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Research Article

Development of Petal Culture Method in Kalanchoe (*Kalanchoe blossfeldiana* Poelnn.) and Investigation of its Potential Use in *In Vitro* Mutation Breeding Studies

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ABSTRACT

Kalanchoe (*Kalanchoe blossfeldiana* Poelnn.), is an important potted indoor plant as well as an outdoor ornamental plant and cut flower in recent years. Studies are being carried out using different breeding methods in order to develop new varieties suitable for market needs. *In vivo* and *in vitro* mutation breeding studies are important for creating different genetic variations for this species, which is highly prone to mutation breeding. In *in vitro* mutation applications, vegetative propagation methods are of critical importance in mass propagation of mutant single individuals while preserving genetic stability. This study consists of two stages: First, development and optimization of petal culture method in kalanchoe to provide *in vitro* vegetative propagation of mutant individuals, and second, morphological observation of genetic stability in clones obtained by propagation via petal culture from M1V4 kalanchoe mutant single plants obtained by using ionizing radiation. *In vitro* petal culture conditions were determined for kalanchoe and it was determined that Murashige and Skoog (MS) nutrient medium containing 2.0 mg L^{-1} thidiazuron (TDZ), 0.5 mg L^{-1} 1-naphthalenacetic acid (NAA), 30 g L^{-1} sucrose, 6 g L^{-1} agar and pH 5.7 provided the best regeneration. In addition, after *in vitro* physical mutagen application, flowers were observed in mutant individuals propagated up to M1V4 stage in laboratory conditions and transferred to external conditions. Petals from plants with 4 different mutant flowers selected from these were cultured and propagated *in vitro*. As control, petals from a commercial variety were used for micropropagation. The flowers of the clones obtained showed homogeneity depending on whether the mutant flowers used as starting material were homogeneous or chimeric in appearance. Following this study, in which the first findings on petal culture in kalanchoe were obtained, studies are continuing to develop it comprehensively.

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Kalanşo'da (*Kalanchoe blossfeldiana* Poelnn.) Petal Kültürü Yönteminin Geliştirilmesi ve *In Vitro* Mutasyon Islahı Çalışmalarında Potansiyel Kullanımının Araştırılması

ÖZET

Kalanşo (*Kalanchoe blossfeldiana* Poelnn.), önemli bir saksılı iç mekân bitkisi olduğu gibi son yıllarda dış mekân süs bitkisi ve kesme çiçek olarak da öne çıkmaktadır. Pazar ihtiyaçlarına uygun yeni çeşitlerin geliştirilmesi amacıyla farklı ıslah yöntemleri kullanılarak çalışmalar yürütülmektedir. Mutasyon ıslahına oldukça yatkın olan bu tür için farklı genetik varyasyonlar oluşturmak amacıyla *in vivo* ve *in vitro* mutasyon ıslah çalışmaları önem taşımaktadır. *In vitro* mutasyon uygulamalarında vegetatif çoğaltım yöntemleri, mutant tek bireylerin genetik stabiliteyi koruyarak kitlesel çoğaltımının yapılmasında kritik öneme sahiptir. Bu çalışma iki aşamadan oluşmaktadır. Birincisi mutant bireylerin *in vitro* vegetatif çoğaltımını sağlamak üzere kalanşo türünde petal kültürü yönteminin geliştirilmesi ve optimizasyonu, ikincisi ise iyonize radyasyon kullanılarak elde edilmiş M1V4 kalanşo mutant tek bitkilerden petal kültürü yoluyla çoğaltım yaparak elde edilen klonlardaki genetik stabiliteyi morfolojik olarak gözlemlemek. Kalanşo için *in vitro* petal kültürü koşulları belirlenmiş ve 2.0 mg L^{-1} thidiazuron (TDZ), 0.5 mg L^{-1} 1-naphthalenacetic asit (NAA), 30 g L^{-1} sukroz, 6 g L^{-1} agar ve ph 5.7 içeren Murashige ve Skoog (MS) besin ortamının en iyi rejenerasyonu sağladığı saptanmıştır. Ayrıca *in vitro* fiziksel mutagen uygulaması sonrasında laboratuvar koşullarında M_1V_4 aşamasına kadar çoğaltılan ve dış koşullara aktarılan mutant bireylerde çiçekler gözlenmiştir. Bunların arasından seçilen 4 farklı mutant çiçeklere sahip bitkilerden petaller *in vitro* kültüre alınmış ve çoğaltılmıştır. Kontrol olarak, mikroçoğaltım için ticari bir çeşidin taç yaprakları kullanıldı. Elde edilen klonların çiçekleri, başlangıç materyali olarak kullanılan mutant çiçeklerin homojen yapılı veya kimerik görünümlü olup olmadıklarına sağlı olarak homojenlik göstermiştir. Kalanşo'da petal kültürü konusundaki ilk bulguların elde edildiği bu çalışmanın ardından, kapsamlı bir şekilde geliştirilmesi konusunda çalışmalar devam etmektedir.

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INTRODUCTION

Kalanchoe (*Kalanchoe blossfeldiana* Poelnn.), originating from Africa and belonging to the Crassulaceae family, is a species that stands out among the potted plant group because of its different flower color, flower and leaf structure. The Kalanchoe genus is naturally distributed in tropical climate regions, mostly in Madagascar, East and South Africa, South America, India, the Arabian Peninsula, and Southeast Asia (Descoings, 2003). The genus Kalanchoe includes about 140 species. The propagation of the *Kalanchoe blossfeldiana* species and the development of new varieties of this species began in the 1930s (Kahraman and Boyacı, 2021; Bejaoui, 2022). It is an ornamental plant with increasing demand among potted plants. Kalanchoe is a sought-after succulent plant with hardy and long-lasting flowers and low-maintenance, thick, glossy and showy leaves. The genus includes numerous cultivars traded in different flower colors such as red, orange, pink, yellow, white and purple. It also shows a very high tolerance to drought due to its succulent structure. In addition, it has been cultivated as a cut flower in recent years and there is a trend towards its use as an outdoor ornamental plant (Kahraman et al., 2022). In addition to *K. blossfeldina*, which has commercial value as an ornamental plant, some other Kalanchoe species are also used in traditional medicine (Vargas et al., 2022), anti-cancer studies (Stefanowicz-Hajduk et al., 2022) or for their insecticidal effects (Kolodziejczyk-Czepas and Stochmal, 2017) throughout the world, according to literature.

Kalanchoe has taken the first place among indoor ornamental plants whose economic value is increasingly remarkable in Europe and America (Sanikhani et al., 2006; Jain and Ochatt, 2010). Kalanchoe is the most traded ornamental plant after orchids. In the Netherlands, which ranks first in the ornamental plant market in Europe, 87.1 million *K. blosfeldiana* hybrids were sold in 2015 (Kazaz, 2016). In the world's largest flower auction (Royal FloraHolland) in the Netherlands, it ranked second after the *Phanelopsis* orchid in the 2018 potted ornamental plant sales ranking with 87 million units and a turnover of 53 million Euros (AIPH, 2019). According to the Plant Variety Community Office (CPVO), there are over 700 registered cultivars of kalanchoe worldwide. The majority of these are *K. blossfeldiana*, but several species such as *K. marmorata* Baker, *K. humilis*, *K. manginii*, *K. laciniata*, *K. uniflora* and *K. thyrsiflora* are also registered (CPVO, 2017).

Breeding studies in ornamental plants are carried out for different purposes in many species (Balkaya et al., 2021). These purposes can be listed as; determination of resistance levels to biotic and abiotic stress conditions, plant odor, color, changes in flower structure, differences in flowering time, duration of flower retention and post-harvest vase life (İzgi Denli and Balkaya, 2023) or growth types of plants (tall or dwarf). At the beginning of *K. blossfeldiana* breeding programs, producers grew plants obtained from seeds resulting from selfing of plants because the gene pool they had was narrow and they did not perform interspecific hybridization, and they selected from these plants to obtain new varieties. However, since these are generally tall plants, the first selection studies were carried out to develop dwarf kalanchoe plants (Boiteau and Allorge-Boiteau, 1995). Interspecific hybridization is the most used method in new cultivars development studies in ornamental plants. Interspecific hybridization is of great importance in developing new hybrid kalanchoe varieties and increasing the existing variation in gene pools. The first interspecific hybrids were obtained from interspecific crossing between *K. blossfeldiana* and *K. glaucescens* in 1939 (Mackenzie et al., 2018). After this stage, genetic variation was increased by interspecific hybrids, and different flower types, development patterns, double-flowered and abundant flowering plants were developed. As a result of hybridization of different kalanchoe species such as *K. flammea, K. grandiflora, K. pumila, K. kirkii, K. manginii* with *K. blossfeldiana*, varieties with very different characteristics and flowers of various colors and structures have been obtained over the years (Descoings, 2006). But also, there are pre-fertilization and post-fertilization barriers that affect and prevent success in interspecific hybridization methods (Kuligowska et al., 2015). Germination ability may be very low or not at all in seeds obtained from interspecific hybridizations of kalanchoe. Although obstacles that arise as a result of interspecies hybridization can be overcome with embryo rescue techniques (embryo, ovule, ovary) (Mackenzie et al., 2018), there are difficulties in breeding interspecific hybrids. While traditional breeding methods still maintain their importance, tissue culture techniques integrating biotechnological methods with molecular methods (Lütken et al., 2010; Fujimoto et al., 2022; Jácome-Blásquez and Kim, 2023) have also begun to be used in breeding studies in recent years. In addition to breeding studies, the use of tissue culture techniques has increased considerably for the commercial propagation and production of this species (Gümüş and Ellialtıoğlu, 2018). Another technique for creating alternative genetic variations is mutation breeding, one of the classical breeding methods. Kalanchoe is a species prone to natural mutations. For example, Vlielander (2002) registered two varieties called Leonardo and Bromo by taking advantage of the

genetic variation that occurred because of natural mutation in the *K. blossfeldiana* species. In addition, there are many kalanchoe varieties registered with the American Patent Institute (Kahraman and Boyacı, 2021). It has been scientifically proven that the kalanchoe species has a high ability to form shoots by the adventitious bud method and that mutant individuals can be successfully produced by this method (Van Harten, 2002). Physical and chemical mutagens were used to create mutagenic variation in Kalanchoe (Broertjes and Leffring, 1972; Krupa-Malkiewicz, 2010). To date, 4 mutant varieties have been developed in Kalanchoe through mutation breeding (Flores, Lombok, Sumba and Harvest Moon cultivars) (MVD, 2024).

Research on *in vitro* mutant breeding has been widely used to produce various variants and give biotic and abiotic stress tolerance in various plant species (Kantoğlu et al., 2021). *In vitro* mutations that will cause changes in the flower (color, size, flowering period, number of petals) and leaves (form, size, color) of the kalanchoe plant as well as plant structure and other morphological characters too. It is seen that mutation breeding methods are used to obtain both disease resistance and morphological differences in kalanchoe plants (Li et al., 2019). Mutation breeding studies are carried out on the *in vitro* propagation of a plant bearing a different flower characteristic of a single mutant with petals isolated from that mutant flower (Anand et al., 2020). Mutant material from *in vitro* or *in vivo* mutation breeding trials can be clonally propagated using petal cultures (Datta et al., 2005). According to the results presented to date, *in vitro* petal culture in chrysanthemum can be easily used for both the isolation of solid mutants and plant production (Prathyusha et al., 2021). Although there are many publications on different tissue culture applications such as leaf, petiole, node, callus and protoplast culture in kalanchoe species (Bejaoui, 2022; Winiarczyk et al., 2024), no study has been found on plant regeneration from petal. However, vegetative propagation from flower tissues is extremely important in terms of maintaining mutations such as flower structure, petal color, and multi-layer flower formation in a permanent way. Moreover, important outcomes were achieved in terms of exposing hidden somaclonal variations in the *in vitro* propagation process following mutation breeding, in addition to propagating homogeneous clonal material with petal culture (Datta, 2023). The *in vitro* petal culture can be used to identify point mutations also.

The main goal of our breeding program is to make homogeneous clonal propagation from single mutant kalanchoe plants obtained by using physical mutagen applications. The aim of this study was to reveal the feasibility of propagation with *in vitro* petal culture, which was attempted for the first time for the kalanchoe, to shorten the breeding process and ensure that the method can be used in practice specific to this ornamental plant species. For this purpose, the study was carried out in two stages: First, development and optimization of petal culture method in kalanchoe to provide *in vitro* vegetative propagation of mutant individuals, and second, morphological observation of genetic stability in clones obtained by propagation via petal culture from M1V4 kalanchoe mutant single plants obtained by using ionizing radiation.

MATERIAL AND METHODS

Material

A kalanchoe genotype with white flowers ‘Calandiva White Kalanchoe’, obtained from a domestic producer, was used. A variety with white and double flowers was chosen to ensure that the morphological differences that would occur after mutagen application could be easily distinguished. Flowers that differ as a single flower on the same plant were determined in plants belonging to mutant clones that were propagated up to the M1V4 stage by *in vitro* physical mutagen (gamma rays) application. The petals taken from these flowers were propagated by taking the *in vitro* petal culture as an example, which was previously successfully applied by Datta et al. (2005) for chrysanthemum but had no application for kalanchoe. For this purpose, conditions that provide *in vitro* regeneration specific to the petals of kalanchoe species were determined. In the first stage of the study, flowers of ‘Calandiva White Kalanchoe’ plants and their petals were used to optimize the *in vitro* regeneration method (Figure 1a). After determining the most suitable growth regulator content for regeneration, petals from M_1V_1 mutant individuals were used *in vitro* propagation in the second stage to see if there is homogeneity among plants belonging to mutant clones.

The main material used in the mutation stage of our research project was obtained by culturing mature kalanchoe leaves *in vitro* and propagating the plant through shoot regeneration. After regeneration obtained in MS medium containing 1.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA; 0.1 mg L^{-1} GA_3 was added to the same composition to provide shoot elongation. The use of 1.0 mg L^{-1} BA was sufficient for proliferation. Obtaining

in vitro shoots for mutation purposes and their multiplication rates were explained in detail by Bejaoui et al. (2023a). After optimization of micropropagation of the genotype, *in vitro* mutation application was carried out using a Cesium-137 (^{137}Cs) gamma ray source (821 Gy/h dose rate) and irradiating the kalanchoe shoots (1000 pieces) with an effective mutation dose of 119 Gy, which was determined for the same genotype in another study (Kantoğlu et al., 2024a). *In vitro* mutant shoots obtained from gamma ray-treated shoot explants were sub-cultured four times by using MS medium including 1.0 mg L^{-1} BA. They identified at the clone level. Clones at the M1V4 stage were transferred to external conditions, according to Bejaoui et al. (2023b). The plant and flower development were watched and the differences observed in the mutant clones in the M1V4 generation were evaluated in terms of features such as petal shape, petal color, number of colored petals on a branch, growth type, intense of branch, and single or multiple layer formation. Applications and results obtained for mutation breeding are explained by Kantoğlu et al. (2024a).

Methods

Establishment of petal cultures and determination of the regeneration medium composition

In the first stage of the study, fully bloomed flowers of 'Calandiva White Kalanchoe' plants (Fig. 1a) and their petals were used as beginning explant sources. White and layered kalanchoe flowers were collected from potted plants grown in the greenhouse (25 ºC) in early spring. The flowers brought to the laboratory were first kept under running tap water for 10 minutes for the purpose of surface disinfection protocol. Then the petals were separated from the flower tray and washed again with tap water for 1-2 minutes. Following this, the petals were kept for 10 minutes by rinsing them 3–4 times with sterile deionized water in a sterile cabinet. In the next stage, the petals were shaken in 70% ethanol (EtOH) for 10 min. and washed three times with sterile water. Subsequently, the samples were shaken for 10 min. in a sterilization solution of 15% commercial bleach (ACE brand) prepared with 1-2 drops of Tween 20 and then washed three times with sterile distillated water (Fig. 1b). The water in the breaker was poured well. The glass-breakers containing the petals were turned upside down on filter paper, and the remaining excess water was drained to prevent contamination (Figure 1c).

Explants were placed on MS (Murashige and Skoog, 1962) basal medium, which had a pH of 5.7 and contained 30 g L^{-1} sucrose, 6 g L^{-1} agar, with the growth regulator combinations presented in Table 1. In determining plant growth combinations, BA x NAA, recommended for chrysanthemum petal culture (ray florets) by Datta et al. (2005), and TDZ x NAA, which highly stimulates regeneration from kalanchoe leaves (Bejaoui, 2022), were preferred. The nutrient medium without plant growth regulators constituted the control group. 30 ml of nutrient medium was filled into 70 ml glass jars with autoclavable plastic lids, and the nutrient media were sterilized in an autoclave at 121 ºC for 20 minutes. Explants were cultured in 5 glass jars for each medium composition and 10 petal leaves per jar. After the agar nutrient media were solidified in the laminar flow cabinet and brought to room temperature, the sterile petal leaf explants were placed in the jars with the help of forceps under aseptic conditions (Fig. 1d).

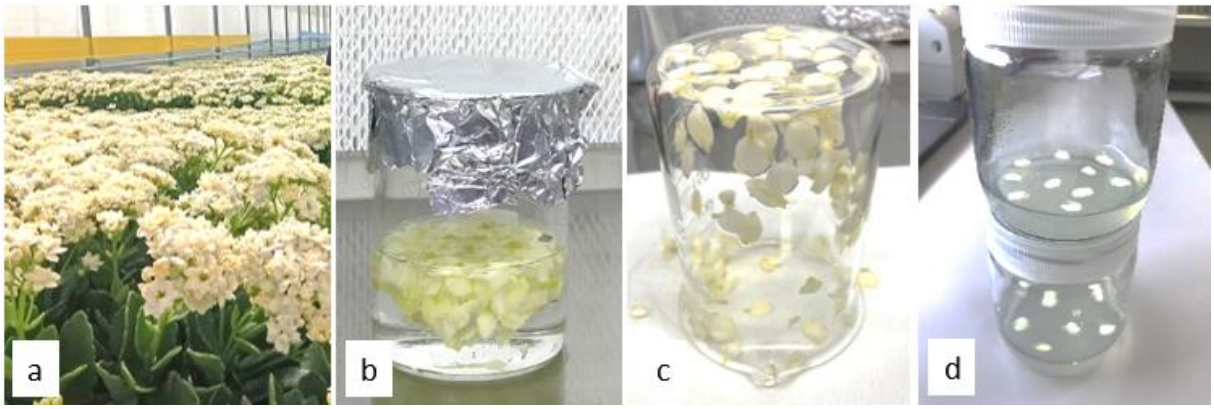

Figure 1. *a. Explant source flowers on the non-irradiated kalanchoe plants in the greenhouse, b. Kalanchoe petals during rinsing by using sterile distillated water, c. Breaker with sterile kalanchoe petals inside, inverted onto sterile filter paper in the laminar flow hood, d. Petals cultured in five different nutrient media contents.*

Table 1. *In vitro petal culture nutrient media combinations*

Medium code	*In vitro* regeneration medium combinations	Sub-culture medium
A1	MS (2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA)	MS (hormone-free)
A2	MS (1 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA)	MS (hormone-free)
B1	MS (1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA)	MS (hormone-free)
B2	MS (4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA)	MS (hormone-free)
C	MS (hormone-free)	MS (hormone-free)

After the explants were planted on the nutrient medium in petri dishes, the cultures were kept for 3 weeks at 25±2 ºC and in constant darkness (Minas, 2007), then in the same temperature regime, 16 hours of light and 8 hours of darkness were adjusted in the photoperiodic regime in the growing chamber that can provide 8 000 lux of light. In the 8th week of incubation, differentiation began to occur on the tissues, and the petals, which were in 3 jars and 10 in each jar, were transferred to fresh media with hormone- free MS content, 5 petals in each jar (6 jars totally for each treatment). Regeneration formation rates were determined in the 10th week of culture. For this, whether there was shoot differentiation on a total of 30 petals belonging to each medium combination was recorded numerically and proportionated as a percentage. Shoot clusters on the explants were separated into small clusters of 1-1.5 cm and transferred to double-layered media in the 14th week. In these media, 30 g L^{-1} sucrose and 7 g L^{-1} agar were used, and pH was adjusted to 5.7. First, MS nutrient media prepared as semi-solid with agar added to 0.25% activated charcoal were added, then 10 mL of MS nutrient media added to 0.3 mg L^{-1} GA_3 was added to each jar in a sterile cabinet. Liquid nutrient media were sterilized using sterile filters and syringes (Ellialtıoğlu and Yanmaz, 1994). After three weeks, the developing shoots were separated one by one and micro-cuttings with 2 nodes were sub-cultured twice in hormone-free MS media, and then rooted in ½ MS media added with 0.1 mg L^{-1} IBA. The acclimatization phase was carried out as applied in previous studies with 100% success (Bejaoui et al., 2023b). The first phase of the experiment ended here.

In vitro regeneration from petals of mutant kalanchoe plants at the M1V4 stage obtained by gamma irradiation and morphological stability control at the flowering stage

In the second stage of the research, fully bloomed flowers and petals of 4 mutant plants obtained by gamma ray irradiation from the mutation breeding project of Has Biotech were used as initial explant sources. One of the 4 different mutants used in the experiment (No: 44) has flowers showing salmon pink homogeneous color. The second mutant has single-layer flowered and white petaled flowers (No: 129), the third is multi-layered and white petaled (No: 65) and the fourth is a mutant with the flower structure of the control plant with white-dark pink variegated petals (No: 42) (Fig. 2).

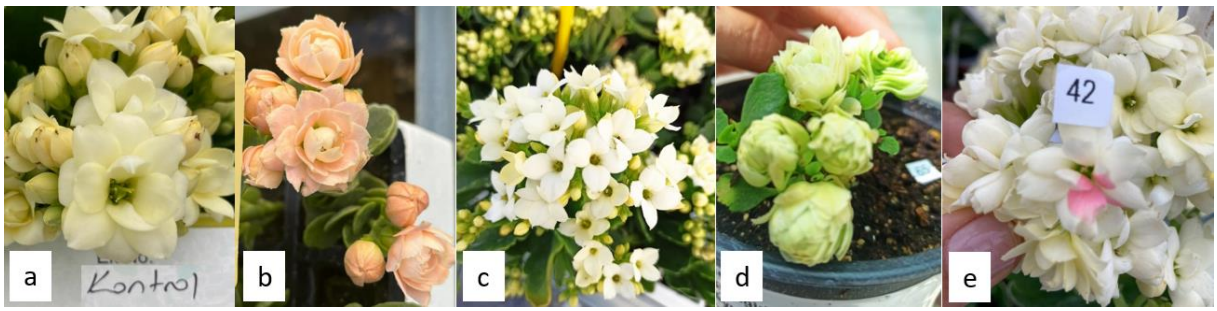

Figure 2. *a. White flowers of control (non-irradiated) plants. b-d: Explant source flowers on the irradiated kalanchoe plants (b. Homogenous salmon pink petals, c. Single layer flowers with white petals, d. Multiple layers flowers with white petals, d. Chimeric heterogenous pink-white petals).*

In the first stage of our study, petals isolated from flowers of 4 different mutants and also petals taken from non-irradiated commercial starting material to form the control group were placed in the most suitable nutrient medium composition determined as MS salts and vitamins included 2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA, 3% sucrose, 0.6% agar and adjusted pH 5.7 for *in vitro* regeneration from petals. General tissue culture conditions,

sterilization of plant material and incubation of cultures were applied as in the first stage. Figure 3 shows petals prepared for cultivation from mutant clones.

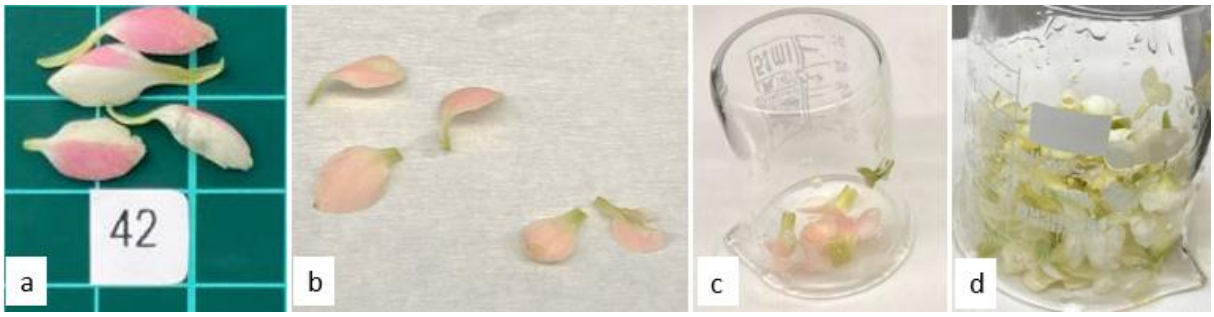

Figure 3. *a. Chimeric leaves of clone number 42, b. Salmon-pink petals of clone number 44, c. Pink mutant petals of clone number 44 before the transfer to culture jars, d. White petals of clone number 65 after the sterilization protocol*

After shoot regeneration was achieved, the petals were transferred to fresh sub-culture media in the 8th week, and the developing clusters were separated in the 14th week and transferred to double layered growth media containing AC (Fig. 4). The developing shoots were sub-cultured twice in hormone-free nutrient media and then transferred to rooting media (0.1 mg L^{-1} + 30 g L^{-1} sucrose + 0.7% agar containing ½ MS medium). The materials that were rooted within a month and continued to form shoots were transferred to peat filled in vials. After irrigation, the seedlings were placed on the benches in the greenhouse and watered twice a day by pulverization for 3 days (Fig. 5). Then, they were acclimated to external conditions by pulverization three times a week and then twice a week.

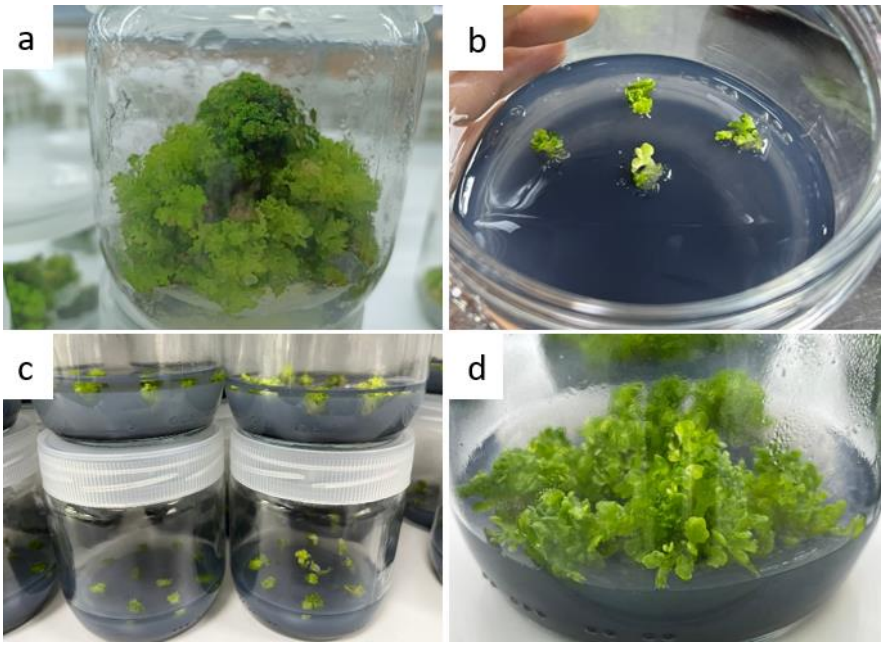

Figure 4. *a. Developed shoot clusters at the 14th weeks of petal cultures, b. Separating the cluster into 10-15 mm pieces and transferring to double layered MS medium, c. Mini plant clusters of mutant clones after transfer into the fresh doubled layered growing medium, d. Growth of healthy shoots from the mini clusters 3 weeks after subculturing.*

Figure 5. *a-c. Transferring kalanchoe mutant clones from in vitro conditions to soil, d. Water pulverization after life water irrigation in the greenhouse.*

During the acclimatization and transfer to the greenhouse of a total of 200 candidate mutant materials within the scope of the project, the plants regenerated from petals in the experiment were planted in vials with 50 plants from each mutant plant. Mutant plants were grown there under optimal conditions at 25 ºC. Flowering occurred after February 20 in the glass greenhouse in Antalya province and observations were made on mutant clones until March 15. Whether flower color was preserved (stability) during the flowering phase in plants regenerated from petal culture was observed under greenhouse conditions. The multiplicity of flowers, petal color and homogeneity rate among 50 plants in total were determined for the plant used as starting material. The breeding program continues and the trials on petal culture were completed with these observations.

Statistical Analysis

To determine the nutrient medium combination that is effective for the regeneration capacity of petals, first stage experiments were set up in three replicates according to a random plot design, with 10 replicates in each repetition. The results were analyzed using MSTACT and MINITAB statistical package programs. The Duncan test was applied for multiple comparisons ($p < 0.01$).

RESULTS and DISCUSSION

Has Biotech Co. has been working on the potential of developing new varieties through kalanchoe breeding since 2019. In this context, it conducts research on developing a mutation breeding protocol. It has carried out pioneering studies in Türkiye on *in vitro* mutation breeding with ^{60}Co-derived gamma ray applications. As is known, breeding studies take a long time. *In vitro* techniques can shorten the time needed for breeding programs. In this study, an *in vitro* petal culture method was applied to mutants with different flower colors on the same plant to propagate mutant individuals with the same color from petals of different colors, as in a previous study conducted in chrysanthemum (Kantoğlu et al., 2024b). Thus, an *in vitro* regeneration protocol through petals specific to the kalanchoe plant was determined. For this purpose, the petal leaves of a commercial kalanchoe variety 'Calandiva White Kalanchoe' used as explants were cultured in A1, A2, B1, B2, and C media. As a result of regeneration studies in the MS medium enriched with four plant growth regulator combinations. In the hormone-free MS medium without any growth regulator additives, no regeneration occurred in the petals at the end of the 14th week. In the study conducted by Bejaoui et al. (2023a; 2024), no development was observed in petiole and leaf explants in hormone-free MS medium, and no shoot formation or callus development occurred in the explants in the 12th week following planting. No callus tissue was formed in the cut areas, and no morphogenesis and shoot or root formation was observed. Indeed, Yuliang et al. (2004), Zhang and Guo (2005), Linjian et al. (2006) also obtained results from nutrient media supplemented with various auxins and cytokinin's for shoot regeneration from leaf explants. Bhuiyan et al.

(2006), in their tissue culture regeneration study from leaf explants not only in *K. blossfeldiana* but also *in K. daigremontiana* species, reported that development was obtained only from explants supplemented with growth regulators, and no development was recorded from hormone-free MS medium. In the cultures with growth regulator additives, color change started to occur from the 4th week onwards, and the white or pink petals started to turn light green and gradually green. In the 8th week, meristematic tissues started to form on the tissues and at their edges, and especially in the A1 and A2 media containing TDZ, compact shoots in the form of clusters were formed (Fig. 6). Data was obtained by counting the meristematic structures formed in the 10th week of culture. However, many shoots could not be expressed numerically due to their compact structure. The tendency to compact cluster formation is less in the media containing BAP and NAA combination. The numerical data obtained are given in Table 2. The control application, in which shoot, and callus formation was not obtained, is not included in the table.

Table 2. *Petal explants' average regeneration values on nutritional media with various growth regulators*

Medium code	Number of flowers isolated	Number of regenerating plants	Regenerated callus number	Mean plantlet number of each explant (petal)
A1	21	191	81	9.10 a
A2	13	84	39	6.46 b
B1	16	0	16	0.00 d
B2	23	6	12	0.26 c

(LSD Val= 0.096, Sx= 0.026, According to the Duncan test, letters define the difference between the means within the limits of $p < 0.01$)

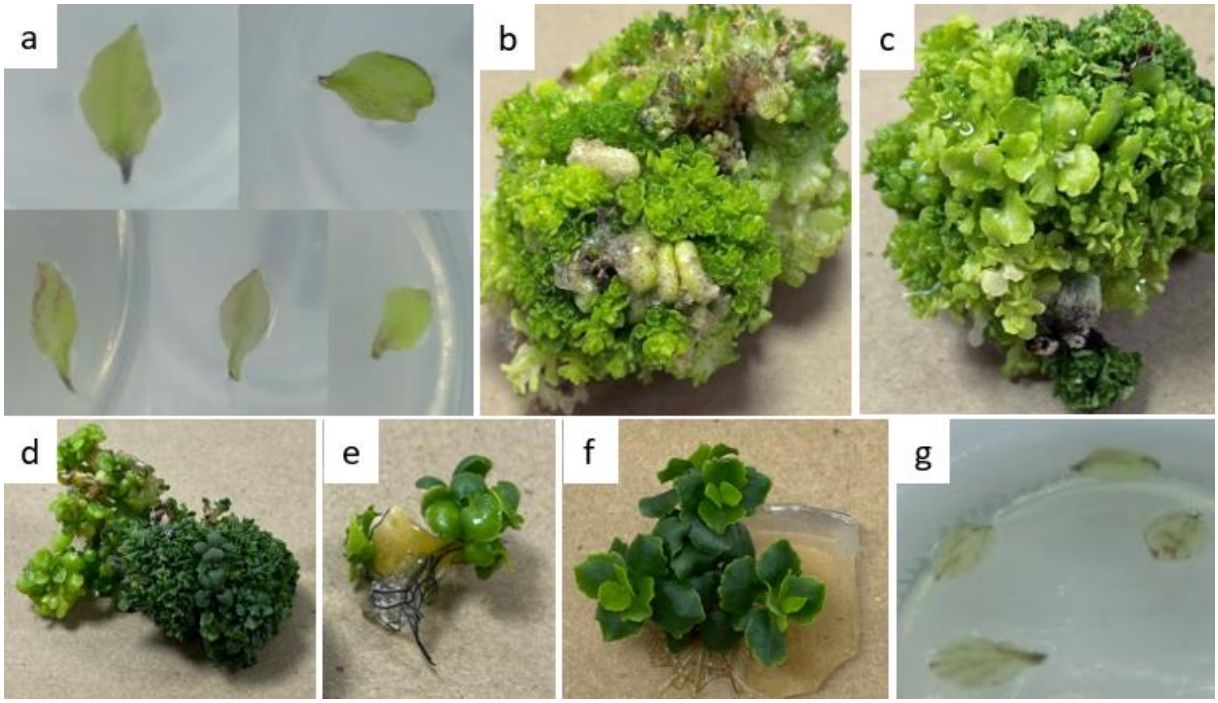

Figure 6. *a. Petal explants starting to turn green in the 8th week of culture, b and c. Shoot organogenesis in the 10th week of culture in A1 medium, d. Meristematic clusters forming in A2 medium, e and f. Shoots and root formation on the explants in B2 medium, g. Petals starting to lose their vitality by necrosis from the 8th week of culture in hormone-free MS medium.*

The best regeneration with an average of 9.10 plantlets per explant was achieved in A1-coded MS nutrient medium containing 2 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA. The study's findings revealed that the A2 nutritional medium was likewise successful in stimulating regeneration, resulting in the growth of 6.46 plantlets per explant. On the other hand, no positive regeneration response was obtained in B1 medium. The combination

of 1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA was insufficient to provide regeneration in kalanchoe petals. The MS medium (B2) to which 4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA was added provided a very low rate of shoot regeneration of 0.26 /petal explants, but these shoots, which were formed in small numbers, developed healthily and strongly during the culture period, indicating that this combination could also be used by increasing the BAP dose. According to the results of the statistical evaluation, the difference between the regeneration capacity provided by the A1 nutrient media and other ones was significant within the error limits of 0.01. After this evaluation made in the 10th week of culture, more than 50 meristematic points and shoot development were detected from each petal starting material that was transferred to fresh media with the same content and kept for development until the 14th week. The development in all explants that reached this period was equalized and no visual or numerical difference was distinguishable between A1 and A2 media (Fig. 4a). After subculturing in double layered media containing activated charcoal, this material was developed healthily and then transferred to media containing IBA as explained in the Method section. The plantlets that were rooted (Fig. 7 a, b) formed healthy kalanchoe plants with 100% success during the acclimatization phase (Fig. 7c). Thus, in addition to the micropropagation method optimized from leaf explants by Bejaoui et al (2023 a, b, c), a successful protocol for kalanchoe plants from petals was presented for the first time in literature with this study. Consequently, it was found that kalanchoes are prone to petal culture and yield good results. Eventually, with the regeneration provided by the cultivated petals, plant production was achieved, and verification could be performed under greenhouse conditions. To date, research on *in vitro* regeneration using petal cultures in kalanchoe species has not been conducted. Although it is not possible to compare the findings for the kalanchoe species, it has been reported that regeneration is achieved in *in vitro* conditions with petal culture in *Chrysanthemum* and *Capparis* plant species; this technique is an effective method, especially in terms of producing a sample plant (Malaure et al., 1991; Datta et al., 2005; Barakat et al., 2010; Carra et al., 2012; Verma et al., 2012; Anand et al., 2020; Asoko et al., 2020; Prathyusha et al., 2021). In Gerbera, which has an important place among ornamental plants, plant regeneration has been achieved from petal explants (Kumar and Kanwar, 2006), and Fritillaria has also entered the literature as a species that can form bulblets directly from its petals (Mohammadi-Dehcheshmeh et al., 2008). Oh and Kim (1994) used *Petunia hybrida* petal explants as starting material for protoplast culture. All of these results demonstrate that *in vitro* petal culture is an alternative technique that makes it possible to reproduce important genetic material (Kantoğlu et al., 2024b). Because it is extremely valuable for breeding work to preserve and propagate the genetic resource that develops only on that plant and cannot be easily reproduced from the flower stem using this method.

Figure 7. *a and b. Rooting of kalanchoe shoots, c. Well acclimatized plants at the flowering stage in the greenhouse.*

Callus development also occurred on explants, although more in media containing TDZ and less in media containing BAP. It was observed that the formed callus was compact and transparent, non-embryogenic, and that the white color turned brown from the 12th week onwards and that it was necrotic at some points. Since the formation of cream-colored embryogenic callus with a fragile and scattered structure did not occur, these structures were not evaluated for breeding or regeneration purposes. In fact, Green and Phillips (1975) reported that different callus types can be induced in plants in tissue culture, i.e. Type I, II and NE callus. Type-I callus is generally white and compact and seems to be a further advanced differentiation step of Type-II callus. This latter type is soft, white or pale yellow, friable, can usually retain totipotency after long periods of time in culture, and is similar to the embryogenic cultures of model plant species (Jiménez and Bangerth, 2001). NE (non-embryogenic) callus is translucent, does not show any sign of organization (Fransz and Schel, 1994). In this study, callus tissue developed from petals did not provide embryogenesis or organogenesis in the media used. It was not planned to obtain regeneration from these structures, which are thought to have a NE callus

appearance. However, in subsequent studies, callus formation from petals and their use in regeneration may be evaluated in the formation of natural or induced somaclonal mutations.

After the gamma irradiation, the kalanchoe plantlets sub-cultured up to the M1V4 stage were transferred to the greenhouse and then observations and evaluations were made within the framework of the mutation breeding program by Has Biotech Co. EMD_{50} (119 Gy) gamma irradiation was successful in creating variations on traits such as flower structure, petal color, plant development and flowering time by providing *in vitro* mutation formation in the studied kalanchoe genotype (Kantoğlu et al., 2024a). Indeed, mutations that will cause changes in the flower (color, size, flowering period, number of petals) and leaves (form, size, color) of the kalanchoe plant as well as plant structure and other morphological characters were observed in previous years too. Broertjes and Leffring (1972) with physical mutagen (200 rad/min X-ray) and Krupa-Malkiewicz (2010) with chemical mutagen (2.0 mM diethyl sulfate (DES) applications, created mutagenic variation in kalanchoe. Fort his study selections of mutant plants were made. In this context, 4 mutant plants were determined to be propagated from petal leaves. These are mutant plants numbered 44, 129, 65 and 42.

Regeneration was provided from petal leaves cultured in A1 medium, 12.20 shoots were determined in the 10th week in plant numbered 44, 10.56 in plant numbered 129, 14.30 in plant numbered 65 and 9.40 in plant numbered 42. The number of shoots consisting of petals taken from the non-irradiated starting material as a control gave a value close to the first stage of the study (11.3/petal explant). However, meristematic regions that continued to develop in the form of numerous green dots in the compact cluster structure were also observed. In the 14th week of culture, a developed cluster structure with over 50 shoot formations was obtained, and these were separated into small clusters as in the first stage and transferred to double layered media containing AC. The plants that went through the rooting and acclimatization stages were allowed to grow in a glass greenhouse in Antalya during the winter period at a minimum temperature of 15 ^{o}C, and they entered the flowering stage in viols at the end of February. The flowers of clones propagated from petals belonging to 4 different mutant plants and the flowers of plants developed from non-irradiated petals were morphologically compared with the flowers of the commercial variety that was the starting plant. In addition, flowering times in the same environment were recorded.

The flowers of the plants obtained from non-irradiated petals were found to have the same morphological structure as the starting material and their flowering times were found to be one-to-one. In the observations made after the mutant clones transferred to the greenhouse had bloomed, four of the five clones produced plants that produced flowers with exactly the same characteristics as the mutant flower in the donor plant. Three of the clones propagated from the petals of the mutant plants (plants no. 44, 129 and 65) had the same flower structures as their mutant donor. While plants no. 44 and 129 did not differ in flowering time from the commercial variety of the starting material, the multi-layer white-flowered mutant no. 65 flowered about a week later. The flowers of the plants resulting from the cultivation of pink-white variegated chimeric leaves also had different colors. These petals were evaluated as chimeric. Pink or pink-white mixed coloration occurred in 8 of 50 plants (16%), and the rest produced white flowers like the starting material (Fig. 8). The results showed that petal culture provides the opportunity to propagate clonally and maintain a single flower mutation. After mutation breeding, studies on the mechanism of the formation of chimeras, especially in flowers, and the mechanisms that stimulate this formation have increased since 2000. Especially in transgenic plants, the change in the gene regions of interest after the application of mutagenesis-inducing chimeras has been among the topics of interest of researchers (Narumi et al., 2008). Applications for tracking chimeric formations for color changes, particularly in liliums, gained prominence following the modeling of Arabidopsis using chimeric repressor gene-silencing technology (CRES-T). Additionally, chimeras were assessed in transgenic technology (Otani et al., 2020). Again, in different studies, cytokinin synthesis on chimeric formations and the functions of the gene regions affected after this synthesis are also examined for transgene technology (Narumi et al., 2008). Therefore, the isolation and reproduction of commercially valuable chimeric formations in mutants is important for breeding studies. Because of this, it is crucial to separate the chimeric forms via petal culture.

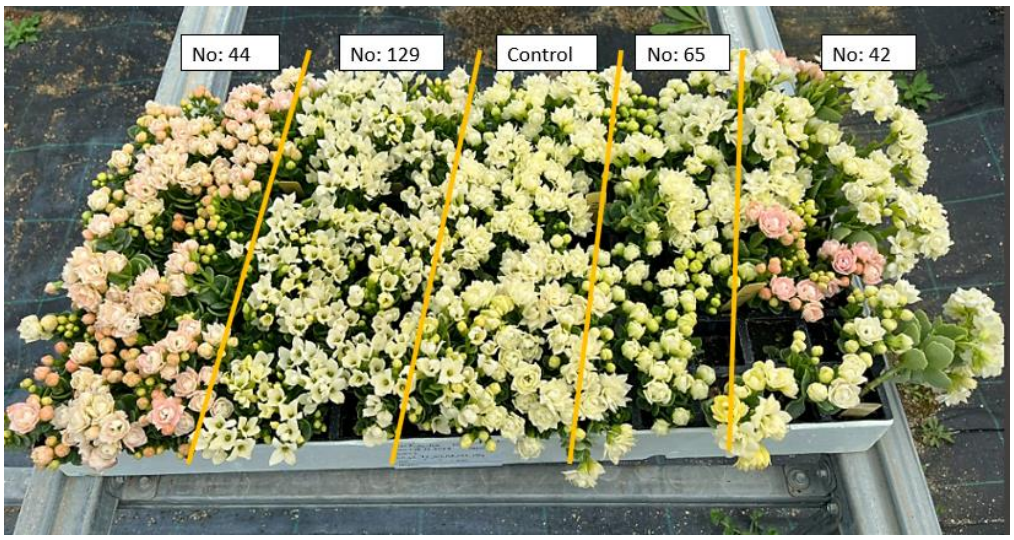

Figure 8. *Stability observation through the clones obtained from petals.*

Regarding *in vitro* mutation applications, Verma et al. (2012) found that petal culture is an effective method for the isolation of solid mutants, whereas in another study conducted on chrysanthemum, mutants selected by this culture multiplied with the same homogeneity at a rate of 98.56%, and 1.44% of somatic mutants were revealed by this method (Kantoğlu et al., 2024b). The results of this research show that it is crucial to introduce petal culture into hybridization or mutation breeding programs for kalanchoe. This research established the viability of ap plying petal culture, particularly in kalanchoe, and showed that a novel explant type could be employed for *in vitro* mutation breeding research studies.

CONCLUSIONS

In vivo and *in vitro* mutation breeding are effective techniques that contribute significantly to the creation of new variations in ornamental plants. Especially in the mutant variation obtained after mutagen application, single flowers with different characters appearing on the same plant are remarkable. It is important to isolate these single flowers, propagate mutant plants with the same structure, and bring them into production as a new variety candidate. As a result of *in vitro* petal leaf culture applications carried out based on this argument in species such as chrysanthemum and capers to date, studies have shown that mutant plants bearing that characteristic can be propagated from petal leaves cultured from flowers with different structures. In this study carried out in kalanchoe, experiments were established based on the argument that the reproduction of single flowers obtained similarly as a result of an *in vitro* mutation breeding study can be done with *in vitro* petal culture. Since petal culture studies have not been carried out for kalanchoe to date, preliminary studies were carried out based on the data previously obtained for chrysanthemum, and different combinations of growth regulators were tried according to the findings obtained. As a result of the treatments, petals regenerated in MS nutrient medium containing 2.0 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA, and plant growth was obtained from explants. The feasibility of *in vitro* petal culture, which was the aim of the research, was revealed in this study. This result shows that it is possible to propagate new mutant clones from single flowers obtained with different characters as a result of mutation or somatic variations in breeding studies carried out in different species. It was found that petal culture is an effective propagation technique that gives results in a short time for *in vitro* mutation breeding studies. With this finding and other successful results obtained for other species in the past, the idea that *in vitro* petal culture may give positive results in other species that have not been tried so far and that preliminary trials should be carried out to adapt it to studies in this context has gained weight.

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AUTHOR CONTRIBUTION

All authors contributed equally.

ETHICAL STATEMENT

During the writing process of the study titled "**Development of Petal Culture Method in Kalanchoe (*Kalanchoe blossfeldiana* Poelnn.) and Investigation of its Potential Use in *In Vitro* Mutation Breeding Studies**", scientific rules, ethical and citation rules were followed; No falsification has been made on the collected data and this study has not been sent to any other academic media for evaluation. Ethics committee approval is not required.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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