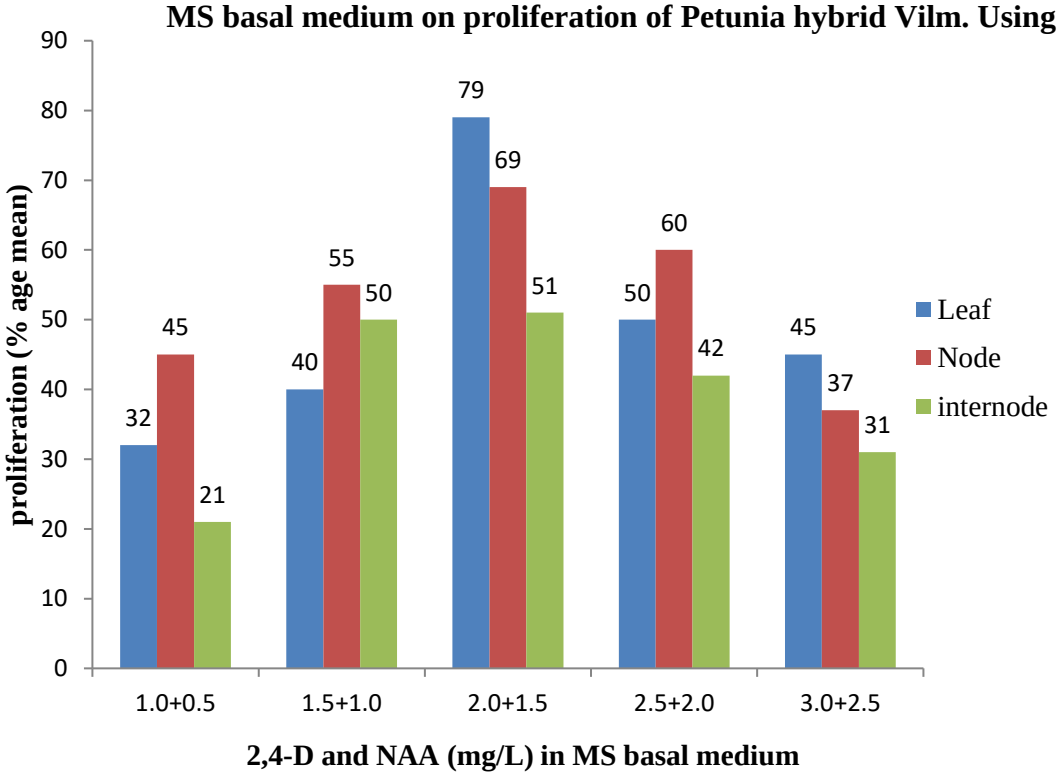


**Figure 6. Effect of different concentrations of BAP and NAA on Petunia hybridaVilm. using different explants.**

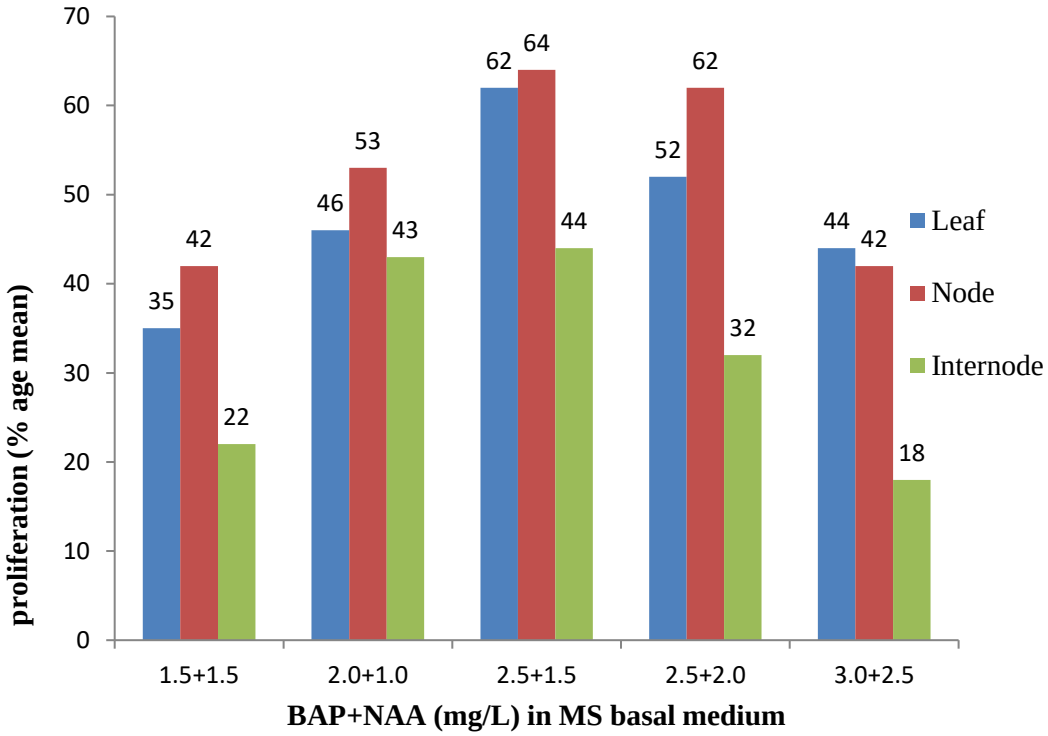
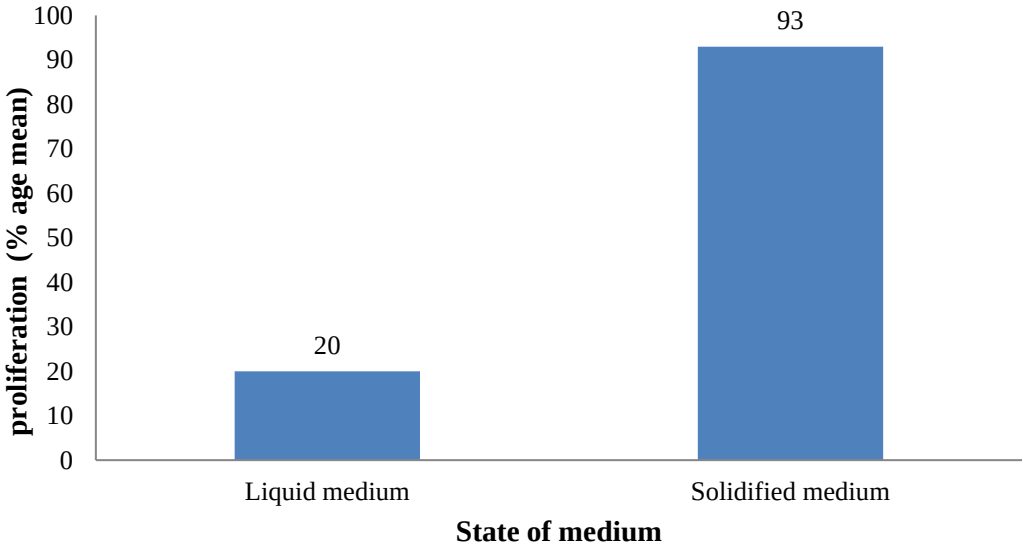



**Figure 7. Effect of Liquid and solidified medium with 2.0 mg/L 2,4-D in MS basal medium on proliferation of *Petunia hybrida*Vilm. using leaf as a explants.**



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

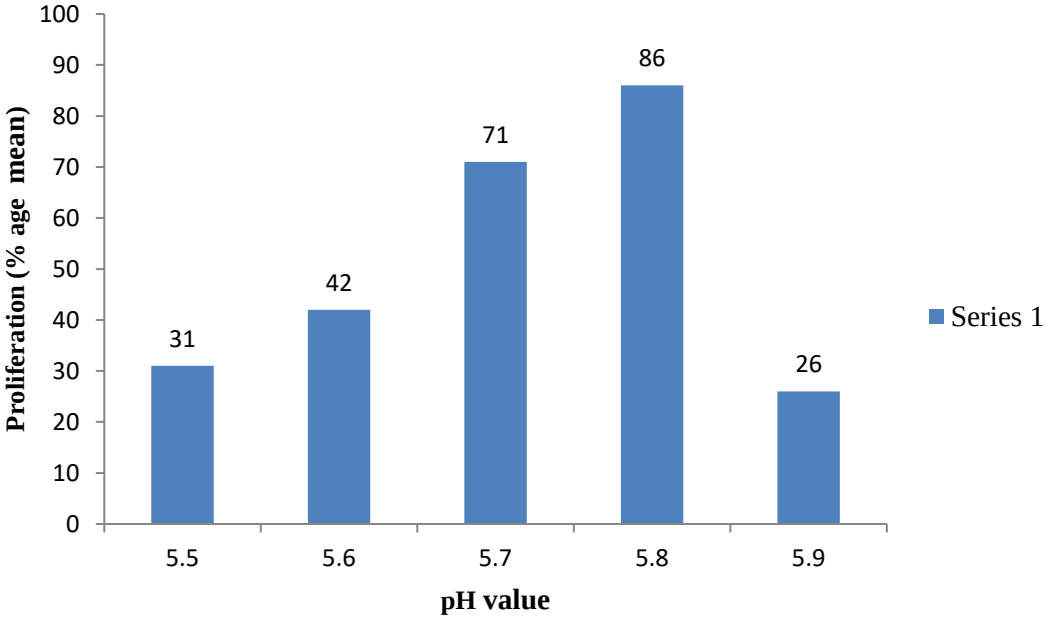



**Figure 9. Effect of different temperatures proliferation of leaf explants of Petunia hybridaVilm. in MS basal medium using 2,4-D (2.0mg/L)**

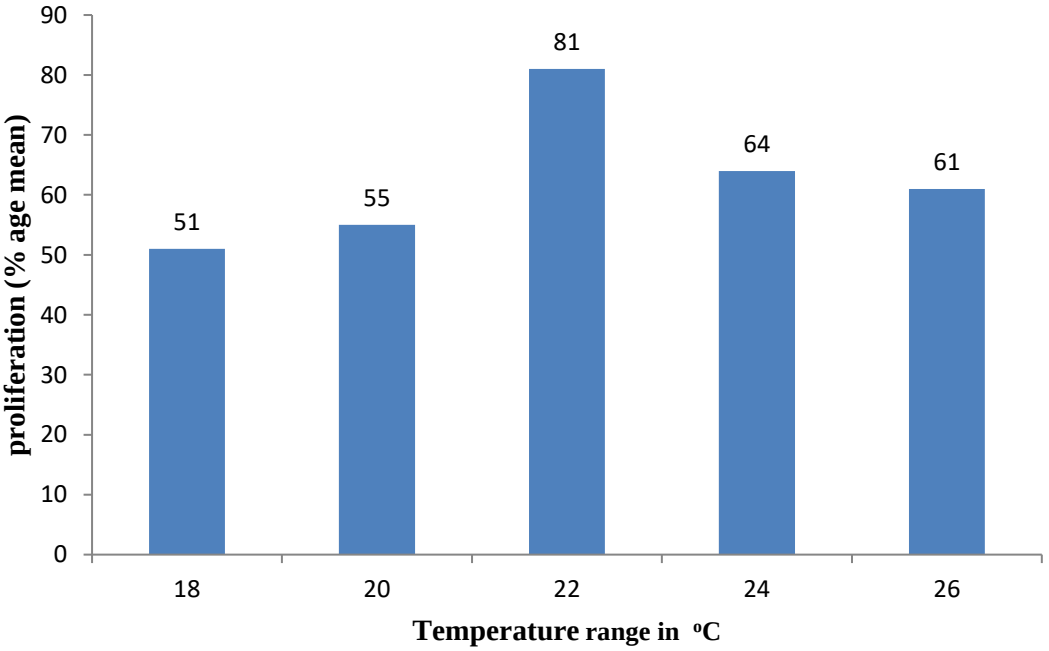
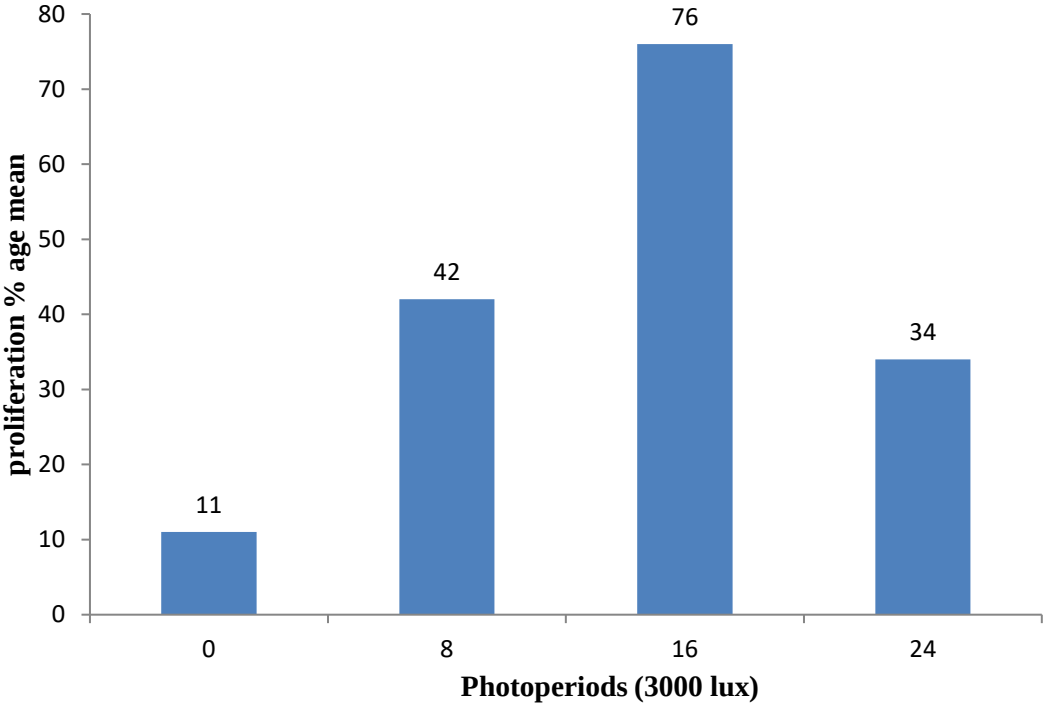


**Figure 10. Effect of different photoperiods on proliferation of Petunia hybrida*Vilm*. in MS basal medium using 2,4-D (2.0mg/L).**

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## CONFLICT OF INTEREST

Authors have no conflict of interest.

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