In vitro propagation of *Allium stamineum*: an endangered medicinal plant

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Abstract

Allium stamineum, an endangered medicinal plant in Iran, requires conservation efforts through in vitro culture techniques. This study established an effective protocol for callus induction and bulblet regeneration in *A. stamineum* using various explant types (radicle, basal plate, and cotyledon) and growth regulators. The results indicated that the best callus formation from cotyledon explants occurred in media containing 1 mg/L 2,4-D, 1 mg/L 2,4-D with 0.5 mg/L BA, 2 mg/L 2,4-D with 0.5 mg/L kinetin, and 1 or 2 mg/L 2,4-D with 1 mg/L kinetin. In terms of regeneration, cotyledon explants showed the highest regeneration rate compared to radicle and basal plate explants, with 3.33 regenerations per explant in MS medium supplemented with 1 mg/L kinetin and 1 mg/L NAA. Additionally, the highest bulblet regeneration rate (11 per explant) was obtained from callus on PGR-free medium. The maximum number of roots (7.90) and root length (10.9 mm) were observed on MS medium containing 3 mg/L IBA. Rooted bulblets were successfully acclimatized in pots filled with a cockpit mixture (3:1 v/v) with a 100% survival rate. This study not only provides a successful in vitro propagation technique for *A. stamineum* but also facilitates its breeding program.

Keywords: Bulblets, Explant, Regeneration, IBA, Rooting

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Introduction

Allium is probably one of the most widespread genera worldwide, including 850 species globally and 114 species in Iran (Razyfard, Zarre et al. 2011, Sharifi-Rad, Mnayer et al. 2016). *Allium stamineum*, from the Amaryllidaceae family, is one of the main native and endemic medicinal Alliums in Iran. This plant grows wild in the southern and southwestern parts of Iran, including Khuzestan, Bushehr, and Fars provinces. Allium species are a major source of organosulfur compounds and flavonoids, which are considered health-promoting phytochemicals (Marrelli, Amodeo et al. 2018). Allicin, as one of the main metabolites of Alliums, has a wide range of pharmabiological activities, such as antimicrobial, anticoagulation, antimycotic, antibiotic, antiparasitic, anti-aging, antiviral, antiplatelet, antitumoral, antioxidant, antihypertensive,

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hypolipidaemic, detoxifying heavy metals, fibrinolysis, and immune-boosting properties (Ebrahimi, Zamani et al. 2009, Bikis 2018, Nabavi, Saeedi et al. 2020). Due to its slow growth and overharvesting, A. stamineum is now considered an endangered species. Thus, using non-conventional methods of multiplication to meet the demand for it in herbal medicine is of high significance for the conservation of this species. A viable alternative strategy for increasing the effectiveness of Allium species growth is in vitro tissue culture (Farhadi, Panahandeh et al. 2017). Therefore, tissue culture techniques, as a well-known method, can enhance traditional A. stamineum breeding programs and produce tolerant, resistant, and higher-yield cultivars. Direct shoot propagation using stem discs and basal plates has been successfully implemented in Allium (Jeong and Yong 2022). Given the medicinal value of A. stamineum in Iran, to our knowledge, no studies have been conducted on this species. The purpose of this work is to examine the effects of explant type and plant growth regulators on multiplication and bulblet regeneration, as well as bulblet regeneration of proliferated callus from A. stamineum.

Materials and Methods

Plant Material

Seedlings of Allium stamineum were used as the initial explants. Seeds were collected from plants growing on clay-loam soil in an open plain in southeastern Borazjan County, Bushehr, in April 2018. After initial washing, the collected seeds were surface sterilized by treating with 2% (v/v) carbendazim for 30 minutes, followed by 75% ethanol for 60 seconds. The seeds were then immersed in a 20% (m/v) sodium hypochlorite (NaClO) solution for 10 minutes and rinsed three times with sterile double-distilled water. All sterilization procedures were carried out in a laminar airflow cabinet (Miao-miao Yan et al., 2009).

Basal Medium and Culture Conditions

Murashige and Skoog (MS) medium (1962) supplemented with different combinations of plant growth regulators (PGRs) [2,4-Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP), Naphthalene acetic acid (NAA), and kinetin (Kin)], 3% (w/v) sucrose, and 0.8% agar was used. The pH of the medium was adjusted to 5.7±0.1 before autoclaving at 121˚C for 15 minutes (Murashige and Skoog 1962; Farhadi, Panahandeh et al., 2017). Cultures were maintained at 22±2˚C in a growth chamber with a 16/8-hour light/dark cycle, with light provided by Philips Luxeon LEDs made in China.

Effect of 2,4-D, Kinetin, and BAP on Callus Induction

The influence of explant type on callus induction in A. stamineum was assessed using three different explant types: radicle, basal plate, and cotyledon. Each explant, measuring around 5–7 mm in length, was placed on MS medium supplemented with various concentrations of growth regulators: 0 and 1 mg/L BAP, 0, 0.5, and 1 mg/L kinetin, and 1 and 2 mg/L 2,4-D.

Effect of Growth Regulators on Bud Proliferation

To assess the impact of explant type on bulblet and shoot regeneration in A. stamineum, three explant types—radicle, basal plate, and cotyledon—were used. Explants, measuring approximately 5 to 7 mm in length, were placed on MS medium supplemented with various concentrations of kinetin (0, 1, 2, and 4 mg/L) and NAA (1 and 2 mg/L). This experiment was conducted as a factorial design based on a completely randomized design.

Effect of Growth Regulators on Callus Proliferation

Calli (approximately 0.4 × 0.4 × 0.4 cm in size) derived from cotyledon explants cultured on media containing 2 mg/L 2,4-D and 0.5 mg/L kinetin were transferred to MS medium supplemented with different concentrations of kinetin (0, 1, 2, and 4 mg/L) alone or in combination with NAA (0 and 1 mg/L) for bulblet induction and multiplication. Data on shoot and

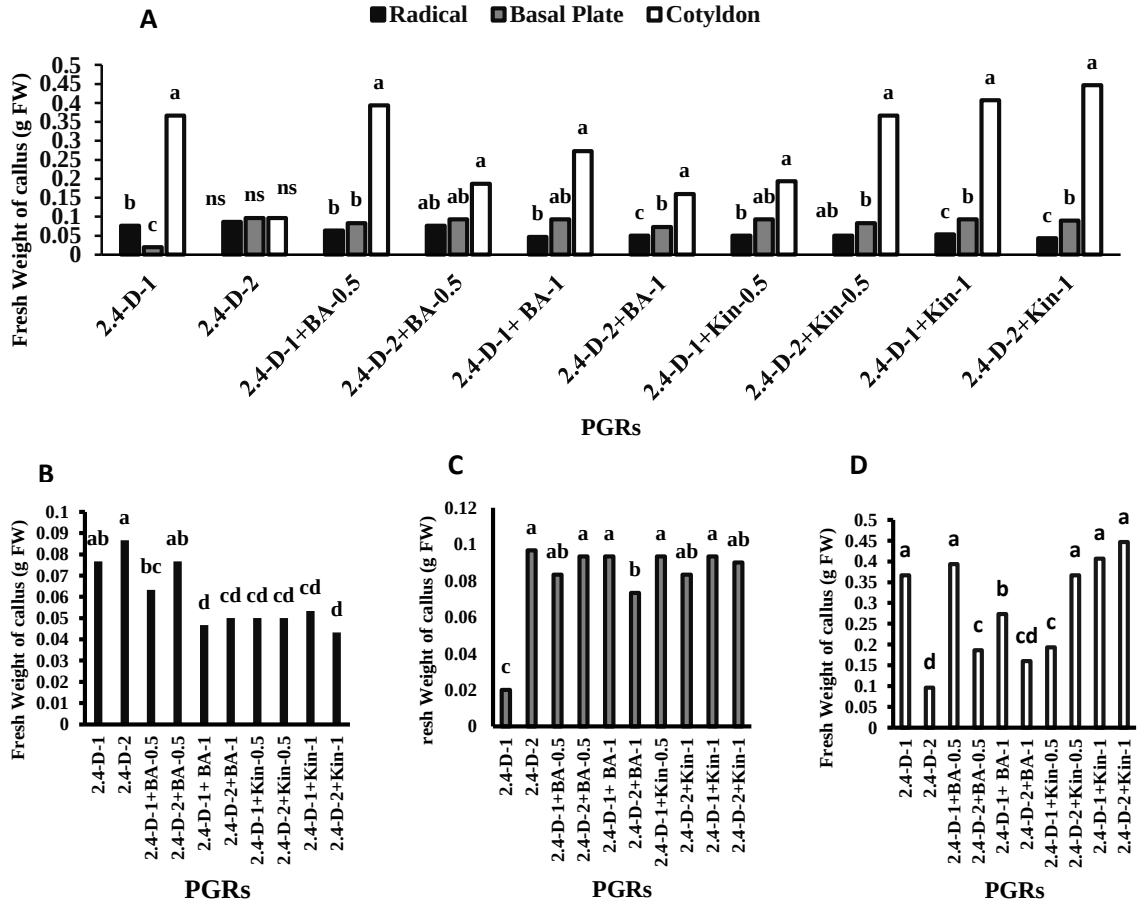

Fig. I. Effects of different growth regulator combinations on callus induction from various explants of A. stamineum. A) Comparisons of different explant types with the same growth regulators. B) Comparisons of different growth regulators on radicle induction. C) Comparisons of different growth regulators on basal plate induction. D) Comparisons of different growth regulators on cotyledon induction. The a-c above the columns indicate significant differences between each group.

bulblet regeneration per callus were recorded after 21 days of culture.

Root Induction

Excised single bulblets from the multiple bulblet clusters (proliferated on MS medium supplemented with kinetin, NAA, and IBA) were transferred to MS medium supplemented with different concentrations of IBA (1, 2, and 3 mg/L) for root induction. The number of roots per explant, the percentage of bulblets producing roots, and root length were recorded after 3 weeks.

Acclimatization

Three-week-old *in vitro* bulblets were gently washed under running tap water and transferred to pots filled with a cocopeat (75:25%) mixture. They were covered with plastic in the culture room. The plantlets were then acclimatized to the natural environment in the greenhouse.

Statistical Analysis

A completely randomized design with 5 replicates per treatment, each containing 4 samples, was used in this study. Significant differences were determined using Duncan's New Multiple Range

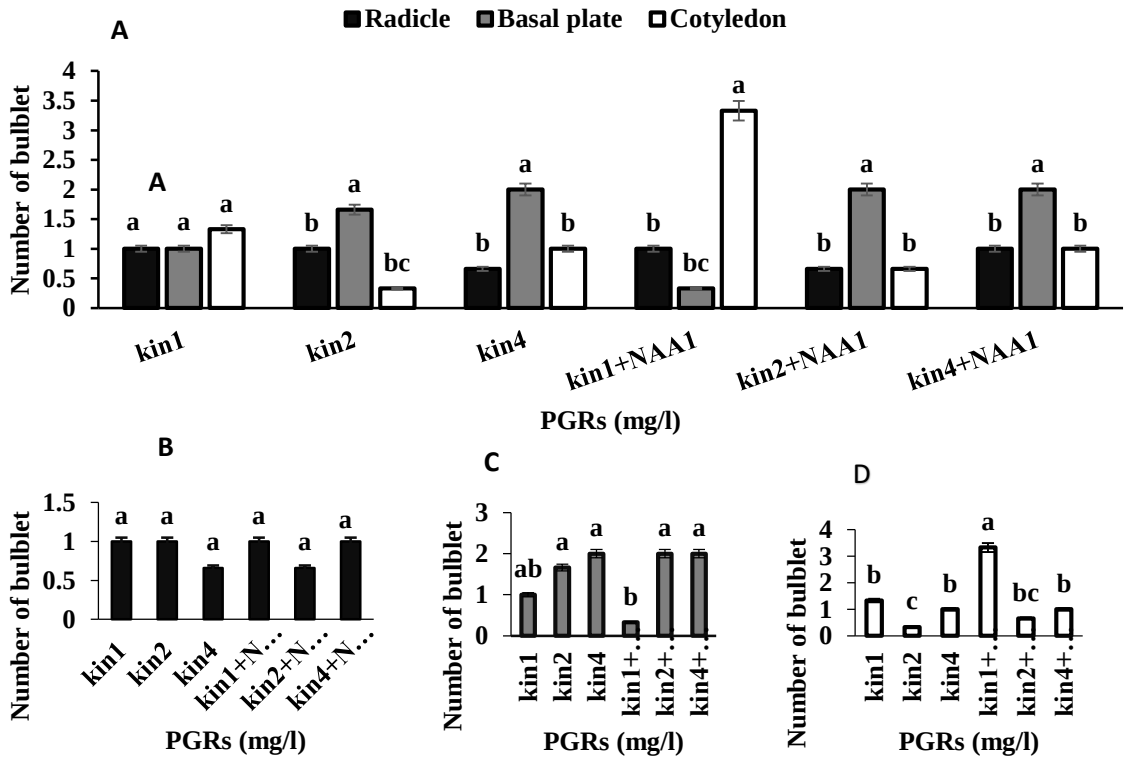

Fig. II. Effects of different growth regulator combinations on bulblet regeneration from various explants of A. stamineum. A) Comparisons of different explant types with the same growth regulators. B) Comparisons of different growth regulators on radicle regeneration. C) Comparisons of different growth regulators on basal plate regeneration. D) Comparisons of different growth regulators on cotyledon regeneration. The a-c above the columns indicate significant differences between groups ($P < 0.05$).

Test at $P < 0.05$. Data were analyzed using SPSS version 16.0 statistical software.

Results

Effect of 2,4-D, Kinetin, and BAP on Callus Induction

The results demonstrated that the composition of growth regulators in the MS medium significantly influenced callus formation in *Allium stamineum*. The best callus formation from cotyledon explants was observed in media containing 1 mg/L 2,4-D, as well as in media supplemented with combinations of 1 mg/L 2,4-D and 0.5 mg/L BAP, 2 mg/L 2,4-D with 0.5 mg/L kinetin, and both 1 mg/L and 2 mg/L 2,4-D with 1 mg/L kinetin. These combinations resulted in dense, friable callus, which is considered optimal for subsequent regeneration experiments. The ability of 2,4-D in combination with cytokinins like BAP and kinetin to induce callus formation highlights the synergistic effects of auxins and cytokinins in promoting cell division and callus proliferation (Fig.I). This observation suggests that cotyledon explants are highly responsive to these specific combinations of growth regulators, making them a suitable explant choice for further tissue culture studies in *A. stamineum*.

Effect of Growth Regulators on Bud Proliferation

The regeneration of *A. stamineum* was significantly influenced by both the type of growth regulators used and the type of explant. Statistical analysis of the data revealed that different concentrations of kinetin and NAA, in combination with varying explant types (radicle, basal plate, and cotyledon), produced varying results in terms of bud proliferation and shoot regeneration (Fig. II and III). For instance, the basal plate explants

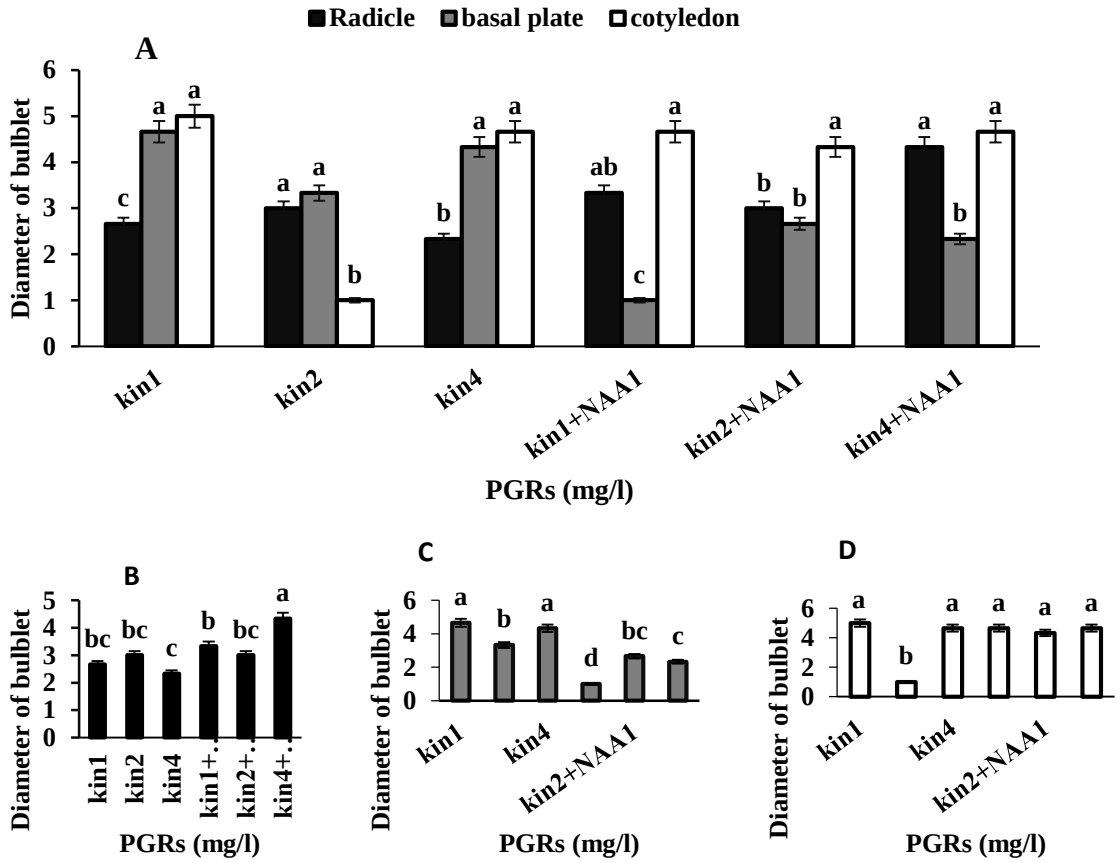

Fig. III. Effects of different growth regulator combinations on bulblet diameter from various explants of A. stamineum. A) Comparisons of different explant types with the same growth regulators. B) Comparisons of different growth regulators on radicle diameter. C) Comparisons of different growth regulators on basal plate diameter. D) Comparisons of different growth regulators on cotyledon diameter. The a-d above the columns indicate significant differences between groups ($P < 0.05$).

responded particularly well to 2 mg/L kinetin combined with 1 mg/L NAA, resulting in the highest number of regenerated shoots. In contrast, cotyledon explants showed moderate bud proliferation in response to kinetin alone, but when combined with NAA, a marked improvement in shoot formation was observed. These findings underscore the importance of optimizing both the type and concentration of growth regulators based on the specific explant used, as the interaction between these factors is critical for successful plant regeneration.

Effect of Growth Regulators on Callus Proliferation

The study found that the presence of growth regulators in the media significantly influenced bulblet regeneration from *A. stamineum* callus. Specifically, media devoid of growth regulators (growth regulator-free) resulted in significantly lower rates of bulblet regeneration compared to media supplemented with kinetin and NAA ($P < 0.05$) (Fig. 4). This suggests that the presence of growth regulators is essential for the successful transition of callus into bulblets. The optimal regeneration was observed in calli that were cultured on MS medium containing 2 mg/L kinetin and 1 mg/L NAA, where robust bulblet formation occurred. The findings highlight the critical role of a carefully balanced hormonal environment in promoting not just callus proliferation, but also its subsequent differentiation into organogenic structures.

Rooting and Acclimatization

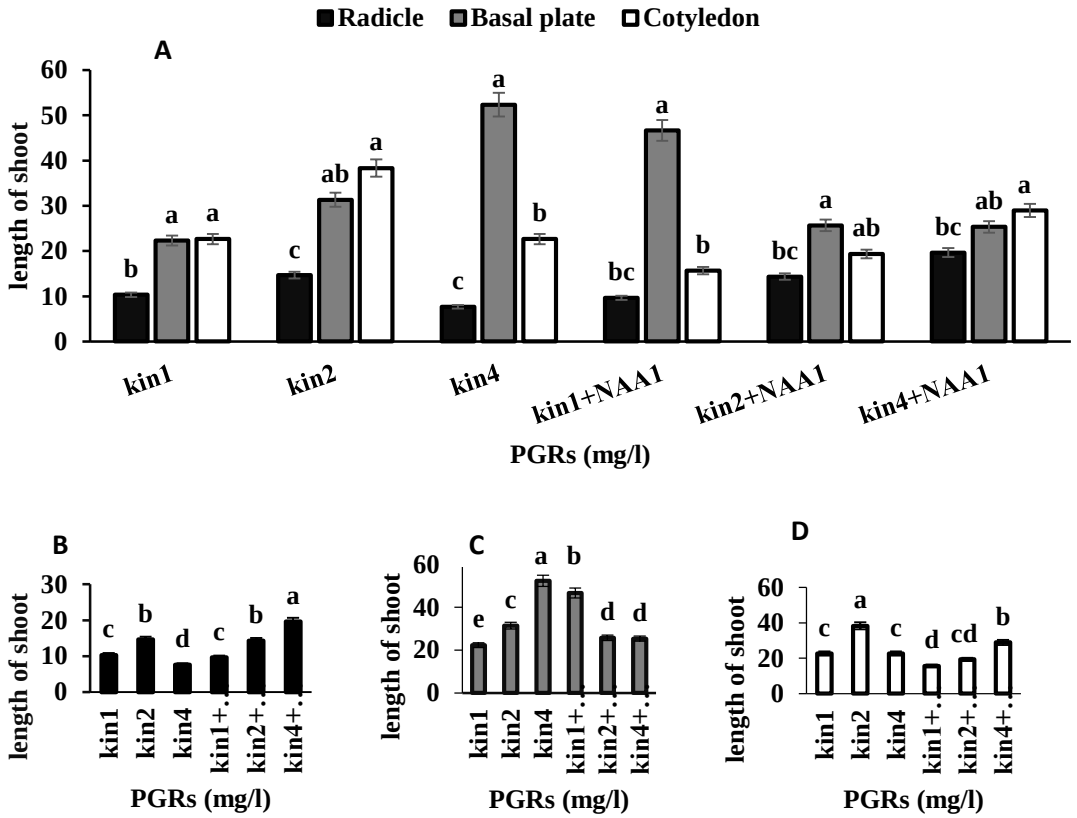

Fig. IV. Effects of different growth regulator combinations on shoot regeneration from various explants of A. stamineum. A) Comparisons of different explant types with the same growth regulators. B) Comparisons of different growth regulators on radicle regeneration. C) Comparisons of different growth regulators on basal plate regeneration. D) Comparisons of different growth regulators on cotyledon regeneration. The a-e above the columns indicate significant differences between groups ($P < 0.05$).

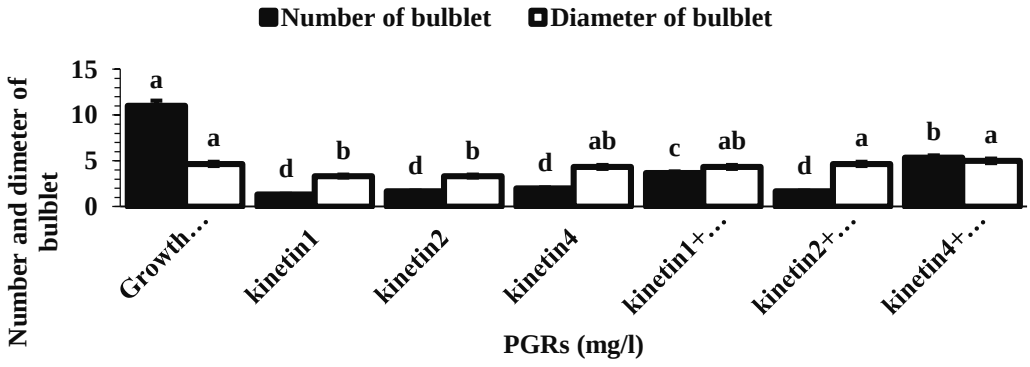

Fig. V. The effect of different combinations of growth regulators on bulblets regeneration of A. stamineum callus. The a-e above the columns show significant differences between each group ($P<0.05$).

All the *A. stamineum* plantlets subjected to the rooting treatments in this study successfully developed roots, although the mean root length and the number of roots varied significantly

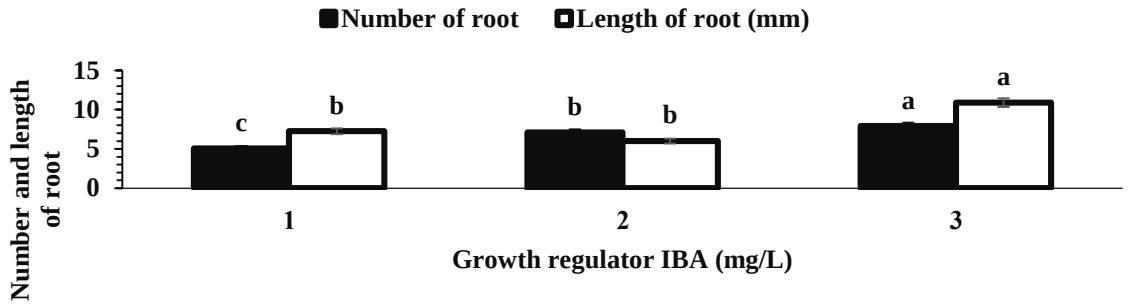

Fig. VI. Effect of IBA on the in vitro rooting of bulblets of A. stamineum. The a-c above the columns show significant differences between each group (P<0.05).

among the different treatments (Fig.V). The highest number of roots per explant (7.9 roots) and the greatest root length (10.9 mm) were observed in media containing 3 mg/L IBA (Fig VI). This suggests that a higher concentration of IBA is particularly effective in promoting both root initiation and elongation in *A. stamineum*. Once a well-developed root system was established, the plantlets were carefully transferred to pots containing a mixture of cocopeat and perlite in a 3:1 (v/v) ratio, which provided a suitable substrate for further root development and acclimatization. These preadapted plantlets were then moved to greenhouse conditions to continue their adaptation to ex vitro conditions. Remarkably, over 95% of the propagated plantlets were successfully acclimatized, indicating that the protocol used in this study was highly effective in producing robust, greenhouse-ready plants. Seed germination was initiated using the paper towel technique, followed by callus formation from cotyledons on 2 mg/L 2,4-D + 0.5 mg/L Kinetin. Bulblets were then formed from the callus on growth regulator-free media, and shoots also developed on the same media. The isolated bulblets were subsequently acclimatized in a cocopeat: perlite (3:1 v/v) mixture (Fig VII).

Discussion

Despite the fact that plants yield a wide range of therapeutic products, the complicated chemical makeup and unique spatial structure of these compounds make their industrial biosynthesis challenging. Nevertheless, given the effect of precursors and stimuli, plant cell culture is a good substitute for the ongoing generation of desirable secondary metabolites (Twaij and Hasan, 2022). Under laboratory conditions, the process of producing secondary metabolites is divided into two stages. The first stage is mass production, while the second stage is secondary metabolite biosynthesis. These steps are independent of each other because each of them has different requirements and can be optimized independently (Scarepa et al., 2022). Elicitors are excellent tools for increasing the synthesis of secondary metabolites in biotechnological systems because they activate secondary biosynthetic pathways that govern carbon flow to obtain various beneficial plant chemicals (Naik and Al-Khayri, 2016). The synthesis pathway of secondary metabolites involves a large number of control points that are regulated by elicitors. This leads to the induction of transcription factors and the expression of particular genes. When a receptor and an elicitor interact, a signal transduction cascade is set off, resulting in the execution of particular actions such as G protein activation, tyrosine kinase activation, calcium-mediated signaling, Janus kinase (JAK) pathway, phosphorylation, jasmonic-mediated early response genes, membrane acidification, DAG, IP3-mediated pathway, and cyclic adenosine-mediated pathway (Zhao et al., 2005). As a growth hormone, JA influences many different physiological and developmental reactions while also modulating the expression of genes. The bioactive form of JA, jasmonoyl isoleucine (JA-Ile), is produced in the cytoplasm of tissues upon elicitation (Li et al., 2017). Jasmonic acid-1 transfer protein (JAT1), which is found in the nuclear and

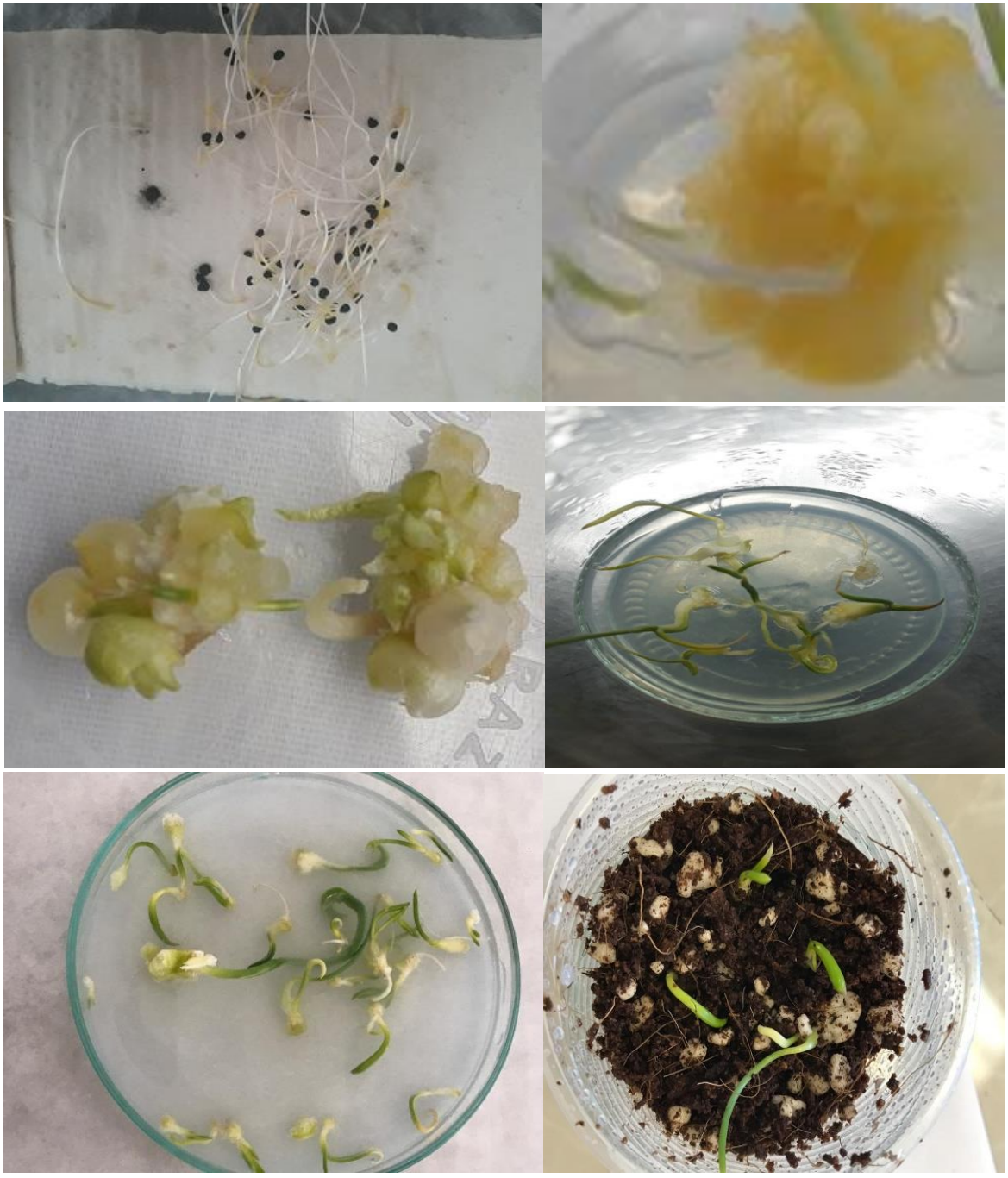

Fig. VII. A) Seed germination using the paper towel technique. B) Callus formation from cotyledons on 2 mg/L 2,4-D + 0.5 mg/L Kinetin. C) Bulblet formation from callus on growth regulator-free media. D) Shoot formation from callus on growth regulator-free media. E) Isolated bulblets. F) Acclimatization on a cocopeat: perlite (3:1 v/v) mixture.

plasma membranes, carries JA and JA-Ile from the cytoplasm to the nucleus and apoplast (Wang et al., 2019). Under normal conditions, transcription factors do not activate jasmonate-responsive genes. On the other hand, under certain conditions, JA specifically induces transcriptional and metabolic reprogramming in cells and tissues to promote the synthesis of secondary metabolites (De Geyter et al., 2012). When methyl jasmonate and SA are used simultaneously in *Rehmannia glutinosa* Libosch, the result is a higher production of secondary metabolites than in the control (Piatezak et al., 2016). In *Portulaca oleracea* suspension cultures, treatment with JA or SA is a suitable alternative method that increases phytochemical production and cell growth. When

cells treated with JA and SA produce high levels of phenolic compounds, flavonoids, and other bioactive compounds, it indicates that the compound has antioxidant potential (Fernandez-Poyatos et al., 2021). Numerous investigations have demonstrated that the stimulant methyl jasmonate increases the activity of a number of antioxidant enzymes (Ho et al., 2020). Probably, one of the reasons for increasing the inhibition of free radicals in the treatment with JA was due to the activity of antioxidant enzymes. Methyl jasmonate regulates catalase activity by increasing reactive oxygen species (ROS) production, thereby increasing the levels of secondary metabolites for large-scale production (Khan et al., 2019; Taj et al., 2019). These are in line with the findings of the present study.

Methyl jasmonate-treated adventitious roots in *Artemisia amygdalina* L. have demonstrated a high level of antioxidant activity, up to 89%. Also, a high antioxidant activity (87%) has been recorded in the methyl jasmonate-treated adventitious root suspension cultures of *Artemisia scoparia* (Taj et al., 2019). When JA is added to the culture medium, *Lavandula angustifolia* tissues develop strong antioxidant capacities (Andrys et al., 2018). SA and JA showed elevated antioxidant activity in elicited cells of *Panax ginseng* L. (Ali et al., 2007), *Artemisia absinthium* (Ali and Abbasi, 2014), and *Momordica dioica* (Chung et al., 2017) compared to the non-elicited cells. Treatment of *Hypericum perforatum* cell suspensions with JA increased phenylalanine ammonium lyase (PAL) and chalcone isomerase (CHI) activity (Gadzovska et al., 2007).

One of the key enzymes in the phenylpropanoid biosynthesis pathway, which is crucial for the synthesis of flavonoids, lignin, phenols, and many other related compounds, is PAL (Zhang et al., 2015). Treating cell cultures with methyl jasmonate was shown to increase the production of PAL enzymes, leading to an increase in secondary metabolite production.

The phenolic content and taxol biosynthesis of cells also increased under SA treatment; therefore, increasing the concentration of SA induced the production of taxol (Caarls et al., 2015). In methyl jasmonate-treated cell suspension cultures with reduced anthocyanin content, the biosynthetic pathways from anthocyanins to phenolic compounds may have changed (Açikgöz et al., 2019). When JA was added to the *Lavandula angustifolia* Mill culture medium, the polyphenol content rose in comparison to the control (Andrys et al., 2018). In line with our findings, applying JA and SA raised the level of total phenols and flavonoids in cell suspension cultures of *Panax ginseng* L. (Ali and Abbasi, 2014) and *Artemisia absinthium* L. (Ali et al., 2015). Optimal concentrations (100 and 200 µM) of methyl jasmonate and SA as well as optimal exposure period resulted in increased total isoflavone in the cell suspension culture compared to the control (Halder et al., 2019). It has been recorded that the phytoecdysteroid levels increased following 14 days of treatment of *Ajuga bracteosa* with methyl jasmonate. Methyl jasmonate and phenylacetic acid (PAA) also increased the total phenolic and flavonoid contents in *A. bracteosa* root suspension (Saeed et al., 2017). PAL activity has been stimulated by the use of JA and methyl jasmonate in the production of high levels of flavonoids (Park et al., 2019).

Panax ginseng cultures were also treated with 500 µM methyl jasmonate and produced 28-fold more saponin than the control (Lu et al., 2001). A threefold increase in saponin production was observed when 0.2 mM SA was applied to adventitious roots of *Panax ginseng* L. (Lu et al., 2001). JA increased the production of ginsenosides in cell suspension leading to an increase in total saponin content by synthesizing ginsenosides (Lu et al., 2001). In *Glycyrrhiza glabra* var. *violacea* (Boiss.), treatment with 2 mM methyl jasmonate and 1 mM SA led to increased saponin production by 3.8 and 4.5 times (Shabani et al., 2009). β-AS, SS, and SE transcript levels in *Medicago* cell culture were unaffected by the inclusion of SA in the culture medium; however, 24 hours after the cultured cells were exposed to 500 µM methyl jasmonate, β-AS transcription increased by about 50 times (Suzuki et al., 2005).

In the 23-day-old hairy root culture of *Rehmannia glutinosa,* the addition of methyl jasmonate (50 µM) and SA (100 µM) in combination increased the production of iridoids (catalpol and harpagide)

and phenylethanoids (verbascoside and isoverbascoside) compared to the control (Piatezak et al., 2016). By treating the hairy root culture of *Rhinacanthus nasutus* with methyl jasmonate and SA, biomass accumulation decreased, and the content of a group of naphthoquinone esters increased compared to the control (Cheruvathur and Thomas, 2014). When methyl jasmonate and/or SA are applied, the expression levels of important genes in the morphine biosynthesis pathway play an imperative role in the accumulation of these alkaloids at different times. Compared to the control, in methyl jasmonate treatment, the expression of key genes coding SalSyn, SalR, SalAT, and CODM increased and caused the accumulation of thebaine, morphine, and codeine in plants. SA treatment increases morphine accumulation by increasing the regulation of SalSyn, T6ODM, and CODM genes (Halder et al., 2019).

The effect of different concentrations of methyl jasmonate (100, 150, and 200 µM) and SA (125, 250, and 500 µM) was investigated on dopamine production in *Portulaca oleracea* root culture. Results showed that treatment with 100 µM methyl jasmonate increased dopamine in cells by 4.3-fold compared to the control. Treatment with SA did not affect dopamine levels (Moghadam et al., 2001).

In *Tripterygium wilfordii* hair root culture, treatment with methyl jasmonate (50 µM) dramatically stimulated the production of epitrophenidol (tryptolide) and wilfurine, although it resulted in a minor reduction in the concentration of a sesquiterpene pyridine alkaloid. On the other hand, treatment with the same concentration of salicylic acid (SA) had no significant effect on hair root growth and had very little effect on the production of this secondary metabolite (Zhu et al., 2014).

SA influences post-translational modifications of transcription factors and regulators, which in turn influence the activity and localization of transcriptional regulators. Through thioredoxin and glutaredoxin, SA alters the transcriptional regulators responsible for the inhibition of JA-dependent genes. SA affects the transcription stimulated by JA. In order to activate JA signaling, JA-responsive transcription factors must first be destroyed and detached from their target genes. SA can then bind to suppressive proteins in the nucleus or bind to the genes in the cytosol. At the DNA level, changes in histones by SA-dependent factors suppress JA-dependent genes (Halder et al., 2019).

Conclusion

Concomitant treatment of *Portulaca oleracea* with JA and SA increased the antioxidant capacity of the cell suspension by enhancing the levels of secondary metabolites such as phenols, flavonoids, alkaloids, and terpenoids.

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