Optimization of an improved, efficient and rapid in vitro micropropagation protocol for *Petunia hybrida* Vilm. Cv. “Bravo”

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**A B S T R A C T**

An efficient protocol for in-vitro propagation of an important ornamental crop, *Petunia hybrida* Vilm. Cv. “Bravo” was developed. The explants that were used to carry out the experiment were Leaf segments, nodal segments and shoot tips. Nodal segments recorded highest per cent asepsis followed by shoot tips and leaf segments. Asepsis was found to be highest when the explants were sterilized with Fungicide (Carbendazim) 0.02% for the duration of 30 min followed by 0.1% HgCl2 for duration of 10 min and then ethanol 70% for 10 s. Longer duration of the sterilant treatment showed more necrotic effects on the explants, thus mercuric chloride treatment when given for 5 min proved to be more effective in terms of survival of the explants. Maximum establishment per cent was recorded in Murashige and Skoog (MS) media fortified with BAP (1.5 mg L⁻¹) and IBA (0.5 mg L⁻¹) in shoot tips and nodal segments, i.e. 97.90 and 95.74% respectively. Callus was efficiently induced and developed when PGR amalgamation of BAP (0.1 mg L⁻¹) and 2,4-D (1.5mg L⁻¹) was used. Kinetin at the concentration of 2.0 mg L⁻¹ along with IBA at 0.5mg L⁻¹ recorded highest callus regeneration in both leaf and internodal segment derived callus. Maximum proliferation percent of shoots (97.90%), 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. Rhizogenesis was recorded to be highest in the MS media containing IBA 1.00 mg L⁻¹. Best hardening media which recorded maximum survival per cent 92.50% was noticed on the media formulation comprised of equal ratio of perlite and vermiculite mix, under poly house conditions.

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**1. Introduction**

*Petunia hybrida* Vilm. is an annual or perennial plant that belongs to the family solanaceae. Petunias have a great profusion of bloom under all conditions which makes them useful and popular aesthetically as well as commercially. It is a decorative plant, grown for its beautiful flowers in beddings, borders, hanging baskets, window boxes, pots and containers. In warm climates petunias are perennials but are used as annuals in temperate zones (Bailey, 1976; Armitage, 1985). Besides, petunias having a significant importance as ornamental crop, these have also been known as one of the most excellent model crops for studies of gene regulation and genome structure, since the system combines innumerable and excellent technical features with a broad range of research possibilities (Singh, 2014). Hybrid petunia is mainly cultivated through seeds and the vigour and quality can be seen degrading in the further generations because of the segregation that takes place. In order to maintain the F1 progeny for further multiplication and to maintain the vigour of particular cultivar, micropropagation plays an important role. Micropropagation refers to the culture of tissues of the selected plants that are grown in an
The present study was carried out with the aim of optimization of growth regulator regimes for in-vitro propagation of Petunia hybrida and ex-vitro standardization of hardening of in-vitro propagated rooted plantlets.

2. Material and methods

2.1. Preparation and sterilization of plant material

The study was carried out at Plant Tissue Culture Laboratory of the Division of Floriculture and Landscape Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar. Actively growing shoots (7–10 cm) of petunia cv. ‘Bravo’ (Fig. 1) were collected from plants grown in polyhouse conditions. The adhering dirt and dust were washed off under running tap water. Younger leaves in shoot tips were retained, as the outer mature leaves were removed with a sharp scalpel. In case of nodal segments, both top and basal re-cuts were given and the explants were reduced to the manageable size of 1.0, 2.0 and 3.0 cm, and leaf explants were prepared as the discs of 1 cm² size. Later the explants were strenuously quivered in Tween-20 surfactant fortified with different concentrations of the fungicide- Carbendazim for different time durations which were later washed off with tap water followed by washing distilled water. Mercuric chloride (HgCl₂) at the concentration of 0.1% was then used to treat the explants for different time durations and later ethanol treatment for 10 s was given under the laminar flow hood. Per cent asepsis of the explants was recorded after two and four weeks of culture and per cent survival was recorded after four weeks of culture.

2.2. Culture media and culture conditions

The nutrient media employed for the development of propagation protocol of Petunia hybrida was MS (Murashige and Skoog, 1962) containing the macro elements, microelements, and vitamins. Sucrose (3%), myo-inositol and Plant Growth Regulators (PGR’s) were put in to the media as per the requirement for each step. Media was maintained at 5.7pH and solidified with agar agar. The test-tubes and flasks containing the prepared culture media were autoclaved for 15–20 min at 121 °C and 15 psi. The cultures were later incubated under controlled conditions in a culture room with 24 ± 1 °C temperature and a 16:8 h, light: dark system. Shoot tip, nodal segment and leaf segment were used as explants for standardization of establishment experiments. Leaf explants failed to regenerate adventitious shoots in exploratory investigations and hence were excluded from the establishment experiments. Explants after the surface sterilization were inoculated on the media carrying different PGR combinations of auxin, IBA (Indole-3-butyric acid) and cytokinin, BAP (6-Benzylamino Purine) for establishment. Callus induction was observed on leaf segments and internodal segments placed on basal MS media consisting of different combinations of BAP, 2,4-D (2,4- dichlorophenoxyacetic acid) and NAA (Naphthalene acetic acid). The percentage of callus induction, callus fresh weight (g explant⁻¹) and type of callus were the parameters documented after 4 weeks of incubation. Callus sections having uniform size and age, originating from leaves and internodal segments were placed on regeneration media boosted with various combinations of cytokinins (BAP and Kinetin) and auxins (NAA and IBA). Percentage of the callus developing shoots and shoot number per callus piecewere then observed. Shoot proliferation was noticed as good number of axillary shoots was induced and multiplied in-vitro from establishment and callus regeneration cultures. Uniform sized microshoots were divided from the shoot clumps and later inoculated on proliferation media containing different PGR combinations of IBA and BAP. Per cent proliferation, shoot length (cm) and shoot number per explant were observed after 6 weeks of inoculation. Rhizogenesis of microshoots was standardized on rhizogenesis media containing various levels of auxins (IBA and NAA). Uniform lateral microshoots were separated from the proliferated clumps and transferred to the media. Rhizogenesis percentage and roots per shoot were studied during the course.

Fig. 1. Petunia cultivar (“Bravo”) selected for the investigation.
Influence of sterilant treatments and time duration on asceptic culture and survival of explants of *Petunia hybrida* Vilm. cv. "Bravo" Figures in the parentheses are the statistically transformed (arc sin and square root) values of percentage data.

### Sterilant Treatments

| Sterilant Treatments | Explant                      | Shoot Tip | Nodal Segment | Leaf                          |
|----------------------|------------------------------|-----------|---------------|--------------------------------|
|                      | Asceptis %                   | Survival %| Asceptis %     | Survival %                    |
|                      | 2 weeks (T1) | 4 weeks (T2) | 2 weeks (T1) | 4 weeks (T2) | 2 weeks (T1) | 4 weeks (T2) | 2 weeks (T1) | 4 weeks (T2) |
| S1                   | HgCl₂ 0.1% for 5 min         | 45.83     | 33.33         | 54.16 | 41.66 | 47.91 | 43.78 | 54.16 | 47.91 | 56.24 | 45.83 | 49.99 | 39.58 |
| S2                   | HgCl₂ 0.1% for 10 min        | 85.41     | 77.08         | 47.91 | 39.58 | 83.33 | 77.08 | 52.08 | 41.66 | 81.24 | 77.08 | 45.83 | 39.58 |
| S3                   | HgCl₂ 0.1% for 5 min + ethyl alcohol 70% for 10 s | 47.91 | 41.66 | 79.16 | 64.58 | 54.16 | 45.83 | 74.99 | 64.58 | 62.58 | 49.99 | 72.91 | 62.58 |
| S4                   | Carbendazim 0.01% for 30 min + S₅ | 49.99 | 41.66 | 91.66 | 83.33 | 60.41 | 47.91 | 93.74 | 85.41 | 64.58 | 56.24 | 89.57 | 79.16 |
| S5                   | Carbendazim 0.02% for 30 min + S₅ | 54.16 | 47.91 | 93.74 | 85.41 | 62.49 | 50.08 | 97.90 | 87.49 | 68.74 | 62.57 | 93.74 | 81.33 |
| S6                   | Carbendazim 0.01% for 30 min + HgCl₂ 0.1% for 10 min + ethyl alcohol 70% for 10 s | 89.57 | 85.41 | 41.66 | 33.33 | 87.49 | 79.16 | 43.74 | 37.49 | 91.66 | 83.33 | 37.49 | 29.16 |
| S7                   | Carbendazim 0.02% for 30 min + HgCl₂ 0.1% for 10 min + ethyl alcohol 70% for 10 s | 93.74 | 89.58 | 39.57 | 31.24 | 95.82 | 89.57 | 41.91 | 31.24 | 91.66 | 87.49 | 35.41 | 29.16 |
| C. Dₜₓₜₛ (0.05) Sterilant (%) | Time (T) | 3.39 | 0.49 | 0.24 | 0.39 | 0.21 | 5.65 | 3.02 | 5.49 | 5.01 | 2.67 |
| TxS                  | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |

### 2.3. Acclimatization

Six formulations of different types of growing media viz., sand, perlite and vermiculite were engaged for the standardization of hardening procedure of the rooted plantlets. The components for particular media formulation were mixed in oven dried containers and then put in cloth bags enveloped in aluminum foil sheets, autoclaved for an hour. Rooted plantlets of petunia obtained were shifted to polypropylene containers for hardening purpose under laminar air flow chamber. Another polypropylene glass was inverted over each container containing hardening media and the rims of both containers were sealed with para film strip. These hardening containers kept in the culture chamber with optimum light duration and intensity. After some time, a sign of establishment was observed as new leaves appeared from the survived plantlets. Small holes were made in inverted glass after 10 days of transfer which were later removed. Observations on number of days taken for establishment and *ex-vitro* survival % of rooted plantlets after 4 weeks were recorded during this transition phase.

### 2.4. Statistical analysis

The data compiled during the current study for different parameters was statistically analyzed under completely randomized design (CRD) with four replications.

### 3. Results

#### 3.1. Culture asepsis and explant establishment

Highest culture asepsis was achieved after 2 weeks of incubation as compared to 4-week duration. The combination of HgCl₂(0.1%) for 10 min and carbendazim (0.02 and 0.01%) for 30 min followed with 70% ethanol gave the best results in culture sterilization in all the three explants i.e., 89.58%, 89.57% and 83.33% for shoot tip, nodal segment and leaf explants respectively (P < 0.05). However, 10 min HgCl₂ dip was proved to produce more asceptic cultures than a 5-minute treatment (Table 1). Nodal segment explant observed significantly higher rate of survival (87.49%) than shoot tip (85.41%) and leaf explant (81.33%). Significantly higher survival was observed in treatments that involved the 5-minute dip in 0.1% HgCl₂ as compared to 10 min dip (Table 1).

Twelve growth regulator combinations were used for standardization of explant establishment in petunia involving IBA at 0.1, 0.2, 0.3 and 0.5 mg L⁻¹ and BAP at 0.5, 1.0 and 1.5 mg L⁻¹ (Table 2). In shoot tips and nodal segments, highest establishment per cent i.e., 95.82% and 89.57% in shoot tips and nodal segments respectively was observed with IBA at 0.5 mg L⁻¹ when combined with BAP at 1.5 mg L⁻¹, followed by IBA at 0.5 mg L⁻¹ in combination with BAP at 1.0 mg L⁻¹ (Fig. 2).

#### 3.2. Callusing, regeneration and organogenesis

Several hormonal combinations were used for callus induction of leaf and internodal segment explants of petunia comprising of (Table 2). Influence of growth regulator combinations on per cent culture establishment in *Petunia hybrida* Vilm. cv. “Bravo”.

| Treatments (MS + PGRs) | Shoot tips | Nodal segments |
|------------------------|------------|----------------|
| IBA (0.1 mg L⁻¹) + BAP (0.5 mg L⁻¹) | 45.83 (6.83) | 31.24 (5.66) |
| IBA (0.1 mg L⁻¹) + BAP (1.0 mg L⁻¹) | 52.08 (7.28) | 43.74 (6.66) |
| IBA (0.1 mg L⁻¹) + BAP (1.5 mg L⁻¹) | 70.83 (8.47) | 64.58 (8.08) |
| IBA (0.2 mg L⁻¹) + BAP (0.5 mg L⁻¹) | 43.74 (6.66) | 37.49 (6.19) |
| IBA (0.2 mg L⁻¹) + BAP (1.0 mg L⁻¹) | 62.49 (7.96) | 58.33 (7.69) |
| IBA (0.2 mg L⁻¹) + BAP (1.5 mg L⁻¹) | 81.24 (9.06) | 72.91 (8.58) |
| IBA (0.3 mg L⁻¹) + BAP (0.5 mg L⁻¹) | 77.08 (8.83) | 77.08 (8.83) |
| IBA (0.3 mg L⁻¹) + BAP (1.0 mg L⁻¹) | 85.41 (9.29) | 79.16 (8.95) |
| IBA (0.3 mg L⁻¹) + BAP (1.5 mg L⁻¹) | 87.49 (9.40) | 83.33 (9.18) |
| IBA (0.5 mg L⁻¹) + BAP (0.5 mg L⁻¹) | 83.33 (9.17) | 79.16 (8.95) |
| IBA (0.5 mg L⁻¹) + BAP (1.0 mg L⁻¹) | 91.66 (9.62) | 87.49 (9.40) |
| IBA (0.5 mg L⁻¹) + BAP (1.5 mg L⁻¹) | 95.82 (9.83) | 89.57 (9.51) |

Figures in the parentheses are square root transformed values of percentage data.
Table 3
Influence of growth regulators on callusing in leaf and internodal segment explants.

| Treatments (MS + PGRs) | Leaf | | Internodl Segment | |
|------------------------|------|------|------------------|------|
|                        | Callus induction (%) | Callus fresh weight (g explant^-1) | Callus type | Callus induction (%) | Callus fresh weight (g explant^-1) | Callus type |
| BAP (0.5 mg L^-1) + NAA (0.5 mg L^-1) | 77.08 (8.82) 1.39 | Compact, yellowish green | 60.41 (7.82) 1.47 | Loose, creamy green |
| BAP (0.5 mg L^-1) + NAA (1.0 mg L^-1) | 72.91 (8.59) 1.28 | Compact, yellowish green | 54.16 (7.42) 1.31 | Loose, creamy green |
| BAP (0.5 mg L^-1) + NAA (1.5 mg L^-1) | 56.24 (7.56) 0.90 | Compact, green | 41.66 (6.51) 1.11 | Loose, creamy green |
| BAP (0.5 mg L^-1) + 2,4-D (0.5 mg L^-1) | 68.74 (8.34) 0.98 | Compact, creamish green | 45.83 (6.83) 1.04 | Compact, cream green |
| BAP (0.5 mg L^-1) + 2,4-D (1.0 mg L^-1) | 74.99 (8.71) 1.32 | Compact, creamish green | 64.57 (8.09) 1.36 | Compact, creamy green |
| BAP (0.5 mg L^-1) + 2,4-D (1.5 mg L^-1) | 79.16 (8.95) 1.44 | Compact, green | 66.66 (8.21) 1.54 | Compact, creamy green |
| BAP (1.0 mg L^-1) + NAA (0.5 mg L^-1) | 83.33 (9.17) 1.58 | Compact, yellowish green | 72.91 (8.59) 1.75 | Compact, green |
| BAP (1.0 mg L^-1) + NAA (1.0 mg L^-1) | 85.41 (9.29) 1.78 | Compact, green | 77.08 (8.83) 1.82 | Compact, green |
| BAP (1.0 mg L^-1) + NAA (1.5 mg L^-1) | 81.24 (9.06) 1.50 | Compact, green | 68.74 (8.34) 1.67 | Compact, green |
| BAP (1.0 mg L^-1) + 2,4-D (0.5 mg L^-1) | 83.33 (9.17) 1.56 | Compact, creamish green | 74.99 (8.71) 1.73 | Compact, creamy green |
| BAP (1.0 mg L^-1) + 2,4-D (1.0 mg L^-1) | 91.66 (9.62) 1.88 | Compact, brownish green | 87.49 (9.40) 2.05 | Compact, creamy green |
| BAP (1.0 mg L^-1) + 2,4-D (1.5 mg L^-1) | 95.82 (9.83) 1.90 | Loose, brownish green | 91.66 (9.62) 2.11 | Compact, green |
| C.D(P0.05) | 0.48 | 0.16 | 0.54 | 0.16 |

Figures in the parentheses are square root transformed value of the percentage data.
* Data recorded after 4 weeks of culture.
** Callus weight recorded after 6 weeks of culture.

Fig. 3. Callusing, (a): Callusing from leaf explants in MS media, BAP + 2,4-D : 1.0 + 1.5 mg L^-1; (b): Callusing from leaf explants in MS media, BAP + 2,4-D : 1.0 + 1.0 mg L^-1; (c): Callusing from internodal segment explants in MS media, BAP + 2,4-D : 1.0 + 1.5 mg L^-1; (d): Callusing from internodal segment explants in MS media, BAP + 2,4-D : 1.0 + 1.0 mg L^-1.
auxins (NAA and 2, 4-D each at the concentrations of 0.5, 1.00 and 1.50 mg L\(^{-1}\)) and cytokinin’s (BAP at the concentration of 0.50 or 1.00 mg L\(^{-1}\)) (Table 3). Maximum induction of callus (85.41% in leaf explants and 77.08% in internodal segment explants) and maximum weight of the callus (1.78 g explant\(^{-1}\) in leaf and 1.82 g explant\(^{-1}\) in internodal segment were recorded on MS media containing higher concentrations of 2, 4-D in combination of BAP at 1.50 mg L\(^{-1}\) (Fig. 3). NAA (0.50 mg L\(^{-1}\)) based treatments significantly regenerated high percentage of callus producing shoots and shoot number callus\(^{-1}\) than IBA (0.50 mg l\(^{-1}\)) containing treatment combinations. This may be attributed to more stability of NAA in autoclave than IBA (Table 4).

Highest per cent of shoot induction and shoot number per callus piece was recorded on media fortified with Kinetin at 2.00 mg L\(^{-1}\) and NAA 0.50 mg L\(^{-1}\) and then on MS fortified with BAP 2.00 mg L\(^{-1}\) and NAA 0.50 mg L\(^{-1}\) in comparison to other treatment combinations (Fig. 4).

### 3.3. Shoot proliferation

Initial shoots raised in vitro from healthy pre-established nodal segments and shoot tips were employed for the purpose. Six growth regulator treatment combinations involving BAP at 0.50 and 1.00 mg L\(^{-1}\) with IBA 0.10, 0.25 and 0.50 mg L\(^{-1}\) were used in this experiment. Significantly high per cent shoot proliferation (97.90%), shoot number (22.25 explant\(^{-1}\)) and maximum shoot length (2.70 cm) was achieved at PGR combination of BAP 0.5 + IBA 0.50 mg L\(^{-1}\), followed by same concentration of BAP combined with IBA 0.25 mg l\(^{-1}\) (Table 5), (Fig. 5).

### 3.4. Rhizogenesis and hardening

The rooting characteristics (per cent rooting and root number shoot\(^{-1}\)) of Petunia hybrida Vilm cv. “Bravo” improved significantly

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**Table 4**

Influence of growth regulator combinations on shoot regeneration in leaf and internodal segment derived callus.

| Treatments (MS + PGRS)                  | Leaf Derived Callus | Interstitial Segment Derived Callus |
|----------------------------------------|---------------------|-------------------------------------|
|                                        | Regeneration (%)    | Shoot number callus\(^{-1}\)       |
| BAP (1.0 mg L\(^{-1}\)) + IBA (0.5 mg L\(^{-1}\)) | 4.16 (2.02)         | 2.08 (1.51)                         |
| BAP (1.0 mg L\(^{-1}\)) + NAA (0.5 mg L\(^{-1}\)) | 18.74 (4.33)        | 16.66 (4.14)                        |
| Kinetin (1.0 mg L\(^{-1}\)) + IBA (0.5 mg L\(^{-1}\)) | 6.24 (2.54)         | 4.16 (2.02)                         |
| Kinetin (1.0 mg L\(^{-1}\)) + NAA (0.5 mg L\(^{-1}\)) | 22.91 (4.84)        | 20.83 (4.65)                        |
| BAP (2.0 mg L\(^{-1}\)) + IBA (0.5 mg L\(^{-1}\)) | 8.33 (2.82)         | 8.33 (2.82)                         |
| BAP (2.0 mg L\(^{-1}\)) + NAA (0.5 mg L\(^{-1}\)) | 27.08 (5.25)        | 27.08 (5.25)                        |
| Kinetin (2.0 mg L\(^{-1}\)) + IBA (0.5 mg L\(^{-1}\)) | 14.58 (3.85)        | 12.49 (3.62)                        |
| Kinetin (2.0 mg L\(^{-1}\)) + NAA (0.5 mg L\(^{-1}\)) | 35.41 (6.02)        | 29.16 (5.47)                        |
| C.D(P\(_{0.05}\)) | 1.46 | 1.31 | 2.63                        |

Data recorded after 8 weeks of culture.
Figures in the parentheses are square root transformed value of the percentage data.

**Table 5**

Influence of growth regulator combinations on shoot proliferation from microshoots of Petunia hybrida Vilm cv. “Bravo”.

| Treatments (MS + PGRS) | Shoot proliferation (%) | Shoot number explant\(^{-1}\) | Shoot length |
|------------------------|-------------------------|-------------------------------|--------------|
| BAP (0.50 mg L\(^{-1}\)) + IBA (0.10 mg L\(^{-1}\)) | 70.83 (8.47)            | 8.25                         | 1.52         |
| BAP (0.50 mg L\(^{-1}\)) + IBA (0.25 mg L\(^{-1}\)) | 91.66 (9.62)            | 21.50                        | 2.53         |
| BAP (0.50 mg L\(^{-1}\)) + IBA (0.50 mg L\(^{-1}\)) | 97.90 (9.94)            | 22.25                        | 2.70         |
| BAP (1.00 mg L\(^{-1}\)) + IBA (0.10 mg L\(^{-1}\)) | 72.91 (8.39)            | 9.50                         | 1.95         |
| BAP (1.00 mg L\(^{-1}\)) + IBA (0.25 mg L\(^{-1}\)) | 77.08 (8.82)            | 18.00                        | 1.87         |
| BAP (1.00 mg L\(^{-1}\)) + IBA (0.50 mg L\(^{-1}\)) | 81.24 (9.06)            | 17.50                        | 2.47         |
| C.D(P\(_{0.05}\)) | 0.46 | 3.04 | 0.39                        |

Figures in the parentheses are square root transformed values of percentage data.
Data recorded after 6 weeks of culture.

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Fig. 4. Shoot regeneration, Kinetin + NAA: 2.0 + 0.5 mg L\(^{-1}\); (a): Shoot regeneration from leaf derived callus; (b): shoot regeneration from internodal segment derived callus.
as IBA and NAA concentration was elevated from 0.25 to 1.00 mg L\(^{-1}\). However IBA was recorded to have more effect than NAA (Table 6). Significantly highest rooting percent i.e., 95.82% and root number shoot \(\times C_{0}\) i.e., 8.00 was recorded on media having 1.00 mg L\(^{-1}\) IBA followed by IBA 0.75 mg L\(^{-1}\) (Fig. 6). Plantlet survival was observed highest (92.50%) in media HM6 (perlite + vermiculite: 1:1) followed by HM5 (vermiculite) and HM4 (perlite) with 86.45 and 83.75 percent, respectively (Table 7). Hardened plantlets have been shown in Fig. 7.

4. Discussions

4.1. Culture asepsis and explant establishment

Choosing the correct explant is essential if the desired outcome of any tissue culture procedure is to be achieved with minimal delays, besides proper sterilization of the explants is the prerequisite step leading to the development of a successful protocol for in vitro propagation. More asepsis was observed in 2 week cultures than the 4 week cultures. This may be attributed to the endophytic pathogens that come out and cause infection after 4 weeks thus increasing the overall contamination. After washing explants with tap water, several workers have used different sterilants like mercuric chloride, sodium hypochlorite or ethyl alcohol for the disinfection singly or in combination with fungicide as Bavistin (a.i. carbendazim). Mercuric chloride being a potent and efficient surface sterilant has been widely and extensively used in petunia

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**Table 6**

| Treatments (MS + PGRS) | Rooting (%) | Root number shoot \(\times C_{0}\) |
|------------------------|-------------|----------------------------------|
| IBA (0.25 mg L\(^{-1}\)) | 35.41 (36.40) | 2.25 |
| IBA (0.50 mg L\(^{-1}\)) | 52.08 (46.17) | 4.75 |
| IBA (0.75 mg L\(^{-1}\)) | 89.47 (71.35) | 7.75 |
| IBA (1.00 mg L\(^{-1}\)) | 95.82 (81.28) | 8.00 |
| NAA (0.25 mg L\(^{-1}\)) | 31.24 (33.85) | 1.50 |
| NAA (0.50 mg L\(^{-1}\)) | 41.66 (40.14) | 3.50 |
| NAA (0.75 mg L\(^{-1}\)) | 62.49 (52.36) | 5.00 |
| NAA (1.00 mg L\(^{-1}\)) | 74.99 (60.13) | 6.25 |
| C.D.\(P_{0.05}\) | 8.17 | 1.43 |

Figures in the parenthesis are arcsine transformed values of percentage data.

*Data recorded after 4 weeks of growth.

**Table 7**

| Hardening Media | Survival (%) |
|----------------|--------------|
| HM1 : Sand     | 36.25 (36.93) |
| HM2 : Sand + Perlite (1:1) | 60.00 (50.76) |
| HM3 : Sand + Vermiculite (1:1) | 62.50 (52.5) |
| HM4 : Perlite | 83.75 (66.38) |
| HM5 : Vermiculite | 86.25 (68.41) |
| HM6 : Perlite + Vermiculite (1:1) | 92.50 (74.29) |
| C.D.\(P_{0.05}\) | 5.14 |

Figures in the parenthesis are arcsine transformed values of percentage data.
the use of 1% HgCl₂ for 8 min as sterilization treatment for shoot tip explants by most of the researchers. Mishra et al. (2006) reported explants of Petunia hybrida on MS media containing BAP at the concentration of 3.0 mg L⁻¹. Sherkar and Chavan (2014) noted that 2, 4-D at the concentration of 3.0 mg L⁻¹ was recorded to be the most effective for induction of callus in potato explants that belongs to the same family. Also, MS media with 2.0-D recorded higher callus weight when compared to NAA fortified MS media. Vidya et al. (2013) and Mahadev et al. (2014) have demonstrated that auxin and cytokinin combination supports organogenesis in the callus. The results obtained were in conformity with many workers who reported shoot regeneration from callus derived from leaf explant of petunia on MS containing NAA and BAP. (Michalczuk et al., 2000; Seema et al., 2003; Wu and Li, 2007; Shrin et al., 2007; Kumar et al. 2014).

4.3. Shoot proliferation

Shoot proliferation from axillary branching method is to be known as most widely successful course for in vitro mass propagation of petunia. Cytokinin’s at appropriate concentration levels enhance cell division and inhibit apical dominance, thereby help in promotion of shoot proliferation from axillary buds. Therefore, for the mass multiplication of any plant species, a suitable cytokinin concentration level is a pre-requisite for optimum shoot production. GhaffariEiszad et al. (2012) have reported positive effect of cytokinin on multiplication and proliferation rate of Lisanthus. A number of workers have tried various PGRs either alone or in combinations with each other for shoot proliferation with diverse results in petunia. Cui et al. (2005) recorded the effects of combination of various concentrations of cytokinin with 0.10 mg NAA L⁻¹ on shoot propagation and proliferation of Petunia hybrida and found the suitable media for shoot propagation as MS + 1.60 mg L⁻¹ BA + 0.10 mg L⁻¹ NAA. Mishra et al. (2006) supplemented MS media with 1.0 mg BAP L⁻¹ and 0.1 mg L⁻¹ of IBA which resulted in significantly highest shoots explants⁻¹ as well as elongation of shoots in Petunia hybrida. Mohamed (2011) has observed shoot proliferation in carnation cv. ‘White Sim’ from nodal explants on MS media containing 8.87 μM BAP.

4.4. Rhizogenesis and hardening

Auxins, especially IBA are known to significantly improve the rooting per cent and its quality. Most of the workers achieved

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**Fig. 7.** (a): Hardening Stage; (b): Rooted plantlets during hardening.
stimulated rooting of petunia on media fortified with auxins as auxins have a good potential to promote root initiation (Wetherell, 1982). Qu and Qu (2001) obtained rooting on stem segments of Petunia hybrida using the treatment combination MS + NAA 0.5 mg L⁻¹ and MS + NAA 0.3 mg L⁻¹ with IBA 0.2 mg L⁻¹. Cui et al. (2005) also determined the effects of auxins on rooting of shoots in Petunia hybrida by using 0.20 mg L⁻¹ IBA, and 0.20 mg L⁻¹ NAA. Mishra et al. (2006) observed rooting in Petunia cv. “Cascade Burgundy” in half-basal MS media that contained auxin combination of 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ NAA. Sabitha et al. (2009) also achieved IBA at 20 μM dissolved in MS media was found to be better for rooting. Atak and Celik (2009) transferred the regenerated shoots of Anthurium andraeanum cv. “Arizona” to the medium supplemented with 1 mg L⁻¹ IBA which produced good quality rooting. In another study, IBA was observed to be more successful in root induction of capsicum plants than NAA even alone or in combination with each other (Otrosy et al. 2011). Plantlets developed in vitro need to be acclimatized for some weeks in low humid conditions before finally transferring them to the field conditions (Bolar et al., 1998). Thus, hardening media has a key importance in ex vitro establishment of the plants developed in vitro. The use of vermiculite as an effective hardening media for in vitro rooted plantlets was reported by many researchers. LiNa et al. (2006) has also observed 90 per cent survival rate when plantlets of carnation were transplanted to the substratum consisting of equal proportions of perlite and vermiculite. All acclimated plants were then transferred to the open conditions which grew normally in the natural environment.

5. Conclusion

For the development of propagation protocol for Petunia hybrida cv. “Bravo”, various steps were followed. Maximum unoinoculated growing cultures of petunia were obtained with 0.02% Carbendazim for 30 min followed by HgCl₂ at 0.1% for the duration of 10 min with a final treatment of 10 second wash with 70% etha-

6. Ethics approval

Not applicable.

7. Consent to participate

All authors consent to participate in this manuscript.

8. Consent for publication

All authors consent to publish this manuscript in Saudi Journal of Biological Science.

9. Availability of data and material

Data will be available on request to corresponding or first author.

10. Code availability

Not applicable.

Author contributions

IF, ZAQ, and SM drafted the experimental design. IF, ZAR, ITN, and NB performed the experiments. AN, HD, SR, KZM, SSA and SM helped in data collection, data analysis and initial draft of manuscript text. All authors read the manuscript before communication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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