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Araştırma Makalesi

# Doku Kültürü Tekniğiyle Çoğaltılan Kalanchoe blossfeldiana Poelln. Sürgünlerinin in vitro veya ex vitro Köklendirilmesi ve Dış Koşullara Alıştırılması

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## ÖZ

Bir süs bitkisi olan kalanşonun (*Kalanchoe blossfeldiana* Poelln.) doku kültürü yoluyla çoğaltılması incelenmiştir. Yaprak parçaları, kalanşo türünde *in vitro* çoğaltım için başlangıç eksplantı olarak kullanılmıştır. Eksplantların çoğaltılmasından elde edilen Kalanchoe sürgünleri, vejetatif olgunluk amacı ile pişkinleştirme aşamasından geçirilmiştir. Pişkinleştirme aşamasında hormonsuz MS + %0.2 aktif kömür ortamı kullanılmıştır. Bu aşamadan sonra köklenme amacıyla 0.1 mg/L IBA ilave edilmiş MS ortamı kullanılmıştır. Ayrıca hormonsuz MS ve ½ MS ortamları da köklendirme denemesinde yer almıştır. Tüm ortamlarda %100 köklenme sağlanmasına rağmen IBA katkısı kök yoğunluğunu artırmış ve köklenme kapasitesini hızlandırmıştır. Vermikülit, kum, vermikülit + kum ortamında dış şartlara geçiş aşamasında; torf ve hidroponik sistem üzerine yerleştirilen viyollere yapılan tüm transfer uygulamalarında %100 başarı ve yaşama oranı sağlanmıştır. Doku kültüründe *ex vitro* köklendirme, üretim maliyetini azaltabilecek bir tekniktir. *Kalanchoe* bitkisinin *in vitro* klonal çoğaltımı, *ex vitro* köklendirme ve dış koşullara alıştırılması işlemleri başarıyla gerçekleştirilmiştir.

**Anahtar kelimeler:** Köklendirme, Kalanchoe, süs bitkisi, in vitro, ex vitro, iklimlendirme

# *In vitro* or *ex vitro* Rooting and Acclimatization of *Kalanchoe blossfeldiana* Poelln. Shoots Propagated by Tissue Culture Technique

## ABSTRACT

The propagation of kalanchoe (*Kalanchoe blossfeldiana* Poelln.), an ornamental plant, through tissue culture has been studied. Leaf pieces were used as initial explant for *in vitro* propagation of kalanchoe. Kalanchoe shoots obtained from the proliferation of explants, have undergone a hardening stage with the aim of vegetative maturity. For hardening of *in vitro* kalanchoe shoots, it was used hormon-free MS medium + 0.2% activated charcoal. After the hardening medium, 0.1 mg/L IBA was added to the MS-medium at the rooting stage were used. Hormone-free MS or hormone-free ½ MS medium were also used as rooting media. Although 100% rooting was achieved in all media, IBA additive increased root density and accelerated rooting capacity. In the media of vermiculite, sand, vermiculite + sand at the stage of transferring to external conditions; 100% success and survival rate was achieved in all applications of transfer to viols containing peat and to the float hydroponic system. *Ex vitro* rooting in tissue culture could be a technique that reduces the production cost. *In vitro* clonal propagation of kalanchoe plant, *ex vitro* rooting, and acclimatization have been successfully carried out.

**Key words:** Rooting, Kalanchoe, ornamental plant, in vitro, ex vitro, acclimatization

## INTRODUCTION

Plant propagation in a tissue culture environment facilitate both micropropagation and lay a groundwork for the use of *in vitro* induction of mutation. *In vitro* cultures can be primarily used in kalanchoe for large-scale reproduction of disease-free clones and maintenance of the gene pool. These options for *in vitro* propagation of *Kalanchoe* is important for the development of new cultivars (Varga et al. 1988). Tissue culture study on *Kalanchoe* plant media using 1.0 mg/L of BAP and NAA each or using 1.0 mg/L BAP and 0.5 mg/L NAA was found balanced and positive in terms of both shoot growth and growth rate (Bejaoui 2022). *Kalanchoe* shoots were incubated twice in regeneration medium that formed 29.19 ± 5.32 to 84.52 ± 24.21 shoots/clusters in the proliferation media (MS medium containing $GA_3$ with or without BAP). For the safe rooting, hardening treatments were applied. In the treatment used 1 mg/L of BAP to induce the highest number of 7.16 ± 0.41 per explant (Bejaoui et al. 2023). The rooting stage is an important stage that prepares the regenerated plants for transport from *in vitro* to *ex vitro* conditions in controlled growth chambers, the greenhouse and then to their final location. This stage may include not only rooting of the shoots but also acclimatization of the plants to increase their acclimatization and survival potential at the time of transplanting. Stimulation of adventitious root formation can be accomplished *in vitro* or *ex vitro* in the presence of auxins (Ostrolucká et al. 2007a, b, Gajdošová et al. 2007). The main advantage of *ex vitro* over *in vitro* rooting is that root damage is less likely to occur during transfer of plants to the soil. It can also provide cost reduction. Root formation rates are generally higher when rooting can be achieved under *in vitro* conditions compared to *ex vitro* conditions. It is also established that root quality can be more easily optimized when rooting occurs under *ex vitro* conditions (Bonga and von Aderkas 1992, De Klerk et al. 1997; 1999, De Klerk 2001). The transfer of regenerated plants to the soil is carried out under natural environmental conditions. Some losses may occur during the transfer of plants formed under *in vitro* conditions to the soil. This situation varies depending on climatic conditions and plant species. Rooted plants that are transferred to the ground and develop roots must be acclimatized in a controlled growth chamber or greenhouse before transfer to their final environment, where they could be grown (Preece and Sutter 1991, Rohr et al. 2003). Plants transferred from *in vitro* to *ex vitro* conditions gradually undergo ontogenetic changes (changes in leaf anatomy and morphology), and their stomata begin to function (the stomata are usually open when plants are in culture). Plants also form a protective epicuticular wax layer on the surface of their leaves. Thus, regenerating plants gradually adapt to survive in their new environment (Donelly and Tisdall 1993). In this study, *in vitro* and *ex vitro* applications were made for rooting of Kalanchoe shoots propagated under *in vitro* conditions. Thus, the study aimed to determine the most suitable rooting conditions for kalanchoe. The maturation and acclimatization stage were also completed and the *in vitro* propagation protocol was for Kalanchoe.

## MATERIAL AND METHODS

During our study of the *in vitro* multiplication of kalanchoe, we used leaf explants. Before the rooting and acclimatization stages, the explants underwent two crucial stages to obtain the regenerating subjects of our studies, which are the regeneration stage and the proliferation stage. In these stages, for the regeneration purposes, 8 different combinations and concentrations of plant growth regulators [four doses of BAP (0.5, 1.0, 1.5 and 2.0 mg/L) and two doses of NAA (0.5 and 1.0 mg/L)] were used. The best treatment used in regeneration was an MS medium reinforced with 1.0 mg/L BAP and 0.5 mg/L NAA. The resulting adventitious shoots were effectively propagated on an MS medium supplemented with 0.3 mg/L $GA_3$ and 0.3 mg/L BAP. Sufficient elongation provided shoots were transferred to a vegetative maturity treatment in order to harden their tissues. This treatment was a ½× MS medium supplemented with 0.2 mg/L $GA_3$ and 0.2% activated charcoal (Bejaoui et al. 2022). Rooting rates, rooting time, and root length at the end of 4 weeks were determined for *in vitro* cultivated kalanchoe shoots, which were transferred to an MS medium enriched with 0.1 mg/L IBA. 1× MS medium and ½× MS medium were compared for rooting after the shoots were hardened. One shoot each was planted in 20 glass tubes for rooting used in both experimental trials. Two different systems were used for acclimatization of kalanchoe plantlets rooted *in vitro* to external conditions: a. Planting in the mortar mixture in viols and acclimatization in mini greenhouses. In this application, three different mortar materials (vermiculite, sand or vermiculite + sand (1:1 v:v)) were filled with plastic violet in mini plastic greenhouses (30 cm width × 50 cm length × 15 cm height) supplied from Bauhaus: (commercial vendor), b. Acclimatization in the climatic chamber under normal atmospheric conditions in Styrofoam viols floating on water.

Rooted kalanchoe shoots were removed from glass tubes, washed under tap water, cleared of nutrient medium with agar, and planted in the mortar mixture. The experiment was established with 6 plants and 3 replications (Figure 1).


Figure 1. Transfer of kalanchoe shoots to mini-greenhouses at the stage of acclimatization to external conditions after rooting

Before the plastic cover was covered, the plants were watered and the leaves were sprayed with a mini hand sprayer. Water spraying was continued by opening the mini greenhouse cover twice a day for 4 days, after which the cover was gradually opened slightly and completely removed at the end of a week. These processes were carried out in the tissue culture laboratory at room temperature (20 °C) and with 16 hours of daily illumination. In the second treatment, Clapa et al. (2013) was taken as a reference and one agar-free rooted plantlet was planted in each compartment of floating Styrofoam trays. The containers were filled with water, which were kept under ambient conditions of room temperature (25±1°C) conditions and darkness for the first 3 days, followed by their transfer to illuminated shelves and kept at 30-32 µMols photons $m^{-2}$ $s^{-1}$ PPFD light intensity under 16/8 h photoperiod arrangement. After 10 days, the number and proportion of living plants was determined. Since the *in vitro* shoots obtained after these applications were transferred to viols containing seedling mortar (commercial peat and perlite mixture) and massively removed to the greenhouse under the misting unit, they developed in a healthy manner close to 100%. The seedlings were transferred to 10x10 cm pots in October 2021 and the plants were grown in an unheated glass greenhouse in Antalya, where the climatic conditions are suitable, until the flowering stage at the end of February. Thus, the replication protocol, of which all stages have been completed, has been put forward.

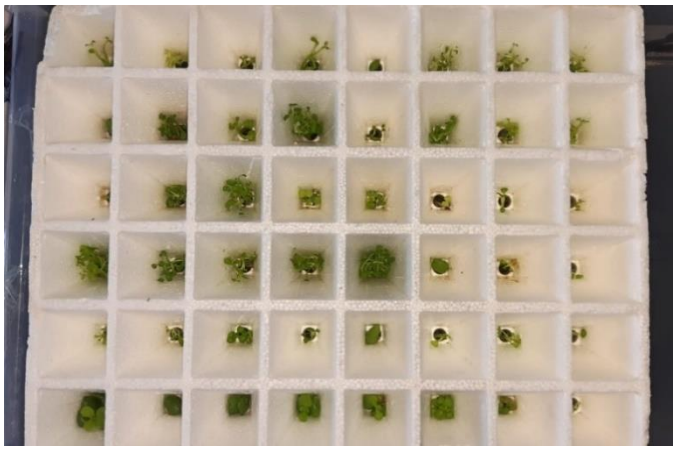

Figure 2. Kalanchoe plantlets acclimatized to external conditions in the hydroponic system in the climate chamber.

## RESULTS AND DISCUSSION

Kalanchoe shoots propagated *in vitro* conditions multiplied very densely and finely. It was grown in hormone-free ½ MS medium with activated charcoal to be homogenized before rooting. In accordance with the opinion of Pan and van Staden (1998) that the shoots obtained *in vitro* should be grown in activated charcoal during the setting stage, in our study, it was possible to grow *in vitro* shoots in a balanced and healthy environment with activated charcoal. In Figure 3, *Kalanchoe* shoots that develop in media containing activated charcoal and can be taken to the rooting stage are seen.

Figure 3. Before the rooting attempt, completion of the curing phase by homogenizing the shoots.

Rooting rates, rooting time, and root lengths at the end of 4 weeks were determined in shoots taken into hormone-free ½ MS, full MS, and MS medium with 0.1 mg/L IBA added during the rooting stage (Table 1) (Figure 4). When kalanchoe shoots were transferred to hormone-free ½× MS, 1× MS and 0.1 mg/L IBA added MS medium during the rooting stage under *in vitro* conditions, no statistical difference was found in terms of rooting percentages, but statistically significant differences ($p \leq 0.05$) were determined among the applications in terms of rooting time and root length. All shoots rooted on 1× MS and ½× MS medium, likewise, this rate was 100% in MS medium with low dose (0.1 mg/L) IBA added. It has been observed that kalanchoe has no problems with rooting, and even rooting occurs when the shoots are kept for a long time during the shoot propagation stage. Rooting medium using 1× MS salt gives positive results in most plant species (Németh 1986).

**Table 1**. Rooting rate, root length and number of days in shoots in 3 different compositions of nutrient media used at the rooting stage.

| Rooting medium | Rooting percentage (%) | Average root length (mm) | Root density | Rooting time (days) |
|---|---|---|---|---|
| 1×MS medium | 100 | 34.60±0.70 b | average | 14.2±0.84 b |
| ½×MS medium | 100 | 45.04±1.50 a | average | 16.4±0.55 c |
| MS medium +1.0 mg/L IBA | 100 | 23.72± 0.78 c | high | 10.00±0.71 a |
| CV% | | 2.88 | | 5.15 |
| nutrient medium | | ** | | ** |

*All means shown by different small letters in a column are statistically different using Duncans test at 0.05 level of significance.


Figure 4. Growth and rooting status of kalanchoe shoots in three different nutrient media during the *in vitro* rooting stage. (a) Hormone-free MS medium, (b) Hormone-free ½ ×MS medium, (c) MS medium + 1.0 mg/L IBA media.

Media where macro and microelements are diluted in ½×, 1/3×, or ¼× can sometimes give better results for rooting (Skirvin et al. 1980, Lineberger 1983). Haifan et al. (2004) recommend a hormone-free ¼× MS medium for kalanchoe at the rooting stage. Bhuiyan et al. (2006) noted that hormone-free ½ × MS medium is ideal for rooting. In terms of root length, ½ × MS medium without hormone gave the highest value (45.04±1.50 mm). This was followed by 1× MS medium (34.60±0.70 mm), and the shortest roots were taken from auxin-doped medium containing 0.1 mg/L IBA (23.71±0.78 mm). The addition of auxin increased root density but decreased root length. Dense and fringe rooting was taken from the IBA-doped 1× MS medium. Auxin group growth regulators have been used to promote rooting under in vitro conditions. For this purpose, IAA, NAA and more intensively IBA are encountered. As a matter of fact, it is seen that IBA and NAA are used in rooting studies of Kalanchoe plant. Peng et al. (2008) reported that ½× MS + 0.2 mg/L IBA medium for rooting were suitable for kalanchoe. Cui and Wei (2003) determined that the best BGD additives for root development in kalanchoe shoots in vitro were 0.1 mg/L NAA and 0.5 mg/L IBA, and the use of nutrient medium (½× MS medium) had a positive effect on plant growth. Cui and Wei (2003) suggested a combination of ½× MS + 0.4 mg/L IBA for rooting. Linjian et al. (2006) provided rooting in 1× MS medium containing 1 mg/L IBA. Xinzheng et al. (2006) preferred 1×MS medium supplemented with 0.4 mg/L IBA, 0.1 mg/L BAP, 1.5 mg/L GA3, and 40 g/L sucrose for rooting instead of using auxin singly. They achieved a success rate of 99% rooting and transfer to external conditions in a white-flowered K. blossfeldiana cultivar. Chen (2007) recommends ½× MS + 0.5 mg/L NAA composition for rooting, Tang (2007), who achieved rooting on 1× MS medium with 0.3 mg/L NAA added, as well as Sanikhani et al. (2006) was one of the researchers who promoted rooting in kalanchoe in medium containing low doses of NAA. Kalanchoe has been observed as a plant species that respond quickly to in vitro

applications and does not require aggressive doses of PGR. This situation also occurred during the rooting phase. Plain media with low strength made it possible to obtain healthier plantlets. However, rooting in IBA supplemented medium occurred 10.00 ± 0.71 days after transplanting. In terms of rooting times, being significant at the p≤0.05 level, 1× MS medium ranked second with a value of 14.2 ± 0.84 days. Rooting in ½ ×MS medium was slower, and rooting was achieved 16.4 ± 0.55 days after the transfer of shoots to the rooting medium. From the stage of propagation, rooting media was preferable according to the needs. All three mediums were found successful, since 100% rooting occurred in all mediums without any difference in rooting rates. Rooted kalanchoe plantlets were planted in (I) Vermiculite, (II) Sand, and (III) Vermiculite + sand (1:1 ratio) and covered for a few days. Transfer counts of acclimatized plants to external conditions are given in Table 2. When these values are examined, it is seen that all of the Kalanchoe plantlets are acclimatized to the external conditions in a healthy way. The survival rate of plantlets transferred to external conditions was determined as 100% in all applications. In terms of average shoot length, it was determined that there was a statistical difference ($p<0.05$) among the applications. Accordingly, the growing medium with the highest average shoot length was "vermiculite" (76,67 ± 0.75 mm). This was followed by the "sand" medium (65.19 ± 1.19 mm), while the "vermiculite + sand" medium took the third place (62.73 ± 0.72 mm). Long shoot length does not indicate that the plant is stronger. On the contrary, in the media where vermiculite alone is used, the hardening was weaker, and the shoots with vermiculite, which has a high water-holding capacity in the root region, were oversized and were weaker as seen in sand substrate. The mature and healthy seedlings were taken from the medium using the same proportions of vermiculite and sand. Figure 5 shows the development of Kalanchoe plantlets transferred to three different substrates. Vermiculite was seen as the least supportive substrate for plant growth in this trial. As a matter of fact, Matsubara and Chen (1989) determined that the weakest development was obtained from vermiculite when they used vermiculite, rock wool and soil media during the acclimatization stage of onion plantlets propagated in vitro. Heliconia spp. using sand, vermiculite and PlantMax® product for acclimatization of in vitro plantlets to external conditions, Rodrigues et al. (2005) similarly report that the use of vermiculite can be preferred in the third place. Vermiculite gives more successful results in the acclimatization phase when it is not used singly, but in a mortar mixture (Wafaa et al. 2017). In our study, the best improvement was achieved when sand and vermiculite materials were mixed in a 1:1 ratio. Indeed, Hong et al. (2002) reported that the addition of 70% vermiculite to the transfer soil during the acclimatization stage showed superior performance in the acclimatization of vine shoots to external conditions.

Table 2. Viability rate and average shoot length after 4 weeks of acclimatization of rooted kalanchoe shoots to external conditions

| Soil mixture medium | Vitality rate (%) | Average shoot length (mm) | The degree of swelling |
|---|---|---|---|
| **Vermiculite** | 100.00 | 76.67±0.75 a | Weak |
| **Sand** | 100.00 | 65.19± 1.19 b | Middle |
| **Vermiculite + Sand** | 100.00 | 62.73± 0.72 c | Good |
| **CV%** | | **1.30** | |
| **Soil mixture medium** | | ** | |

*All means shown by different small letters in a column are statistically different using Duncans test at 0.05 level of significance.

This study also induced rooting through liquid culture successfully. Since the high success achieved in the rooting and acclimatization stages of the kalanchoe plant; it raises the idea that the rooting could also be induced by taking shoots to liquid cultures to root them for three weeks. For the first time, Clapa et al. (2011), induced roots successfully and acclimatized them to external conditions. Thus, for the first time in the production of kalanchoe by tissue culture, successful acclimatization to external conditions was performed in liquid culture. Figure 6 shows some images from this study. Liu (2010) and Duan et al. (2020) used the development method in liquid Hoagland solution using hydroponic system, which is a new application in the acclimatization phase, on *in vitro* shoots of *Trichosanthes kirilowii*. When the researchers examined the survival rate, photosynthetic capacity, pigment content, stomatal density and morphological parameters; they concluded that rooting and acclimatization to external conditions in the hydroponic system is a technique that can be preferred for growing healthy plants. Zhang et al. (2019) also acclimatized the shoots propagated *in vitro* in *Caladium* plants to external conditions in hydroponic systems containing Hoagland solution and stated that this prevents plant loss and provides rapid growth. Ibrahim (2022), carried out to determine the use potential of the float hydroculture in the acclimatization of *in vitro* rooted plantlets and *ex vitro* rooting of microcuttings in AH-32 and AH-75 wild pear types (*Pyrus elaeagrifolia* Pall.) and Old Home x Farmingdale 333 (OHxF 333) pear clonal rootstock (*P. communis* L.). In the study, *ex vitro* rooting could not be achieved in *Pyrus* microcuttings. However, using the float hydroculture technique, *in vitro* plantlets were successfully acclimatized in a 4 weeks period.

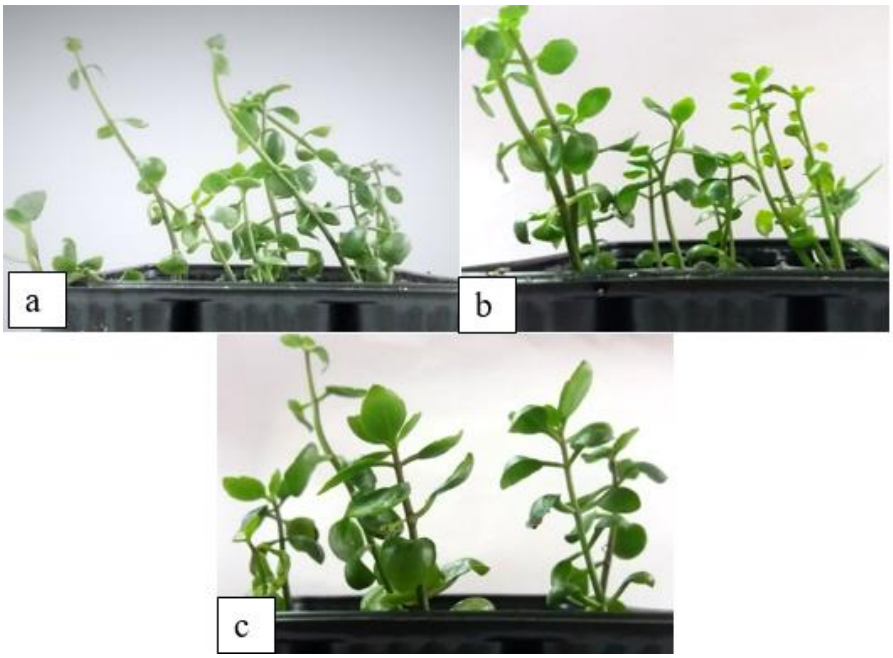


Figure 5. The development status of *in vitro*-obtained kalanchoe shoots at the end of 4 weeks during the acclimatization stage to external conditions in (a) Vermiculite, (b) sand and (c) Vermiculite + sand media.

Figure 6. Kalanchoe plants acclimatized to external conditions in the float hydroponic system containing water.

*Kalanchoe,* in all media continued their healthy growth when transferred to pots. Another application is to transfer the rooted shoots directly to the seedling mortar in the viols and acclimatize them to the external conditions within a week under the misting unit in the greenhouse. In this application, kalanchoe seedlings with 100% establishing rate were grown in a healthy way. As a matter of fact, Hepaksoy and Aksoy (2006) determined that the acclimatization success obtained under mist and in peat medium as a substrate during the acclimatization of the shoots grown *in vitro* to external conditions is high. Intermittent spraying of fine water droplets, is easier for the plantlets emerging from the tissue culture to adapt to the atmospheric relative humidity, making it easier for the plantlets to maintain their vitality until new leaves develop. For this reason, the method of transferring kalanchoe to soil or mortar mixtures and allowing them to acclimate by placing this material under the fogging unit in greenhouses has also been seen as another option for transferring kalanchoe to external conditions. Deng et al. (2005), Zhang and Guo (2005), Peng et al. (2008), Kordi et al. (2013), and Bhuiyan et al. (2006), working on *in vitro* propagation of kalanchoe indicate that rooted *in vitro* shoots have a

viability rate of 85-100% under suitable conditions if they are successfully acclimatized. In vitro rooted kalanchoe plantlets were successfully acclimatized in a mixture of perlite + peat (1:1), vermiculite, and a commercial seedling-growing soil by Altındağ Çelik (2022) also. The results obtained in our study and the information in the sources are compatible. The seedlings developed in our research were transferred to larger pots and flowered in closed greenhouses in February 2022. Thus, a complete cycle was achieved (Figure7).

Figure 7. Kalanchoe plants acclimatized to external conditions on peat substrate in viols followed by transferred to pots to flower.

## CONCLUSIONS

In this study in which *in vitro* and *ex vitro* rooting experiments and acclimatization of *in vitro* regenerated shoots of kalanchoe were investigated, and the results obtained are summarized below:

i. Kalanchoe shoots root easily in vitro conditions. Hormone-free MS, ½× MS or IBA-added MS media can be used for this purpose. Rooting rate was 100% in all media.
ii. New studies can be done on rooting and acclimatization to external conditions in still water hydroponic conditions, the water culture technique was found to be successful in acclimatization to external conditions.
iii. 1:1 vermiculite:sand substrate medium was found suitable for acclimatization to external conditions.
iv. The plants have been healthy grown and have reached the flowering stage.

**Conflict of Interest Statement:** The authors declare that they have no conflict of interest.

**Contribution Rate Statement Summary of Researchers:** The authors declare that they have contributed equally to the article.

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