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In vitro direct and indirect regeneration of plants from nodal and petiole explants in *Pelargonium odoratissimum* (L.) Herit.

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***In vitro* direct and indirect regeneration of plants from nodal and petiole explants in *Pelargonium odoratissimum* (L.) Herit.**

Abstract: Nodal and petiole explants were employed to study the direct and indirect regeneration from *Pelargonium odoratissimum in vitro*. Direct shoots regeneration of nodal segments was tried in MS medium containing 1 and 2 mg l^{-1} BAP and 0.5 mg l^{-1} IBA. The highest mean shoots number and the greatest shoots per explant number were obtained in the medium containing 2 mg l^{-1} BAP. Nodal segments were the source of petiole explants and the resulting petioles were cultured in ½ MS medium supplemented by 1, 1.5, 2 and 4.5 mg l^{-1} BAP enriched with 0.1, 1 and 1.5 mg l^{-1} NAA. With the petiole explants, the lowest browning percentage, the highest callus induction and also, the top number of shoots per explant were recorded in 2 mg l^{-1} BAP + 0.1 mg l^{-1} NAA medium. The medium supplemented with 0.2 mg l^{-1} NAA exhibited the desired effect on rooting percentage and mean root number and length. The rooted young plants were transferred to the pots containing peat-moss and perlite (1:1) and the acclimatization was successful since, more than 90 % of plants adapted-well in the greenhouse conditions. This *in-vitro* propagation methodology would be advisable to the plant production systems and to whom wish to produce the clonal homogenous plants for the commercial ideas and for the detailed molecular studies.

Key words: callus induction; MS medium; nodal segments; *Pelargonium odoratissimum*; shoot regeneration

Neposredna in posredna *in vitro* regeneracija rastlin iz izsečkov nodijev in listnih pecljev pri muškatki (*Pelargonium odoratissimum* (L.) L Herit)

Izvleček: V raziskavi so bili uporabljeni izsečki nodijev in listnih pecljev za preučevanje neposredne in posredne vzgoje rastlin muškatke (*Pelargonium odoratissimum* (L.) L Hér.) *in vitro*. Neposredna tvorba poganjkov iz izsečkov nodijev je bile preiskušena na MS gojišču, ki je vsebovalo 1 in 2 mg l^{-1} BAP in 0,5 mg l^{-1} IBA. Največje poprečno število poganjkov in največje število poganjkov na izseček je nastalo v gojišču, ki je vsebovalo 2 mg l^{-1} BAP. Nodiji so bili vir izsečkov listnih pecljev, ki so bili gojeni na polovičnem MS gojišču z dodatkom 1, 1.5, 2 in 4,5 mg l^{-1} BAP, obogatenega z 0,1, 1 in 1,5 mg l^{-1} NAA. Z uporabo izsečkov listnih pecljev je bil dosežen najmanjši odstotek porjavitev, najboljša indukcija kalusa in največje število poganjkov pri dodatku 2 mg l^{-1} BAP + 0,1 mg l^{-1} NAA v gojišče. Gojišče, kateremu je bil dodano 0,2 mg l^{-1} NAA je pokazalo zaželjen odstotek ukoreninjenja in največje poprečno število in dolžino korenin. Vkoreninjene mlade rastline so bile posajene v lončke z mešanico šotnega mahu in perlita (1:1). Aklimatizacija je bila uspešna, saj se je več kot 90 % rastlin uspešno prilagodilo razmeram v rastlinjaku. Takšno metodologijo *in vitro* razmnoževanja rastlin bi bilo primerno uvesti v sisteme razmnoževanja, v katerih želimo vzgojiti homogene klonske rastline za komercialne namene in podrobnejše molekularne raziskave.

Ključne besede: indukcija kalusa; MS gojišče; nodijski izsečki; *Pelargonium odoratissimum*; regeneracija poganjkov

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1 INTRODUCTION

Pelargonium odoratissimum is an important aromatic plant belonging to the Geraniaceae family and is in common growth in the tropics and sub-tropics (Ghanem et al. 2008). This is a perennial herbaceous plant and, is propagated by the semi-hard wood stem cuttings. However, the success rate with this propagation method is quite limited. The oil extracted from the above ground parts of the plant is in great need with the cosmetic, fragrance and hygienic industries and preparations from the plant have remarkable antimicrobial, antifungal and insecticidal activities (Ghanem et al. 2008; Gupta et al. 2002).

Plants regeneration and propagation by the *in vitro* culture methods have been defined as reliable ways to the breeding and the economical production of medicinal plants. Shoot regeneration with both the direct and indirect ways were experienced to have enough plant materials with the traits of interest. The need for the disease-free plants besides mass-production have been the main ideas to resort to *in vitro* propagation of *Pelargonium odoratissimum*. However, there is no universal protocol suitable for *Pelargonium* spp *in vitro* propagation (Wojtania et al. 2004). In the *in vitro* conditions, regeneration rate is dependent upon genotype, explants type, media components, plant growth regulators (PGRs), light intensity and quality and, the interaction of these factors (Arshad et al. 2012). *In vitro* direct and indirect regeneration of *Pelargonium graveolens* L'Hér. has been reported by leaf and nodal explants (Satyakala et al. 1995; Saxena et al. 2000) as well as shoots formation from calli samples derived from the leaf sections of 'Bipuli' cultivar (Gupta et al., 2002).

With *Pelargoniun radula* L Herit micropropagation (Zuraida et al. 2013), the utmost shoots regeneration rate was acquired for nodal explants in a medium containing 0.5 mg l^{-1} BAP+1 mg l^{-1} IBA. Saxena et al. (2000) were successful to obtain the highest shoots number per leaf explants in a medium enriched with 5 mg l^{-1} Kinetin+1 mg/L NAA. For the nodal segments, the best results were acquired from the combination of 8 mg l^{-1} Kinetin +1 mg l^{-1} NAA. Krishna Raj et al. (1997) reported that somatic embryogenesis of *P. odoratissimum* 'Frensham' cultivar was successful with the young leaf petioles. Hammerchlag and Bottino (1981) noted that callogenesis from the young seedlings of nodal segments was dependent upon the phenological stage of growth and the younger seedlings produced the highest number of shoots *in vitro*. Adventitious shoots regeneration from the petiole explants of *Pelargonium* × *hederaefolium* 'Bonette' cultivar was stimulated by the high amounts (9-18 µM) of thidiazoron and the related embryogenesis and organogenesis crosstalk was studied in detail.

Rooting behavior *in vitro* is another criterion in plants micropropagation which is mainly dependent on auxin and cytokinin equilibrium with the dominancy of auxin type PGRs (Zuraida et al., 2013). Rooting in *P. graveolens and P. radiattum* (Andrews) Pers was the best in half strength MS medium with 0.1 mg l^{-1} NAA (Zuraida et al., 2013). In spite of great attempts on *Pelargonium* species *in vitro* cultures; there are scattered information on *P. odoratissimum* propagation methods *in vitro*. The improvements in tissue culture method with the *Pelargonium* species would be the basal steps in the enhanced production of high valued secondary metabolites from these species. Moreover, the *in vitro* cultural methods will assist molecular biologists and biotechnologists to manipulate the species for the optimized production of the desired constituents. The present experiment was planned to study the potential direct and indirect regeneration capability of nodal and petiole explants of *P. odoratissimum in vitro*. This methodology would be possibly a reliable procedure for the commercial in vitro production of this high-valued crop for the coming molecular and breeding programs.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND STERILIZATION

Nodal explants were taken from mother plants grown under a reference university research greenhouse in Maragheh, Iran. The homogenous mother pot plants (2 years old) available in our greenhouse were employed for sampling of the uniform nodal section of about 5 to 8 mm in diameter from the middle part of shoots. 30 nodal segments (3 treatments and 10 replications per treatment) were rinsed in running water for 45 minutes. Superficial sterilization of nodal cuttings was completed in a laminar-airflow cabinet as followed: immersion in 70 % ethanol for one minute, followed by sodium hypochlorite, 15 % containing 2 drops of Triton X-100 for 13 minutes. Subsequently, the explants were rinsed three-times in distilled water and treated with 70 % alcohol for 20 seconds. Finally, the samples were placed on sterile paper to get dried. The dried sterile plant material was cut into 0.5-1 cm segments and transferred to MS medium. Petiole explants were acquired from the shoots grown from nodal segments *in vitro*. The sterile petiole samples were divided into 0.5 cm explants.

2.2 REGENERATION

The basal medium was MS supplemented with 3 % sucrose and 0.7 % agar along with 500 mg l^{-1} myoinositol. For some instances, we used also ½ MS medium. Nodal segments were cultured in MS medium supplemented by BAP (1 and 2 mg l^{-1}) and IBA (0.5 mg l^{-1}). Petiole explants were cultured in ½ strength MS medium containing BAP (1, 1.5, 2 and 4.5 mg l^{-1}) and NAA (0.1, 1, 1.5 mg l^{-1}). To prevent tissue browning, 50 mg l^{-1} citric acid was added to the medium. Three explants were placed in every 9 cm petri-dish. The cultures were placed under dark conditions in a growth chamber for two weeks under 16:8 hours photoperiod regime at 23 ± 1 °C and 20 ± 1 °C.

2.3 PROLIFERATION STAGE

Two weeks after the cultures establishment; the nodal segments were subcultured in the MS basal medium with the initial treatment combinations. Three successive subcultures were done at two week intervals. The number and length of shoots were recorded twice per week. In the second phase; and 4 weeks after establishment of the petiole explants, the tender creamy colored calli were developed. Callus formation percentage was measured for each sample. Calli were subcultured on the ½ MS medium with the initial treatment combinations as well. Two weeks after calli were sub-cultured; the shoots were produced. Later, the number and length of the shoots were measured. Petiole explants were taken from the shoots produced from the nodal segments *in vitro*. For the indirect regeneration stage, the derived calli were cultured on the initial ½ MS medium.

2.4 ROOTING

The shoots derived from the proliferation stage were cut from the distal ends and were cultured on ½ MS medium containing 0.1 and 0.2 mg l^{-1} NAA combined with 2 g l^{-1} activated charcoal. Rooting percentage and the number and length of the roots were recorded about ten days after transferring to the rooting medium.

2.5 ACCLIMATIZATION OF THE NEWLY-FORMED SEEDLINGS

Warm (50-60 °C) water was used to remove the agar debris from the young roots and, the plantlets were transferred to the pots containing peat moss: perlite (1:1) and were placed in a growth chamber under 16:8 photoperiod regime and 23±1 °C and 20±1 °C temperature regime during the day and night, respectively.

2.6 STATISTICAL ANALYSIS

Experimental design for the nodal segments was arranged with 3 treatments and 10 replications in a completely randomized design (CRD) and for petiole explants with 6 treatments and 5 replications with 3 experimental units as factorial experiment based on CRD. Each experiment was repeated at least twice and the reported data are the means of two experiments. Data were analyzed by SAS (version 9) and mean comparisons were done by LSD (Least Significant Difference) test at the 0.05 probability level.

3 RESULTS

3.1 DIRECT SHOOT REGENERATION

Two weeks after the explant cultures; the shoots were visible on nodal explants. For the multiplication/proliferation, shoots were cultured with the initial treatments. Treatments and growth regulators concentration and combinations had inevitable effects on the growth and developmental response of plant samples.

Mean comparisons revealed that 2 mg l^{-1} BAP+0.5 mg l^{-1} IBA and 2 mg l^{-1} BAP (alone) had significant effects on reducing the browning percentage in samples (Figure 2). With the nodal section explants, the proximal ends were responded to the media and, we observed the browning disorder.

BAP proved to be the desirable compound for initiating the shoot growth in terms of total shoot number per explant and mean shoot number in nodal segments of *Pelargonium odoratissimum* (Figure 1)

Calli were produced on ½ MS medium containing different combinations of BAP and NAA. Calli were induced from the explants during 25 to 30 days under dark conditions. All treatment combinations (NAA+BAP) had positive effects on the calli production from petiole explants. Mean comparisons revealed that 1.5 and 2 mg l^{-1} BAP+0.1 and 1 mg l^{-1} NAA had the highest callus induction potential (Table 1). In the present experiment, the explant browning was overcome with using 50 mg l^{-1} of citric acid in ½ MS medium. Our results shows that the lowest browning rate in petiole explants was achieved in the medium containing 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Table 1).

The results revealed that the survival rate and regeneration potential in petiole explants were related to

Table 1: Mean comparison for the effects of treatment combination on callogenesis, and shoots induction in petiole explants of Pelargonium odoratissimum in vitro

Treatment combination	Mean shoots length (cm)	Mean shoots number	Browning percentage (%)	Callogenesis (%)
1.5 mg l^{-1} BAP +1.5 mg l^{-1} NAA	0.45^{c}	1.78^{bc}	79.36^{ab}	45.66^{de}
2 mg l^{-1} BAP + 1 mg l^{-1} NAA	0.21^{c}	0.66^{c}	92.66^{a}	25.32^{e}
2 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3.33^{a}	19.14^{a}	15.35^{d}	100^{a}
1mg l^{-1} BAP + 1 mg l^{-1} NAA	1.22^{b}	4.36^{b}	58.96^{bc}	73.03^{bc}
1.5 mg l^{-1}BAP + 1 mg l^{-1} NAA	1.35^{b}	2.78^{bc}	40.66^{c}	93.24^{ab}
4.5 mg l^{-1} BAP + 1 mg l^{-1} NAA	1.12^{b}	2.02^{bc}	56.38^{c}	59.63^{cd}

Different letters in columns are significant *($p \geq 0.01$)* based on LSD test

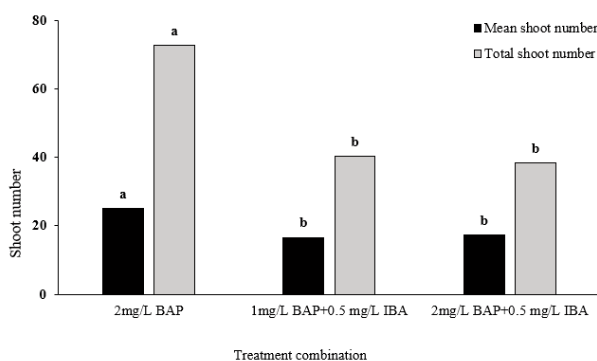

Figure 1: Treatment combination effects on total shoot number per explant and mean shoot number in nodal segments of Pelargonium odoratissimum in vitro (based on LSD test)

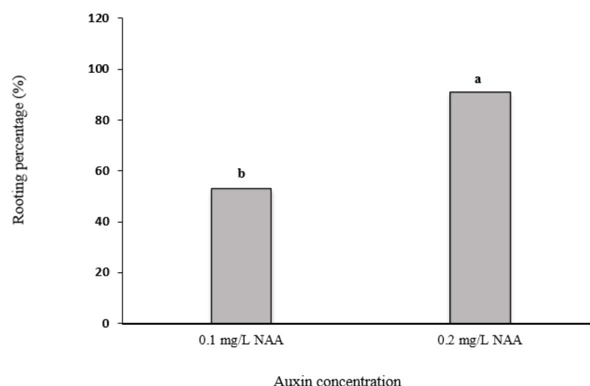

Figure 3: The effect of NAA concentrations on rooting percentage of *Pelargonium odoratissimum* in vitro ($p \geq 0.01$)

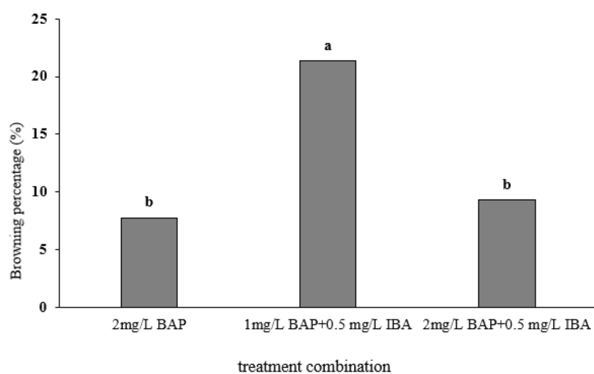

Figure 2: Treatment combination effects on browning rate of nodal segments of Pelargonium odoratissimum in vitro (based on LSD test)

the ratios and amount of plant growth regulators applied. Two weeks after subculture, the shoots were developed. The result showed that the highest number and length of the shoots was obtained in the medium containing 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Table 1).

The greatest proliferation rate with nodal explants was recorded in the medium containing 2 mg/L BAP and with petiole explant in the medium supplemented by 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Figure 2 & Table 1)

3.2 ROOTING AND ACCLIMATIZATION

The shoots derived from the nodal and petiole explants were cultured in ½ MS medium enriched with 0.1 and 0.2 mg l^{-1} NAA plus 2 g l^{-1} active charcoal. Figure 3 shows that the highest rooting percentage was observed in 0.2 mg l^{-1} NAA. 0.2 mg l^{-1} NAA was the concentration of choice for both root number and rooting percentage as well.

Furthermore, mean comparisons revealed that, for the root number, there was a difference between NAA concentrations, and explant type. So that, with 0.2 mg l^{-1} NAA, the top root number was belonged to the nodal explants (Figure 4). The results in figure 5 shows that with both nodal and petiole explants; 0.2 mg l^{-1} NAA had more root length.

Eventually, the intact rooted plantlets were selected for the acclimatization stage. The plantlets were trans-

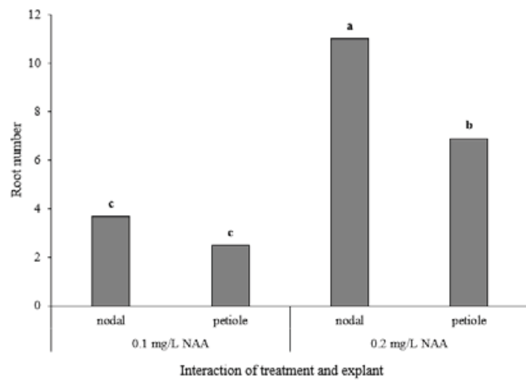

Figure 4: The effect of NAA concentrations on root number of *Pelargonium odoratissimum* in vitro (p ≥ *0.0)*

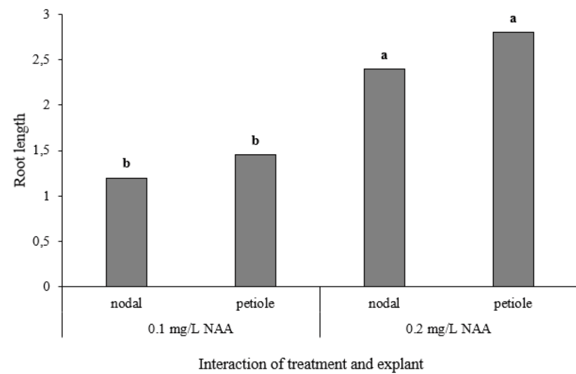

Figure 5: The effect of NAA concentrations on root length of Pelargonium odoratissimum in vitro (p ≥ 0.)

ferred to the pots containing peat moss-perlite (1:1), and were placed in a growth chamber under 16:8 hrs. light: darkness period, and 23±1 °C and 20±1 °C day: night temperature regime. After one month, the survived plantlets percentage was recorded and later, the acclimation of the potted plants was quite successful (more than 90 %).

4 DISCUSSION

The results showed that the sole cytokinin compared to the co-application of cytokinin:auxin had more promising effect on mean shoots number. It seems that cytokinins at higher concentrations, weaken the apical dominance and hence, stimulate auxiliary shoot induction.

These findings are concomitant with the reports of Zuraida et al. (2013) with diverse *Pelargonium* species. From the production viewpoint, *in vitro* micropropagation is a crucial step for the optimized proliferation rates, and furthermore, the fast and mass multiplication of the shoots provide the plant material needed for the coming stages of propagation route. The internal hormonal ratios and, especially auxin (IAA) amount were the major factors affecting the multiplication in direct organogenesis from *Jasmine* nodal segments and the MS medium having 3 mg l^{-1} BAP and 1 mg l^{-1} NAA was the best treatment (Farzinebrahimi et al., 2014). In *Pelargonium capitatum* plants incubated under dark conditions for 4 weeks and cultured in the medium containing 0.5 mg l^{-1} NAA+1 mg l^{-1} BAP+1 mg l^{-1} Zeatin; the full (100 %) direct shoot regeneration was achieved (Hassanein and Dorion, 2005). Epinosa et al. (2006) produced the highest indirect organogenesis of *Prunus serotina* Ehrh. plants from the leaf explants incubated under dark conditions for three weeks. Possibly, darkness affects the internal hormonal balance and positively interacts with the auxin:cytokinin exogenous applications in favor of higher organogenesis potential. Furthermore, darkness ameliorates the deteriorative effects of phenolics and hence declines the browning rates in the plant samples *in vitro* (Sukhumpinij et al., 2010).

Our results showed that for the scented geranium, BAP alone was enough for the shoot multiplication. The idea is that for the optimized shoots proliferation in geranium, the high amounts of cytokinins per auxins are functional and essential.

Calli production is dependent upon the morphological characteristics of the plant tissues and the type and concentration of growth regulators employed. Thiruvengadam et al. (2006) reported that MS medium supplemented with 1 mg l^{-1} 2, 4-D attained the maximum callus induction.

Khodadadi et al. (2015) reported the improved calli production and shoot regeneration of *Momordica charantia* L. leaf explants by increasing NAA and kinetin concentrations up to 1 mg l^{-1}. However, high concentrations of the growth regulators mentioned had quite negative effects on callogenesis. Benazir et al. (2013) realized that the greatest callogenesis percentage in *Pelargonium graveolens* was obtained by 20 µM IBA+10 µM KIN and 20 µM IBA+10 µM BAP as well as by 20 µM IAA+10 µM KIN. Overall, the balanced auxin to cytokinin ratio is the essential factor impacts calli production and the further proliferation of leaf explants of scented geranium (Benazir et al., 2013). Furthermore, Charlwood and Charlwood (1991) noted that the callogenesis was improved in scented geranium whenever they used more than 1 mg l^{-1} of auxins and cytokinins. Callogenesis response in different organs and tissues is dependent on the endogenous and exogenous hormonal balance, so, the logical cytokinin:auxin ratios with the dominancy of cytokinins are vital for the petiole explants callogenesis.

The diverse responses of the leaf and petiole explants to the varying concentrations of BA and NAA in the me-

dium possibly are due to the differences in the internal hormonal content or because of the different sensitivity of the organs to the growth regulators employed (Koroch et al., 2002). Arshad et al. (2012) demonstrated that the highest survival rate in *P. capitatum* was obtained in a medium enriched with 2 mg l^{-1} BA+1 or 2 mg l^{-1} NAA. In another study, Saxena et al. (2000) said that using 0.5 mg l^{-1} BAP instead of kinetin along with 0.1 mg l^{-1} NAA drastically increased the survival rate in *P. graveolens*.

Shoots proliferation and growth need suitable rates and amounts of auxin and in this case, NAA. The need for the low concentrations of auxins (0.1 mg l^{-1} NAA) for the formation and elongation of the shoots is maybe due to the near sufficiency of internal auxin. Saxena et al. (2000) reported that cytokinin/auxin rate plays a pivotal role in both direct and indirect organogenesis process of the plants *in vitro*.

The same idea has been reported by Brown and Charlwood (1986). The later scientists noted that organogenesis responses and shoot growth in scented geranium tissue culture was dependent on the exogenous applications of growth regulators and specially was responsive to BAP and NAA. Generally, shoot induction *in vitro* is highly responsive to the cytokinins type and concentration. This bio-molecules stimulate the organogenesis in the potentiate calli cells and hence, encourage the shoots induction and initiation. Cytokinins also increase cell division rates and more specially enhance the adventitious shoots formation via combating the apical dominancy.

Koroch et al. (2002) reported that the medium containing 4.4 µM BAP+0.05 µM NAA achieved the highest regeneration rate and the greatest number of shoots. Moreover, Saxena et al. (2000) demonstrated that the maximum number of shoots in rose-scented geranium was obtained by a medium enriched with 0.5 mg l^{-1} BAP+0.1 mg l^{-1} NAA. Ghanem et al. (2008) wrote that leaf and petiole explants of *P. graveolens* had the suitable regeneration rate at the MS medium filled with 1 mg l^{-1} BAP+0.1 mg l^{-1} NAA. The essentiality of using at least 0.5 to 1 mg l^{-1} BAP for the shoots elongation in the nodal explants of scented geranium has been emphasized by Zuraida et al. (2013).

Satyakala et al. (1995) noted that the maximum number and length of the nodal cutting for the same plant need 1 mg l^{-1} BAP and IAA. The highest regeneration percentage with *P. hortorrum* was obtained in the medium supplemented with 1 mg l^{-1} BAP and/or Zeatin plus 0.2 mg l^{-1} NAA (Hassanein and Dorion, 2005). Generally, the direct and indirect regeneration in rose–scented geranium is responsive to the endogenous and exogenous PGR_S, explant type and the chemical properties of the growing medium.

The most probable idea is that root formation in *Pelargonium* species *in vitro* is most dependent upon the shoots related attributes. Zuraida et al. (2013) noted that 0.2 mg l^{-1} auxin (IAA and IBA) was the best for the highest rooting percentage in *Pelargonium citrosum* and *P. radula* which is in line with our results. Auxins alone or in combination with the low concentration of cytokinin are able to initiate the root primordia (Zuraida et al., 2013). It seems that, deciding on the type and concentration of exogenous auxin application for *in vitro* proliferation and production is quite dependent on the internal hormonal content of the plant tissue. So, owing to the results obtained from the present experiment, 0.2 mg l^{-1} NAA produced the highest root number as well as the longest roots.

5 CONCLUSION

The current experiment was conducted to reach a reliable fast and easy propagation method for the direct and indirect regeneration of *Pelargonium odoratissimum* nodal and petiole explants under *in vitro* conditions. Overall, an established *in vitro* culture regeneration protocol is dependent on several factors such as; plant species, explant type and media composition. In the present study, the best treatment for direct regeneration from the nodal segments was obtained by 2 mg l^{-1} BAP and for indirect regeneration from the petiole explants. The optimized treatment combination was 2 mg l^{-1} BAP+1 mg l^{-1} NAA. Moreover, the results showed that nodal explants compared to petioles had greater regeneration rate and have been considered more suitable for *Pelargonium* regeneration purposes. All in all, difficulty in petioles regeneration rate, along with the highest probability of somatic mutations and the low rate of shoots proliferations, makes this explant not suitable candidate for the *in vitro* proliferation of *Pelargonium* and even makes the indirect regeneration method more time-consuming and expensive. However, since the calli are the initial and easy starting materials for the production of secondary metabolites with suspension cultures, so, the study of callogenesis and calli production and optimization for the accelerated secondary metabolites production will be a motivation for using the petiole explants with *in vitro* commercial insights. Moreover, the results showed that explant type and PGR_S concentration were the main factors influencing regeneration potential and rooting behavior in *Pelargonium in vitro* culture. In such a way, the highest regeneration rate was traced and recorded with nodal segments. The easy protocol experienced with the present experiment could be employed for the clonal propagation and suspension cultures of this high-valued ornamental medicinal species.

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