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Effect of Plant Growth Regulators and Explant Type on Direct Regeneration and Root Induction of Zarrin-Giah (*Dracocephalum kotschyi* Boiss) as a Medicinal Plant

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ABSTRACT

Dracocephalum kotschyi Boiss. (Lamiaceae), known in Persian as “Zarrin-giah” or “Denaei Lemon Balm”, is a medicinal plant native to Iran. It is used in traditional remedies and as a flavoring in tea and doogh (a yogurt-based drink). Extracts of this species contain bioactive compounds with reported therapeutic properties. Efficient micropropagation protocols can support its conservation and large-scale production. This study evaluated plant growth regulator combinations and explant types for direct regeneration and rooting of *D. kotschyi*. A completely randomized experiment with three replications was conducted in 2025 to investigate the effects of cytokinin (BAP), auxin (NAA), and explant type (leaf, hypocotyl, and cotyledon) on regeneration, as well as the effects of Indole-3-butyric acid (IBA) and culture media (MS and ½ MS) on rooting. Measured traits included regeneration percentage, number of shoots per explant, shoot length, number of leaves per shoot, rooting percentage, number of roots, and root length. Results showed that the combination of 1 mg/L BAP and 1.5 mg/L NAA applied to hypocotyl explants produced the highest regeneration percentage and improved shoot quality. In the rooting stage, the interaction between medium type and IBA concentration significantly influenced root formation. The best rooting response, including 100% rooting, occurred in ½ MS medium supplemented with 1–2 mg/L IBA. These results provide a practical protocol for efficient in vitro regeneration and rooting of *D. kotschyi* and support its micropropagation for medicinal use and conservation.

Keywords: *Dracocephalum kotschyi*, BAP (6-benzylaminopurine), NAA (α-naphthaleneacetic acid), Regeneration

INTRODUCTION

Plant tissue culture is a crucial tool in both basic research and commercial applications. Numerous studies dedicated to plant tissue culture have demonstrated its great importance. Despite nearly half a century of research on producing plants through tissue culture, many aspects remain unknown. Through plant growth regulators in tissue culture techniques, explants can be reprogrammed to induce morphological, physiological, biochemical, or ultimately genetic and epigenetic changes in the target plant [1]. Furthermore, the widespread use of secondary metabolites in various fields underscores the need for focused research on increasing their production using plant tissue culture techniques [3].

Plant tissue culture has proven to be an invaluable tool in both basic research and commercial applications, showcasing its vast potential in the field of plant biology. Countless studies have been dedicated to this area, emphasizing the crucial role that tissue culture plays in understanding and manipulating plants. Despite significant progress made in the past decades, there are still many aspects of this technique that remain unknown and require further research. Plant growth regulators, used in tissue culture techniques, can reprogram explants and induce crucial changes in plant morphology, physiology, biochemistry, and even at the genetic and epigenetic level [1]. Moreover, with the increasing use of secondary metabolites in various fields, the demand for focused research on enhancing their production through tissue culture techniques is more pressing than ever [3]. By exploring and enhancing the potential of plant tissue culture, we can further understand and utilize the complex processes of plant growth and development, leading to significant advancements in both scientific research and practical applications.

Local names such as Mishk, Misheh, Ghooch-ooti, Azmireh, and others are used for Zarrin-giah, and in the Iranian plant name culture, it is also referred to as Badranjboyeh Denai, Palang Meshk, and Faranjmeshk [5]. This plant grows in highland and mountainous areas in northern, northeastern, western, and central Iran, is rich in essential oils, and has thus attracted significant attention. Zarrin-Giah holds a special place in traditional medicine and has historically been used as a flavoring agent for tea and doogh (a traditional yogurt-based drink) [5, 7]. The plant’s requirement for specific microclimatic conditions—such as high-altitude, rocky areas with relatively high rainfall—has resulted in small, isolated populations. Consequently, there is a risk of genetic drift and inbreeding depression, leading to reduced genetic diversity. As a result, even minor environmental changes pose a threat of extinction for these populations [5]. The essential oil of Zarrin-giah contains more than 55 medicinal compounds, which explains its extensive use in traditional medicine. The main chemical constituents include myrtenol (30.8%), limonene (23.6%), and geranial (14.3%), along with methyl geranial, flavonoid rosmarinic acid, and monoterpene glycosides [7, 8].

The Lamiaceae family is recognized as one of the largest and most diverse plant families, encompassing over 3,200 species and 282 genera, with a prominent presence in the Mediterranean region. In Iran specifically, the Dracocephalum genus is home to eight herbaceous, annual, or perennial species, renowned for their medicinal and aromatic properties. One such species, *D. kotschyi*, is endemic to Iran and commonly referred to as "Zarrin-giah" or "Dena Lemon Balm" in Persian [8].

The therapeutic effects of Zarrin-giah include treatment of rheumatoid diseases and inflammation, immune system regulation, multiple sclerosis treatment, anti-tumor and anti-cancer properties, antispasmodic, expectorant, lipid-lowering, fever-reducing, wound-healing, antiviral, antifungal, and antibacterial activities, as well as strong analgesic effects [7]. Limonene exerts its antiviral, antifungal, antibacterial, and antispasmodic effects by inhibiting the angiotensin-converting enzyme and also provides analgesic effects. Geraniol is effective in inhibiting polyamine synthesis and cancer cell growth. Additionally, the essential oil contains a compound called spathulenol-Z, which has been used for cancer treatment for many years [11, 13]. As previously mentioned, Zarrin-Giah faces the risk of extinction due to factors such as overgrazing, hard seed coats, irregular germination caused by complex seed dormancy, and steep habitat slopes leading to increased soil erosion and shallow soil depth, along with poor establishment of the plant during the first year. Given its significant therapeutic effects, propagation and cultivation of this plant are considered essential measures [7].

The medicinal properties of Zarrin-giah are highly regarded in herbal medicine. Its therapeutic effects have been found to range from treatment of rheumatoid diseases and inflammation to immune system regulation and even the treatment of multiple sclerosis. It also boasts numerous other benefits, such as being anti-tumor and anti-cancer, antispasmodic, expectorant, and fever-reducing [7]. The compound known as limonene found in Zarrin-giah is responsible for its strong antiviral, antifungal, antibacterial, and analgesic effects by inhibiting the angiotensin-converting enzyme. Furthermore, the presence of geraniol in the essential oil has been found to inhibit cancer cell growth through the inhibition of polyamine synthesis. Another compound found in the plant, spathulenol-Z, has been used in traditional cancer treatments for many years [11, 13]. However, despite its numerous benefits, Zarrin-giah faces the risk of extinction due to various factors such as overgrazing, complex seed dormancy, and shallow soil depth. It is imperative that measures be taken to propagate and cultivate this plant to preserve its therapeutic effects for future generations [7].

In a study on micropropagation of Zarrin-giah, nodal segments from germinated plants were cultured using the shoot organogenesis method on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA. After 30 days, the highest rooting efficiency was observed with this combination [17]. The rooted plantlets were then transferred to the greenhouse for acclimatization, with a reported survival rate of 90–95%. Researchers studied micropropagation of Zarrin-giah through direct organogenesis and concluded that the best response was obtained from shoot tip explants cultured on MS medium containing 5 mg/l BAP and 0.2 mg/l NAA. Regeneration from hypocotyl and cotyledon explants was unsatisfactory, as they turned into callus after 12 days. Elongation of shoot organs was achieved on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA. The regenerated shoots produced roots optimally on the same medium, and after rooting, the plantlets were transferred to the greenhouse, where they reached the flowering stage with a 95% success rate [16]. Jonoubi *et al.* (2017) investigated indirect regeneration of Zarrin-giah and found that all treatments applied to the hypocotyl axis resulted only in callus formation, with no regeneration observed. Indirect regeneration results from 14-day-old cotyledon explants showed the highest callus induction percentage on MS medium containing 0.5 mg/l BAP and 5 mg/l NAA. Calli transferred to regeneration medium containing 2 mg/l BAP and 0.2 mg/l NAA exhibited a 33.3% regeneration rate [13]. Additionally, to induce shoots via indirect regeneration, cotyledon explants cultured on medium with 1 mg/l BAP and 1 mg/l NAA showed a 53.3% regeneration rate. The best rooting percentage was 75% in rooting medium containing 2 mg/L NAA. Regenerated plants were transferred to the greenhouse for acclimatization after root development. Razavizadeh and Adabavazheh (2018) studied the application of chitosan under laboratory conditions and its effects on the physiological traits and essential oil content of Zarrin-giah. They cultured 14-day-old plantlets on MS medium containing 0, 5, 10, and 20 mg/l chitosan. After four weeks, the essential oil components thymol, p-cymene, and caryophyllene oxide increased by 16.2%, 20.4%, and 34%, respectively [19]. Taghizadeh *et al.* (2020) investigated the optimization of callus induction and cell suspension culture for secondary metabolite production in Zarrin-giah. For callus induction, explants from hypocotyl, stem, and leaf were used with 1 mg/l NAA and 4.5 mg/l BAP, while root explants were cultured with 1 mg/l NAA and 6 mg/l BAP. The cell suspension culture showed the highest growth rate in B5 medium supplemented with 1 mg/l NAA and 2.5 mg/l BAP [22].

In a recent study on micropropagation techniques for the Zarrin-giah plant, researchers have discovered a high success rate using a combination of 2 mg/l BAP and 0.5 mg/l NAA on MS medium for shoot organogenesis of nodal segments. After a period of 30 days, the rooted plantlets were then successfully transferred to a greenhouse with a promising survival rate of 90-95% [17]. Further investigations have been conducted to compare the effectiveness of direct and indirect organogenesis methods. Shoot tip explants cultured on MS medium containing 5 mg/l BAP and 0.2 mg/l NAA were found to have the best response, while regeneration from hypocotyl and cotyledon explants proved to be unsuccessful, resulting in callus formation. However, elongation of shoot organs was achieved on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA. Subsequent rooting of the regenerated shoots was successfully achieved on the same medium, with a 95% success rate [16]. Other studies have explored indirect regeneration techniques, but results from treatments applied to the hypocotyl axis only resulted in callus formation [13]. The highest callus induction percentage was achieved with 14-day-old cotyledon explants on MS medium containing 0.5 mg/l BAP and 5 mg/l NAA. The calli were then transferred to a regeneration medium containing 2 mg/l BAP and 0.2 mg/l NAA, resulting in a 33.3% regeneration rate [13]. In addition, through the use of indirect regeneration, it has been reported that cotyledon explants cultured on medium with 1 mg/l BAP and 1 mg/l NAA exhibited a high regeneration rate of 53.3%. Moreover, optimal results for rooting were achieved when the rooting medium contained 2 mg/l NAA, resulting in a 75% rooting percentage. After successful root development, the regenerated plants were acclimatized in the greenhouse. Researchers Razavizadeh and Adabavazheh (2018) have explored the impact of chitosan on the physiological traits and essential oil content of Zarrin-giah under laboratory conditions [19]. They found that culturing 14-day-old plantlets on MS medium containing 0, 5, 10, and 20 mg/l chitosan resulted in increased thymol, p-cymene, and caryophyllene oxide by 16.2%, 20.4%, and 34%, respectively [19]. In a separate study, Taghizadeh et al. (2020) have focused on the optimization of callus induction and cell suspension culture to enhance secondary metabolite production in Zarrin-giah [22]. Their results indicated that the use of 1 mg/l NAA and 4.5 mg/l BAP for hypocotyl, stem, and leaf explants, and 1 mg/l NAA and 6 mg/l BAP for root explants, resulted in successful callus induction [22]. Additionally, the highest growth rate for the cell suspension culture was observed in B5 medium supplemented with 1 mg/l NAA and 2.5 mg/l BAP. These findings contribute to the efforts in understanding effective methods for tissue culture and secondary metabolite production in Zarrin-giah [22].

However, findings across studies show considerable variation, and regeneration success has been strongly dependent on explant type and growth regulator combinations. Importantly, inconsistent results—especially regarding regeneration from hypocotyl and cotyledon explants, as well as optimal hormonal balances for rooting—indicate that the tissue culture protocol for *D. kotschyi* remains insufficiently optimized. Therefore, the main purpose of this study is to develop an improved and reliable tissue culture protocol for *D. kotschyi* by identifying the most effective combinations of plant growth regulators (cytokinins and auxins) and explant types for both shoot regeneration and root induction. By optimizing these parameters, the study aims to support large-scale propagation, conservation efforts, and future biotechnological applications of this medicinally valuable plant.

In light of numerous studies, it has become evident that the success of regeneration in *D. kotschyi* is significantly influenced by the type of explant used and the combination of growth regulators applied. However, inconsistencies in results, particularly regarding regeneration from hypocotyl and cotyledon explants, as well as the optimal hormonal proportions for root induction, indicate the inadequacy of current tissue culture protocols for this plant species. As such, the primary objective of this study is to devise an improved and dependable tissue culture protocol for *D. kotschyi* by determining the most effective combinations of plant growth regulators, including cytokinins and auxins, and explant types for both shoot regeneration and root formation. The ultimate aim of this investigation is to optimize these parameters to facilitate large-scale propagation, conservation efforts, and potential biotechnological applications of this valuable medicinal plant.

MATERIALS AND METHODS

The experiment was conducted in the tissue culture laboratory of the Faculty of Agriculture, Campus of Agriculture and Natural Resources, Razi University of Kermanshah in 2025. In this study, seeds of *D. kotschyi* obtained from Bazram Company were used as the source for explant preparation.

The experiment undertaken in the tissue culture laboratory of the Faculty of Agriculture at the Campus of Agriculture and Natural Resources, Razi University of Kermanshah, in the year 2025, aimed to explore the potential of *D. kotschyi* seeds obtained from Bazram Company as a source for explant preparation. With meticulous care and methodological precision, researchers experimented in a controlled environment, ensuring scientific integrity and accuracy in their approach.

Preparation and Planting of Seeds

To break seed dormancy, the seeds were first soaked in 98% sulfuric acid for 15 minutes. For sterilization, the seeds were disinfected with 70% ethanol for two minutes, followed by treatment with 2% sodium hypochlorite for five minutes with constant shaking, and then rinsed with distilled water. The sterilized seeds were cultured on MS medium containing 750 mg/l gibberellic acid. The plants derived from seed germination and growth were used as the source of explants (Fig. 1).

The breaking of seed dormancy is a crucial step in the successful propagation of plants. To ensure optimal growth and development, proper measures such as soaking the seeds in 98% sulfuric acid for 15 minutes and sterilization with 70% ethanol for two minutes, followed by treatment with 2% sodium hypochlorite for five minutes and constant shaking were taken. The seeds were then rinsed with distilled water to remove any residual chemicals. Once sterilized, the seeds were cultured on MS medium supplemented with 750 mg/l gibberellic acid. The resulting seed germination and growth produced healthy plant specimens that were used as the source of explants for further experimentation (Fig. 1).

Fig. 1 Seeds of *D. kotschyi*, Sterilized seeds and Plants derived from seed germination Respectively from left to right.

Subculturing of Plantlets in Direct Regeneration Medium

The direct regeneration experiment was conducted in a completely randomized design (CRD) with three replications. In this experiment, different explants, including leaf, cotyledon, and hypocotyl, were cultured on MS medium containing various concentrations of NAA (0.25, 0.5, 1, and 1.5 mg/l) and BAP (0.5, 1, and 1.5 mg/l). Depending on the type of explant, the cultured samples were subcultured every two weeks to one month.

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Rooting Medium

In the rooting experiment, to evaluate the rooting ability of the *in vitro* regenerated plantlets, the effects of different culture media (MS and ½ MS) combined with various concentrations of the plant growth regulator IBA (1 and 2 mg/l) were assessed. For this purpose, shoots measuring 1–2 cm in length were transferred to the mentioned media, and after about one month, traits such as root length and rooting percentage were recorded. A control was also included for each treatment.

In the rooting experiment, the rooting ability of *in vitro* regenerated plantlets was evaluated by assessing the effects of different culture media (MS and ½ MS) combined with varying concentrations of the plant growth regulator IBA (1 and 2 mg/l). For this, shoots measuring 1-2 cm in length were transferred to the mentioned media, and after approximately one month, traits such as root length and rooting percentage were recorded. Additionally, a control group was included for each treatment.

Acclimatization and Transfer to the Greenhouse

For acclimatization of the rooted plantlets to environmental conditions, the planting substrate, a mixture of cocopeat and peat moss in equal proportions (1:1), was autoclaved before use. After transferring the rooted plantlets to soil, the area around them was covered with plastic domes to maintain humidity. During the acclimatization period, the plastic covers were gradually perforated using a syringe, and the plants were irrigated with regular water. Finally, after the complete removal of the plastic covers, the plants were transferred to the greenhouse.

To acclimatize the plantlets to their new environment, a planting substrate consisting of equal parts cocopeat and peat moss (1:1) was autoclaved before use. The rooted plantlets were then transferred to the substrate, and plastic domes were placed over the soil to maintain humidity. Over time, small holes were made in the plastic covers using a syringe, and the plants were watered with regular water. Once the plants had adjusted, the plastic covers were removed, and the plants were moved to the greenhouse.

Measured Traits

The measured traits included Regeneration percentage, Number of shoots per explant, Shoot length, Number of leaves per shoot, Root length, Number of roots, and Root percentage.

Statistical Analysis

Analysis of variance (ANOVA) was performed using MSTAT-C software, and the mean values were compared using LSD at a 5% probability level.

RESULTS AND DISCUSSION

The analysis of variance (ANOVA) results for the direct regeneration traits of the *Zarrin-Giah* under tissue culture conditions revealed significant interaction effects between auxin and cytokinin concentrations (Table. 1). Specifically, the interaction between these two classes of plant growth regulators had a statistically significant effect ($p < 0.01$) on the number of shoots and number of leaves per explant, and a significant effect ($p < 0.05$) on shoot length (Table. 1). These findings indicate that the combined influence of auxin and cytokinin plays a critical role in modulating organogenic responses in Zarrin-giah, more so than their individual effects alone.

The significance of the interaction effects suggests that the optimal morphogenic response is not achieved by simply increasing one hormone type, but rather by achieving a specific balance between auxin and cytokinin. This aligns with the classical model proposed by Skoog and Miller (1957) [21], which emphasizes the importance of the auxin-to-cytokinin ratio in determining the developmental fate of plant tissues *in vitro*. High cytokinin in the presence of low auxin typically promotes shoot formation, whereas higher auxin levels tend to favor root development or callus formation. The highly significant interaction effect on the number of shoots and leaves suggests a strong synergism between these hormones in promoting shoot organogenesis and leaf development. This implies that certain combinations may activate meristematic activity more effectively, leading to increased shoot initiation and leaf expansion.

It is clear that the interplay between auxin and cytokinin is crucial for optimal morphogenesis, and further research is needed to fully understand and harness these interactions for plant tissue culture and regeneration efforts. The significance of the interaction effects between auxin and cytokinin cannot be understated. These findings suggest that the optimal response for morphogenesis is not achieved by simply increasing one hormone, but rather by achieving a delicate balance between the two. This concept is in line with the classical model proposed by Skoog and Miller (1957), which highlights the crucial role of the auxin-to-cytokinin ratio in determining the developmental fate of plant tissues *in vitro*. Generally, a high level of cytokinin in the presence of low auxin promotes shoot formation, while higher levels of auxin tend to favor root development or callus formation. The greatly significant interaction effect observed in the number of shoots and leaves implies a strong synergistic relationship between these hormones in promoting shoot organogenesis and leaf development. This suggests that certain combinations could effectively activate meristematic activity, resulting in a higher frequency of shoot initiation and leaf expansion.

Similar interaction effects have been reported in several medicinal and horticultural species, such as *Withania somnifera* [20], *Stevia rebaudiana* [12], and *Ocimum sanctum* [18], where optimized auxin–cytokinin interactions enhanced shoot multiplication rates and foliage quality. The significant interaction at the 5% level for shoot length also highlights that not only shoot initiation but also elongation is influenced by hormone synergy, albeit to a slightly lesser degree. This may be due to shoot elongation being influenced by other factors such as endogenous hormone levels, explant physiological status, or media composition, in addition to exogenously applied hormones. These results underscore the importance of considering hormone interaction effects, rather than evaluating auxin and cytokinin concentrations in isolation. For effective protocol development in Zarrin-giah tissue culture, attention must be given to both the type and ratio of plant growth regulators used. The significance of these interactions provides a basis for further refinement of hormone combinations to maximize regeneration efficiency and plantlet quality.

Similar interaction effects have been reported in several medicinal and horticultural species, such as *Withania somnifera* [20], *Stevia rebaudiana* [12], and *Ocimum sanctum* [18]. In these cases, optimized auxin-cytokinin interactions have resulted in increased shoot multiplication rates and improved foliage quality. Interestingly, this study also revealed a significant interaction at the 5% level for shoot length, indicating that not only shoot initiation, but also elongation, is influenced by hormone synergy. However, this effect may be slightly less pronounced, as shoot elongation can be affected by other factors such as endogenous hormone levels, explant physiological status, and media composition, in addition to exogenously applied hormones. These findings emphasize the importance of considering hormone interaction effects, rather than solely evaluating individual auxin and cytokinin concentrations. Therefore, for the successful development of protocols in Zarrin-giah tissue culture, careful attention must be paid to both the type and ratio of plant growth regulators used. These significant interactions provide a foundation for further refinement of hormone combinations, ultimately leading to higher regeneration efficiency and better plantlet quality in this species.

Table 1 Analysis of variance for traits in direct regeneration of *D. kotschyi* in tissue culture conditions

S.O.V	df	Regeneration Percentage	Number of shoots per explant	Shoot length	Number of leaves per shoot
BAP	2	0.01 **	28.08 *	2.01 *	4284.69 **
NAA	3	4.93 ns	68.99 **	1.45 *	7425.29 **
BAP × NAA	6	14.82 ns	63.49 **	1.42 *	8286.10 **
Error	24	8.64	7.50	0.46	81.11
C.V%		37.84	55.73	46.05	22.86

*, ** and ns, Significant at the 5%, 1% level and Non-significant Respectively.

In this study, various concentrations of the cytokinin and combined with the auxin were added to the basic MS medium to evaluate their effects on the growth of Zarrin-giah explants. After approximately one month of culturing the explants in different media, each explant exhibited a distinct response to regeneration (Fig. 2). The current study highlights the critical role of plant growth regulator balance, particularly the interaction between cytokinins and auxins, in the *in vitro* regeneration of Zarrin-giah explants. After evaluating 12 different hormonal combinations in MS-based media, it was evident that auxin, specifically naphthaleneacetic acid (NAA), is indispensable for explant survival and regeneration. The failure of any explants to survive in media containing cytokinin alone (BAP) underscores the necessity of auxin in maintaining cell viability and inducing organogenic responses. This finding is consistent with the widely accepted concept that auxin, and cytokinin balance regulate organogenesis in plant tissue culture systems [21].

In this investigation, a range of concentrations of cytokinin, either alone or in combination with auxin, was incorporated into the fundamental MS medium for the purpose of studying their influence on the growth of Zarrin-giah explants. After a period of approximately one month during which the explants were cultured in various media, it was observed that each explant displayed a distinct response to regeneration (Fig.2). This study highlights the vital role that the balance of plant growth regulators, particularly the interaction between cytokinins and auxins, plays in the *in vitro* regeneration of Zarrin-giah explants. After examining 12 different hormonal combinations in MS-based media, it became evident that auxin, specifically Naphthaleneacetic acid (NAA), is crucial for both the survival and regeneration of explants. The failure of any explants to survive in media containing cytokinin alone (BAP) emphasizes the necessity of auxin in maintaining cell viability and inducing organogenic responses. This finding is in accordance with the widely accepted concept that the balance between auxin and cytokinin regulates the process of organogenesis in plant tissue culture systems [21].

Auxins like NAA are known to promote cell division and elongation, particularly in the early stages of callus formation and shoot development [6]. The absence of NAA likely disrupted the endogenous hormonal signaling required for dedifferentiation and subsequent regeneration, leading to complete loss of explant viability in cytokinin-only treatments. Interestingly, the results also showed that neither leaf nor cotyledon explants exhibited regenerative potential under any hormonal condition tested. The regeneration percentage for these tissues remained at zero, suggesting that the developmental stage and physiological condition of the explants significantly influence their totipotency. This observation is supported by prior studies indicating that juvenile tissues such as hypocotyls often possess higher regenerative capacity due to their greater meristematic activity and hormone responsiveness [10, 23]. As a result, only hypocotyl explants were selected for subsequent stages of the tissue culture process, including rooting and acclimatization. This strategic selection is methodologically sound, as focusing on the most responsive explant type can improve the overall efficiency and reproducibility of *in vitro* propagation protocols. Collectively, these findings not only confirm the crucial role of auxin in the regeneration process of the Zarrin-giah but also emphasize the need for careful selection of explant type and hormone combinations. Future studies may explore the addition of other auxins, such as IAA or IBA, or varying the cytokinin type and ratio, to further optimize regeneration responses in recalcitrant explants like leaves or cotyledons.

Auxins are a type of plant hormone that play a key role in promoting cell division and elongation, particularly in the early stages of callus formation and shoot development [6]. In the case of the Zarrin-giah plant, the absence of NAA has been found to disrupt the endogenous hormonal signaling required for dedifferentiation and subsequent regeneration, resulting in a complete loss of explant viability in treatments with only cytokinins. Interestingly, the study also revealed that leaf and cotyledon explants showed no regenerative potential under any hormonal conditions tested, with a regeneration percentage of zero. This suggests that the developmental stage and physiological condition of explants are crucial factors influencing their totipotency. This finding is consistent with previous research indicating that juvenile tissues, such as hypocotyls, possess a higher regenerative capacity due to their greater meristematic activity and responsiveness to hormones [10, 23]. As a result, only hypocotyl explants were selected for subsequent stages of the tissue culture process, including rooting and acclimatization. This deliberate selection of the most responsive explant type is a methodologically sound approach that can improve the overall efficiency and reproducibility of *in vitro* propagation protocols. These results not only confirm the significant role of auxin in the regeneration process of Zarrin-giah but also highlight the crucial importance of careful explant selection and hormone combinations. In future studies, the potential for using other auxins such as IAA or IBA, or varying the type and ratio of cytokinins, may be explored as a means of further optimizing regeneration responses in recalcitrant explant types such as leaves or cotyledons.

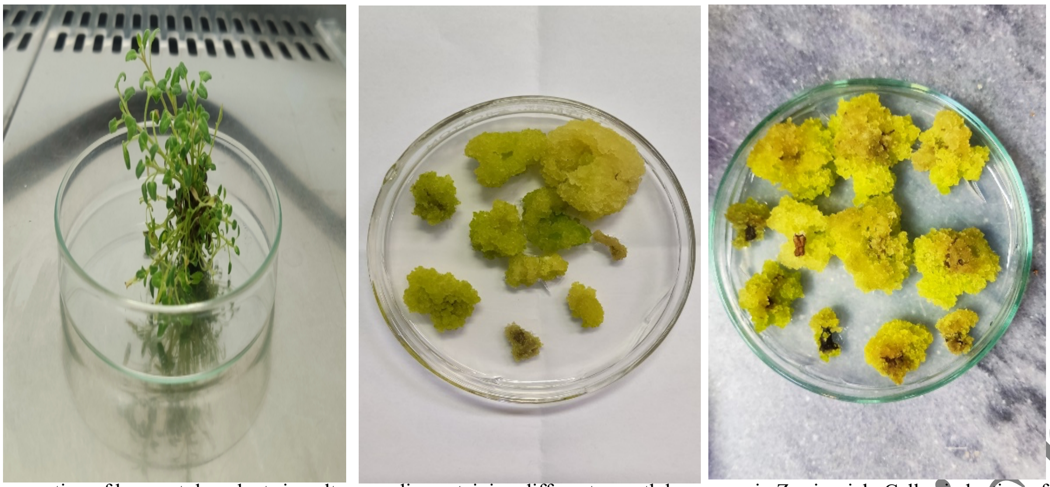

Fig. 2 Direct regeneration of hypocotyl explants in culture media containing different growth hormones in Zarrin-giah, Callus induction of leaf explants in culture media containing different growth hormones in Zarrin-giah and Callus induction of cotyledon explants in culture media containing different growth hormones in Zarrin-giah Respectively from left to right.

The type and concentration of plant growth regulators (PGRs), specifically BAP and NAA, significantly influence direct regeneration traits in *D. kotschyi*. The findings reveal a clear synergistic effect between BAP and NAA, particularly at concentrations of 1.0 mg/l BAP and 1.5 mg/l NAA, which produced the most favorable outcomes across multiple growth parameters. This specific hormone combination resulted in the highest number of shoots (17), the greatest shoot length (2.833 cm), and the highest number of leaves per shoot (163) (Table 2). These results suggest that this balance of cytokinin and auxin effectively promotes both shoot initiation and elongation. BAP is a widely used synthetic cytokinin that stimulates axillary shoot proliferation and inhibits apical dominance [6], while NAA, a synthetic auxin, supports cell elongation and organogenesis when applied in suitable concentrations [14].

The utilization of plant growth regulators (PGRs), specifically BAP and NAA, is crucial in the successful regeneration of *D. kotschyi*. The type and concentration of these hormones have been shown to have a significant impact on direct regeneration traits, as demonstrated by the findings of this study. Of note is the synergistic effect between BAP and NAA, with the most favorable outcomes observed at the concentrations of 1.0 mg/l BAP and 1.5 mg/l NAA. These optimal levels resulted in the highest number of shoots (17), greatest shoot length (2.833 cm), and highest number of leaves per shoot (163) (Table 2). These results highlight the effectiveness of this specific balance between cytokinin and auxin in promoting both shoot initiation and elongation. BAP, a synthetic cytokinin, is renowned for its ability to stimulate axillary shoot proliferation and inhibit apical dominance [6], while NAA, a synthetic auxin, supports cell elongation and organogenesis when applied in appropriate concentrations [14]. Therefore, the utilization of this particular combination of PGRs proves to be advantageous in promoting successful and robust regeneration of *D. kotschyi*.

The interaction between auxins and cytokinins plays a key role in determining the developmental pathway *in vitro*. When these PGRs are present in optimal ratios, they trigger the activation of specific morphogenetic pathways that result in direct shoot regeneration [21]. A higher cytokinin-to-auxin ratio generally favors shoot induction, whereas a higher auxin-to-cytokinin ratio tends to promote root formation or callus development. The optimal ratio observed in this study aligns with similar findings in other medicinal plants such as *Withania somnifera* [20] and *Ocimum sanctum* [18], where comparable BAP–NAA combinations significantly enhanced shoot multiplication and growth. Furthermore, excessive concentrations of these PGRs can have inhibitory effects. For instance, elevated levels of NAA may promote callogenesis or abnormal tissue growth, while high BAP concentrations can lead to vitrification or reduced shoot quality [23]. Thus, the optimal concentrations observed in this study likely maintained the delicate hormonal balance necessary for efficient morphogenesis. The combination of 1.0 mg/l BAP and 1.5 mg/l NAA can be considered an optimal treatment for promoting direct organogenesis in *D. kotschyi*. These findings contribute valuable insight for the development of efficient *in vitro* propagation and conservation protocols for this species.

The complex interaction between auxins and cytokinins plays a crucial role in determining the developmental pathway *in vitro*. Through precise control of the concentrations of these PGRs, specific morphogenetic pathways can be activated, resulting in successful direct shoot regeneration [21]. It has been noted in various medicinal plants, including *Withania somnifera* [20] and *Ocimum sanctum* [18], that a higher ratio of cytokinin-to-auxin favors shoot induction, while a higher ratio of auxin-to-cytokinin promotes root formation or callus development. The optimal concentrations of 1.0 mg/l of BAP and 1.5 mg/l of NAA observed in this study closely align with similar findings in other medicinal plants, highlighting the importance of maintaining a delicate hormonal balance for efficient morphogenesis [23]. Excessive concentrations of PGRs can have inhibitory effects, as evidenced by the promotion of abnormal tissue growth or reduced shoot quality. Therefore, the optimal BAP-NAA combination in this study serves as a valuable treatment for promoting direct organogenesis in *D. kotschyi*, providing valuable insight for the development of efficient *in vitro* propagation and conservation protocols for this species.

Table 2 The effect of BAP and NAA on various traits in direct regeneration of *D. kotschyi* in tissue culture conditions.

BAP (mg/l)	NAA (mg/l)	Number of shoots per explant	Shoot length (cm)	Number of leaves per shoot
0.5	0.25	2.33 d	0.833 cd	8.33 e
0.5	0.5	4.00 cd	0.933 bcd	11.67 e
0.5	1	3.00 d	1.96 abc	6.00 c
0.5	1.5	7.66 bc	22.00 ab	63.67 c
1	0.25	4.66 bcd	2.66 a	52.00 c
1	0.5	1.66 d	20.76 d	7.33 e
1	1	3.33 cd	1.36 bcd	16.67 e
1	1.5	17.00 a	2.83 a	163.0 a
1.5	0.25	3.00 d	1.03 bcd	33.67 d
1.5	0.5	2.00 d	1.13 bcd	4.33 e
1.5	1	8.66 b	1.26 bcd	102.00 b
1.5	1.5	1.66 d	0.933 bcd	4.00 e

Means with the same letters in each column are not statistically significantly different.

The analysis of variance for rooting traits (Table 3) revealed that the interaction effect between hormone levels significantly influenced root length at the 1% probability level and root formation at the 5% level, while it had no significant effect on the number of roots. These findings suggest that the combined application of different hormone concentrations plays a crucial role in determining certain aspects of root development during *in vitro* culture, but not all rooting parameters respond equally to hormonal interactions. The significant interaction effect on root length indicates that specific combinations of hormones synergistically promote the elongation of roots. Root length is an important indicator of root vigor and overall plantlet quality, as longer roots generally contribute to better nutrient and water uptake once transplanted. This result is consistent with previous studies where optimized auxin and cytokinin ratios enhanced root elongation in tissue culture systems [14, 23].

The results of the analysis of variance for rooting traits, presented in Table 3, demonstrate a significant interaction effect between hormone levels on root length and root formation, at the 1% and 5% probability levels, respectively. However, there was no significant effect on the number of roots, indicating that not all rooting parameters respond equally to hormonal interactions. These findings emphasize the importance of combined hormone application in determining certain aspects of root development during *in vitro* culture. The significant interaction effect on root length suggests that specific combinations of hormones act synergistically to promote root elongation. As root length is a critical indicator of root vigor and overall plantlet quality, it is noteworthy that the optimized auxin and cytokinin ratios in previous studies have also been shown to enhance root elongation in tissue culture systems. This reinforces the impact of proper hormonal balance on promoting desirable root traits, which contribute to efficient nutrient and water uptake post-transplantation [14, 23].

Rooting was also significantly affected by hormone interactions, though at a lower significance level (5%). This suggests that hormone combinations can influence the ability of explants to initiate roots, but this trait may be governed by additional factors such as explant type, endogenous hormone levels, or culture conditions. Interestingly, the number of roots did not show a significant response to the interaction of hormone levels, indicating that root proliferation might be more influenced by either individual hormone effects or other environmental and physiological factors rather than the hormone interaction per se. This pattern has been reported in some studies where root number remained relatively stable despite variations in hormone treatments [6].

The influence of hormones on rooting is a subject of great significance in plant tissue culture research. In our study, the results revealed that hormone interactions had a significant impact on rooting, albeit at a lower significance level of 5%. This finding implies that hormone combinations may play a crucial role in determining the ability of explants to initiate roots. However, it is worth noting that other factors, including explant type, endogenous hormone levels, and culture conditions, could also be involved in this trait. Interestingly, the number of roots did not exhibit a significant response to hormone interactions, indicating that root proliferation may be more influenced by individual hormone effects or other environmental and physiological factors, rather than the interaction of hormones themselves. This finding is in line with previous studies, which have reported the relative stability of root number despite variations in hormone treatments [6]. Therefore, while hormones certainly play a vital role in rooting, their interactions might not be the sole determining factor, and other complex processes might also be at play.

These results emphasize the importance of optimizing hormone combinations to improve specific rooting parameters, particularly root length and initiation, which are critical for successful acclimatization and growth of regenerated plants. Future studies could explore additional factors that regulate root number to further enhance rooting efficiency in tissue culture protocols. The effect of BAP and NAA hormones on various traits in rooting of *D. kotschyi* in tissue culture conditions (Table 4) showed that plant height is low in the half-strength MS medium, and the plants exhibit limited vegetative growth, with leaves and stems becoming somewhat yellow. However, the rooting speed in the ½ MS medium is higher than in full-strength MS. Overall, the percentage of rooting, as well as the length and number of roots, is greater in the ½ MS medium.

These findings underscore the necessity of carefully selecting and optimizing hormone combinations in order to achieve specific rooting outcomes, particularly in terms of root length and initiation. These factors are critical for the successful acclimatization and growth of regenerated plants. For future research, it would be beneficial to consider other variables that may influence root number and further refine and improve the efficiency of rooting in tissue culture protocols. Analysis of the impact of BAP and NAA hormones on various traits in rooting of *D. kotschyi* under tissue culture conditions (Table 4) reveals that plant height is notably stunted in half-strength MS medium, with limited vegetative growth observed and visible signs of yellowing in the leaves and stems. Despite this, the rooting process appears to occur at a faster rate in the ½ MS medium compared to full-strength MS. On the whole, the percentage of successful root development, as well as the length and quantity of roots, is higher in the ½ MS medium.

The results of this study indicate that ½ MS medium is more effective for root induction than full-strength MS, although it supports less vigorous vegetative growth. Plants cultured in ½ MS exhibited reduced shoot height, along with signs of nutrient deficiency such as chlorosis of leaves and stems. This outcome aligns with previous findings that reduced macronutrient concentrations can limit vegetative growth due to insufficient availability of essential nutrients like nitrogen and potassium [6]. Despite the diminished shoot growth, the ½ MS medium significantly enhanced rooting performance. Both the percentage of rooting and the number and length of roots were higher in half-strength MS compared to full-strength MS. This is consistent with reports suggesting that high salt concentrations in full-strength MS medium may inhibit root formation due to osmotic stress or ion toxicity [2, 4]. Reduced salt strength in the medium may create a more favorable environment for root initiation and elongation by lowering osmotic pressure and improving water uptake.

The findings of the study demonstrate that ½ MS medium is a more effective medium for root induction compared to full-strength MS. Despite supporting reduced vegetative growth, plants cultured in ½ MS exhibited improved rooting performance, as seen through a higher percentage of rooting and longer and more numerous roots. This aligns with previous research showcasing the negative impact of reduced macronutrient concentrations on vegetative growth due to insufficient availability of critical nutrients like nitrogen and potassium [6]. These results also support the notion that high salt concentrations in full-strength MS medium can hinder root formation due to osmotic stress and ion toxicity [2, 4]. Lower salt levels in the ½ MS medium create a more favorable environment for root initiation and elongation by reducing osmotic pressure and facilitating water uptake. Overall, the findings suggest that while full-strength MS may be better for promoting vegetative growth, the use of ½ MS may be more advantageous for inducing roots in plant tissue culture.

Furthermore, the highest rooting speed was observed in the ½ MS medium supplemented with 2 mg/l of indole-3-butyric acid (IBA), supporting the role of auxins in root induction. IBA is known to be one of the most effective auxins for stimulating adventitious root formation in many plant species [9, 15]. Its effectiveness is often enhanced in reduced-strength basal media, where the balance of hormones and nutrients more closely mimics in vivo rooting conditions. Overall, the results suggest that while full-strength MS medium may be more suitable for shoot proliferation and vegetative growth, ½ MS medium, particularly when supplemented with IBA, provides optimal conditions for rooting. These findings can be valuable in refining tissue culture protocols for species where efficient rooting is critical for successful acclimatization and transplantation.

The results of this study show that the ½ MS medium supplemented with 2 mg/l of indole-3-butyric acid (IBA) yielded the highest rooting speed, confirming the involvement of auxins in root induction. IBA, known for its effectiveness in promoting adventitious root formation in various plant species, is shown to be most effective in reduced-strength basal media [9, 15]. This is attributed to the balance of hormones and nutrients in these media, which resemble in vivo rooting conditions. While full-strength MS medium may be better suited for shoot proliferation and vegetative growth, the results suggest that supplementing ½ MS medium with IBA provides the optimal conditions for rooting. These findings hold significance in improving tissue culture protocols for species that require efficient rooting for successful acclimatization and transplantation.

Table 3 Analysis of variance for traits in rooting of *D. kotschyi* plant in tissue culture conditions

S.O.V	df	Rooting percentage (%)	Number of roots	Root length (cm)
BAP	1	75.00 *	0.01 **	0.18 ns
NAA	1	675.00 **	48.00 **	1.68 **
BAP × NAA	1	75.00 *	12.00 ns	1.68 **
Error	8	12.50	1.33	0.08
C.V %		3.82	11.17	7.29

*, ** and ns, Significant at the 5%, 1% level and Non-significant Respectively.

Table 4 The effect of BAP and NAA on various traits in rooting of *D. kotschyi* in tissue culture conditions.

Medium	IBA(mg/l)	Root length (cm)	Number of roots	Rooting percentage (%)
MS	1	3.83 b	9.33 bc	90.00 b
½MS	1	3.83 b	11.33 ab	100.00 a
MS	2	3.33 b	7.33 c	80.00 c
½MS	2	4.83 a	13.33 a	100.00 a

Means with the same letters in each column are not statistically significantly different.

Following successful rooting of seedlings in the rooting media, the subsequent hardening phase (Fig. 3) was carried out under controlled conditions in the phytotron, followed by transfer to the greenhouse for further acclimatization and adaptation. This two-step hardening process proved effective, as evidenced by the high survival rate of 85 to 90% among the transferred seedlings. The transition from *in vitro* to *ex vitro* conditions is a critical phase in plant tissue culture, where plantlets must adapt to lower humidity, fluctuating temperatures, and natural light intensity. The use of a phytotron for initial hardening provides a controlled environment with regulated temperature, humidity, and light, which helps to reduce transplant shock and improve seedling vigor. Gradual exposure to ambient greenhouse conditions further supports acclimatization by allowing seedlings to adjust to more variable external conditions.

After successfully rooting seedlings in the designated media, the hardening phase was carried out meticulously in a controlled environment phytotron as shown in Fig. 4. This was followed by the transfer of the seedlings to a greenhouse for further acclimatization and adaptation (Fig. 5). The carefully designed two-step hardening process proved to be highly effective, with an impressive survival rate of 85% to 90% among the transferred seedlings. This phase is a crucial transition in plant tissue culture, where the seedlings are exposed to lower humidity levels, fluctuating temperatures, and natural light intensity. The utilization of a phytotron for initial hardening provides a precisely controlled environment with regulated conditions of temperature, humidity, and light, which ultimately helps to minimize transplant shock and promote the overall vigor of the seedlings. Furthermore, the gradual exposure to variable external conditions in the greenhouse provides a perfect environment for the seedlings to gradually acclimatize and adjust to their surroundings, leading to their successful growth and development.

The high survival rate observed indicates that the rooting and hardening protocols established in this study effectively prepared Zarrin-giah seedlings for successful establishment in soil conditions. Similar survival percentages have been reported in other species where stepwise acclimatization strategies were employed [6]. This high survival rate is critical for the overall efficiency of micropropagation protocols, ensuring that *in vitro* gains translate into viable plants in the greenhouse or field. The combination of rooting *in vitro* followed by phased hardening in the phytotron and greenhouse provides a robust approach to producing healthy, well-adapted seedlings. This method can be recommended for large-scale propagation and conservation efforts of Zarrin-giah and potentially other related species.

The results of this study revealed that the rooting and hardening protocols utilized for Zarrin-giah seedlings were highly effective in producing viable plants that were able to successfully establish in soil conditions. This is evidenced by the high survival rate observed, which is consistent with similar percentages reported in other species when employing stepwise acclimatization strategies [6]. This finding is significant as it demonstrates the successful translation of *in vitro* gains into healthy and well-adapted seedlings in the greenhouse or field. Furthermore, the combination of *in vitro* rooting followed by phased hardening in the controlled environment of the phytotron and greenhouse has proven to be a reliable method for large-scale propagation and conservation efforts of not only Zarrin-giah but also potentially other related species. Overall, these results highlight the importance of stringent protocols in micropropagation for ensuring high survival rates and ultimately, the success of propagation and conservation efforts for valuable plant species.

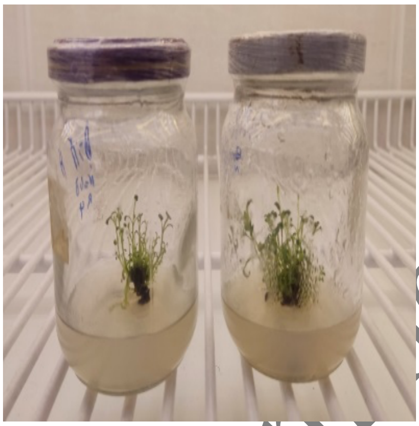

Fig. 3 Plant cultivation in rooting medium

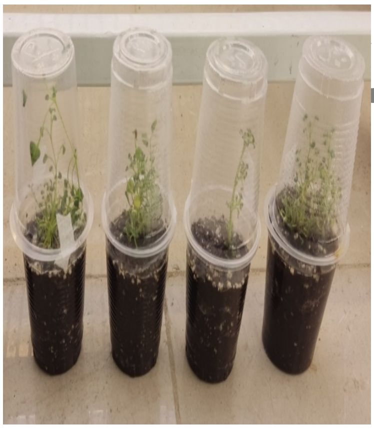

Fig. 4 Acclimatization stage of the propagated plantlets

Fig. 5 *D. kotschyi* plant after acclimatization

CONCLUSION

The findings of this study showed that callus production in this plant was successful; however, these calli did not regenerate into whole plants, so we focused the research program on direct regeneration and succeeded in producing whole plants in this way. Among the explants used, the hypocotyl achieved the best results. On the other hand, the combined use of cytokinin (BAP) at 1 mg/l and auxin (NAA) at 1.5 mg/l had the highest regeneration. To produce roots in the shoots, auxin (IBA) is required. The shoots did not produce roots in an environment lacking auxin. According to the final results obtained, it is possible to maintain and propagate the golden plant through *in vitro* propagation and prevent the extinction of this valuable medicinal plant.

The results of our study have revealed promising advancements in the tissue culture techniques for the propagation of the golden plant. It was found that callus production was successful; however, further observations showed that these calli were unable to regenerate into whole plants. In light of this, our research program shifted its focus towards direct regeneration and was successful in producing whole plants through this method. Notably, the hypocotyl was found to be the most effective explant for this process. Additionally, the simultaneous application of cytokinin (BAP) at 1 mg/l and auxin (NAA) at 1.5 mg/l proved to be the optimal combination for regeneration. Furthermore, in order to induce root formation in the produced shoots, auxin (IBA) was found to be crucial. The absence of auxin in the growth environment hindered root development in the shoots. These significant findings demonstrate the potential of *in vitro* propagation for the maintenance and propagation of the golden plant, thus aiding in the prevention of its extinction.

Conflict of Interests

The authors declared no potential conflicts of interest.

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