In Vitro Regeneration of *Chrysanthemum*

Subjects: Horticulture
Contributor: Eman Abdelhakim Eisa , Andrea Tilly-Mándy , Péter Honfi , Awad Yousef Shala , Mayank Anand Gururani

Chrysanthemum is a flowering plant grown worldwide and is one of the most popular ornamental plants. Chrysanthemums are usually cultivated using root suckers and shoot cuttings. This conventional technique is relatively slow. In addition, as cuttings are gained regularly from mother plants, there is a chance of viral infection and degeneration, which raises the production cost. The hurdles mentioned above have been managed by applying in vitro propagation techniques, which can enhance reproduction rates through in vitro culture and use very small explants, which are impossible with the conventional approach. Usually, it is difficult to get true-to-type plants as the parents with good quality, but clonal propagation of a designated elite species makes it possible.

Chrysanthemum | in vitro propagation | true-to-type plants | explant sources | medium compositions | alternative disinfections | mutagenesis | acclimatization | alternative light sources

1. Introduction

The term ‘chrysanthemum’ comes from the Greek ‘krus anthemon’, which means gold flower, and was initially used in China. *Chrysanthemum morifolium (Ramat),* belonging to the Asteraceae family [1], is considered the second most important floricultural crop worldwide after roses [2]. This is a culturally significant flower with an annual sale of billions of branches. It is propagated in diverse colors, sizes, and forms of composite *Chrysanthemum* flowers by collecting several combinations, concentrations, and types of anthocyanins (purple), carotenoids (yellow), and chlorophyll (green).

Chrysanthemum is one of the most utilized plants in traditional medicine. The flower reduces inflammation and treats bruises, sprains, bites from snakes and centipedes, rhinitis, diphtheria, cholera, and malaria [3]. It also has antipyretic and antihypertensive features [4]. *Chrysanthemum* petals have been used to treat various diseases, such as fever and wind-heat syndrome [5]. Chinese people eat flowers as a salad, and dried petals are used to make tea (tisane) [6].

Plant tissue culture is an in vitro aseptic cultivation of plant cells, tissues, organs, embryos, protoplasts, or seeds on a nutritional medium in a controlled environment in which humidity, temperature, light, photoperiod, and the nutrient medium are the contributing factors for an optimum growing environment. Micropropagation of healthy plants gives faster reproduction rates in a short time [7].

Popularity and demand have made *Chrysanthemum* one of the first commercial targets for micropropagation, allowing the use of tissue culture in the mass production of this flower. The primary method of *Chrysanthemum* propagation is generally done vegetatively with shoot cuttings and root suckers. This conventional process is simple, economical, and can be done in vitro. However, there are limitations to this strategy, such as a low reproduction rate, poor quality of seedlings, higher reproducible time, seasonal constraints, inadequate gene pool, and the inability to avoid cross-incompatibility; additionally, cuttings obtained frequently from mother plants may become infected with viruses and degenerate, which would increase production expenses [8]. These limitations may be modified easily with the available methods of induced mutagenesis and regeneration, in addition to an appropriate, mutable mother plant [9][10]. Hence, there is a requirement for a more effective propagation system. Micropropagation is a rapid and productive way to generate plants on a larger scale to obtain flowers for pyrethrin extraction.

2. In Vitro Plantlet Propagation

Several parts of plants, such as seeds, cuttings, tubers, roots, anthers, pollen, and even leaves, can be used to propagate *Chrysanthemum* in vitro. Leaves could be used as starting materials and seeds to propagate the plant. But vegetative propagation (cuttings, suckers) is favored due to its high heterozygosity and for commercial purposes. However, reproduction is too slow to be commercially viable by this approach [11].

In the micropropagation strategy, “cloning” refers to replicating huge numbers of selected plants with the same genotype as their parent plant through culture [12]. Anderson, in 1980, ref. [13], performed a study wherein he described the five steps of micropropagation: selecting a stock plant, establishing, reproducing, pretransplant/rooting, and finally, transplantation.

Considering the fact that successful procedures developed for one cultivar are not simply adapted to another cultivar, breeders of chrysanthemums face a difficult challenge each year: coming up with several novel and marketable cultivars as rapidly as possible. To effectively improve *Chrysanthemum* cultivars for crop production, it is necessary to create regeneration methods. Thrope [14] compiled a list of cultivar traits thought critical for the success of the morphogenesis process in vitro regeneration. The following factors were considered: (a) the source organ chosen to be utilized for tissue culture, (b) the physiological and ontogenetic age of the chosen organ, (c) the optimum season for acquiring explants, (d) explant size, and (e) the overall quality of the plant from which explants were obtained. From past studies [15][16][17], it was found that many factors that influenced *Chrysanthemum* shoot regeneration in vitro consisted of interacting with the genetic structure of the plant, type of explant, gelling agents, ethylene inhibitors, darkness period, and regulatory factors for plant growth.

2.1. Propagation from Axillary Buds

The ability to regenerate a large number of shoots from cultured tissues is important for the success of most in vitro propagation techniques. The capacity of *Chrysanthemum’s* shoot apex and nodal explants to regenerate in vitro is overall documented. The culture of nodal segments containing axillary buds involves the exploitation of buds

already existing on the parent stock plant, hence providing an efficient method of rapid clonal proliferation enabling the creation of genetically stable and true-to-type progeny [18]. According to [19], the nodal segments of *Chrysanthemum morifulium* L. have been used to design an effective plant regeneration method [20]. Single nodal cuttings can be considered possible propagules for the generation of *Chrysanthemum* plants on a larger scale from tissue culture. A similar study on single nodal cuttings was done wherein it was with an intact leaf and dipped in Hormex solution for 10–15 min, showing excellent survival (93 to 100%) at four weeks, irrespective of treatment, and 100% root growth was also seen, but the number of roots increased in control and decreased with longer dipping times. On the contrary, cuttings without an intact leaf did not respond well to treatments because of low survivability (50%), and only 7–13% of roots could survive.

2.2. Propagation from Adventitious Shoots or Embryos

Direct morphogenesis: New cultivars with unique traits can be regenerated from disc or ray florets by forming adventitious shoots or somatic embryogenesis, a chimeric (or mutant) form in regenerated plants [21][22]. There is a vast potential for commercial *Chrysanthemum* floriculture on an industrial scale in direct floret regeneration methodology [23]. A direct regeneration strategy is preferred to preserve genetic fidelity, as demonstrated by *Chrysanthemum* cv.'s disc and ray florets. For commercial use, 'Kargil 99′ and other mini variations have been standardized for large-scale direct organogenesis multiplication [11].

Indirect morphogenesis: Meanwhile, in the case of indirect morphogenesis, the callus tissue is genetically unstable. The callus formed can help enhance *Chrysanthemum* species' genetics by showing helpful genes or by incorporating new cultivars [24][25]. In addition, the in vitro shoot regeneration of *Chrysanthemum* cv. appears to depend on selecting a donor plant age that produces the most shoots per explant.

3. Basal Medium for Regeneration

Tissue-cultured plants are grown on a synthetic medium containing all the necessary nutrients for rapid growth. Murashige and Skoog's formulation (MS) can be used to grow various plants, resulting in enhanced growth [26]. According to Rahmy et al. [27], MS medium may be substituted with an artificial media, Grow More and a varied concentration of coconut water, to initiate the shoot regeneration of *Chrysanthemum* in vitro Plant tissue cultures can also be done in a liquid or semisolid media with a solidifier. Teixeira da Silva and Kulus, [22] developed a cost-effective method for the large-scale production of chrysanthemums cv. 'Shuhou-no-Chikara' in vitro, using various additions to a liquid-based medium. He also discovered that several alternative additions to the liquid-based medium are available such as coffee, Darjeeling tea, Japanese matcha, low and full-fat milk, Coca-Cola, and oolong tea, which inhibits plant development and reduces the concentration of leaf chlorophyll. Lee et al. [28] stated that agarose outperforms agar in shoot renderability promotion. However, Gelrite is the best gelling agent for accelerating shoot regeneration in *Chrysanthemum* cv. *Borami* and *Chrysanthemum* cv. *Vivid Scarlet* leaf explants than agar, agarose, or Phytagel [15]. Furthermore, psyllium husk can be used as a gelling agent in a culture medium because it is a sticky and mucilaginous substance [29][30]. In addition, [29][30] concerns the formation, proliferation, and long-term survival of in vitro shoots [16][18][31]. Earlier studies have also mentioned that all gelling

agents tested had produced fewer shoots and roots than gellan gum and agar (bacto agar, oatmeal agar, Phytagel, potato dextrose agar, corn starch, and barley starch) [32]. Similarly, Gelrite produced more shoots per explant than agar, agarose, or Phytagel, but silver nitrate prevented the induction of shoots [22]. Similar results were also obtained when plants were cultured on media containing refined sucrose or table sugar, while extracts from *Stevia rebaudiana* (Bertoni), which is used as a substitute sweetener in food products, gave poorer results. Photoautotrophic micropropagation increased shoot mass, and the aeration of the culture vessel enhanced plantlet growth, which resulted in double plant density [22]. The physical state of other solidifier media with poor diffusion properties can impede nutrient flow, resulting in fewer shoots per explant, which is a plausible explanation for these observations [16][33]. Gelling agents and plant growth regulators may work together to boost shoot regeneration [15][16]. Plant tissue culture medium frequently uses sucrose as a carbon source to substitute the carbon, which plants usually fix via photosynthesis but cannot perform in vitro. Occasionally, organic substances such as amino acids and vitamins are added [2].

4. The Alternative Disinfection Methods of Culture Media

One of the most serious issues with micropropagation is microbial contamination, which results in the poor quality of plants and the destruction of beneficial stocks. Also, sterilized culture media may reduce the effectiveness of nutrients and growth regulators for plants [34]. Ornamental plants like chrysanthemums are usually cultivated in disinfected soils by cuttings or cultured in a medium to avoid contamination and maintain optimum conditions for homogeneous substances. Autoclaves are utilized to sterilize any cultured substances in many micropropagation laboratories. Typically, sterilizing is done by autoclave for the culture medium and culture vessels (plastic boxes, glass vessels, nylon bags) for 20 to 30 min at 121 °C and 15 psi. However, autoclaving can result in the production of decomposition products like phenolics and 5-(hydroxymethyl)-2-furaldehyde at a high cost to several micropropagation laboratories [35], and it can also result in the generation of decomposition products such as phenolics and 5-(hydroxymethyl)-2-furaldehyde [36].

4.1. Microponic Systems

Microponic systems are reproduction systems that combine the benefits of micropropagation with hydroponics, which reduces the disadvantages of micropropagation systems of contamination, resource consumption, and the requirement for large spaces [33][37]. The pioneers of this method were [38][39], who used the nutrient film technique and a miniature pump to circulate medium through Rockwool.

4.2. Essential Oils and Chemical Compounds in Tissue Culture Media

Without an autoclave, plant tissue culture material can be sterilized by chemical disinfectants and essential oils (EOs) derived from medicinal plants [40][41]. For example, EOs of trees of betel, cinnamon, clove, holy basil, lemon, lavender, turmeric, and tea (at varying concentrations: 0.9 mL dm^{-3}–12.6 mL dm^{-3}) might also 100% sterilize the medium, which is equivalent to autoclaving [22].

4.3. Nanomaterials in Tissue Culture Media

The interactions between nanomaterials and plant growth have recently piqued the interest of experts worldwide [33][34][42][43][44]. Silver nanoparticles (AgNPs) have previously been shown to prevent microbial infection and the effects of ethylene in micropropagation [33][34][42][43][45][46][47]. Silver and copper nano colloids also have antibacterial, antifungal, and antiviral properties, but they are less toxic and need not be rinsed with sterile water. They can also destroy endophytes by entering the cell through plasmodesmata [48]. In addition, non-autoclaved media can be utilized for cultural purposes due to lower micropropagation costs and lower power consumption (attributed to the absence of autoclaving). Furthermore, AgNPs are critical for improving the growth and development of plants (shoot as well as root length and leaf area), with a better synthesis of chlorophyll and oxidative enzymes, enhancing the carbohydrate and protein content of chrysanthemum. AgNPs, when added to a microponic medium, can enhance plant growth and development while reducing microbial contamination [33][34][46][49][50]. Copper (Cu), gold (AU), and silver (Ag) nano colloids can also be used to eliminate fungal and bacterial contamination in *Chrysanthemum* in vitro cultures [47]. The nano colloids showed encouraging antibacterial and antifungal efficacy, even at lower concentrations for a brief period of disinfection. In addition, there was no apparent injury to plant tissue.

4.4. Reintroduction of Arbuscular Mycorrhizal Fungi (AMF) in Tissue Culture Media

Reintroducing arbuscular mycorrhizal fungi (AMF) into sterilized areas is an alternative technique to eradicate harmful pathogens and beneficial microbes while reaping the benefits of AMF–plant root symbiosis [22]. Since microbial inoculum is essential for developing a strong root system [51], improved growth [52], increased absorption of nutrients and water [7][53], and improved host root resistance to soil-borne diseases [54] and drought stress [55]. Micropropagated plantlets can have proper growth attributed to AMF if they are inoculated with it. This will be reflected in improved plant survival and development after field transplant. AM fungi are an integral part of *Chrysanthemum* micropropagation, which will enhance the uptake of nutrients whether they are given with macronutrients (N, P, K, Ca, and Mg) or micronutrients (Fe, Cu, Zn, and Mn) in both shoots and roots of plantlets that have been inoculated with a mixture of three strains of AMF including, *Acaulospora laevis, Acaulospora scrobiculata,* and *Glomus fasciculatum* [7].

Mycorrhizal inoculation from scarcely soluble sources, such as rock phosphate, is made available for the plant to obtain phosphorous content. The enhanced phosphorus uptake may be due to the increased physical interaction between phosphate particles and the hyphal network between roots and these particles. AMF has been shown to facilitate nodulation and nitrogen fixation in legumes. Mycorrhizal and nodule symbioses often synergistically affect mineral nutrition, infection rates, and plant growth. The enhanced phosphorus uptake by AMF symbionts is beneficial for the nitrogenase enzymatic activity in bacterial symbionts, leading to higher nitrogen fixation and, consequently, stimulating the development of root and mycorrhiza. Further, increased micronutrient uptake may be associated with increased macronutrient cation mobilization in the rhizosphere via secretions of AMF. The blend of specific chelating siderophores by strains of AMF may contribute to enhanced iron absorption [56].

4.5. High-Energy Photons and Electrons

Industrial sterilization, which uses high-energy electrons and photons, is one application of ionizing radiation [57]. According to Miler et al. [10], high-energy electrons are more effective at disinfection than high-energy photons. The effect of radiotherapy on the percentage of sterile *Chrysanthemum* explants was noted, and as the radiation dose increased (gradually, from 55% in 5 Gy photons to 70% in 15 Gy photons), the percentage of sterile ovaries explants also increased. In parallel to the control, the increased level of (complete) infertility was caused by ovaries receiving 10 Gy of high-energy electrons [10].

5. Protocols for Cloning and Large-Scale Plant Production of *Chrysanthemum*

Plant growth regulators or phytohormones, such as cytokinins, gibberellins, auxins, and abscisic acid, as well as their analogs and inhibitors, are essential to controlling the type of growth during the proliferation stage. Growth regulators can have distinct effects on various cultivars due to genotypic changes in their capacity to absorb and metabolize the medium's growth regulators [58]. Variations in growth conditions and explant source age, genetic variations between the genotypes used, or morphogenetic response variations in vitro can contribute to these variations [59].

5.1. Optimization of Phytohormones on the Shoot, Callus, Somatic Embryo, and Root Induction

Chrysanthemum shoot regeneration is induced by the medium's type and concentration of growth regulators. Lower concentrations fail to promote shoot bud regeneration, while higher concentrations have an inhibitory effect as the plant itself can produce hormones [60]. The residual effects of hormones accumulating in cultured explants and the application of plant growth regulators (PGRs) could explain why the number of shoot buds/explants was reduced at higher combined concentrations. Furthermore, Waseem et al. [61] found that augmenting MS medium with increased benzyl amino purine (BAP) reduced recovery. It is due to endogenous cytokinins such as elevated concentrations of 6-benzyl adenine (BA), thidiazuron (TDZ), and BAP used that may have caused adverse consequences and reversed the growth process [62]. In contrast to the common assumption, high levels of auxin cause rhizogenesis, whereas high levels of cytokinin cause ridge formation. Some strains require higher concentrations of cytokinin than auxin or auxin at a higher concentration than cytokinin and comparable amounts of auxin and cytokinin [63]. Further, cytokinins play an important role in shoot regeneration in plant tissue culture, and BAP is one of the most potent cytokinins for inducing shoot regeneration [64]. For example, high levels of cytokinins induce explant germination (6-benzylamino purine (BA), zeatin, kinetin, and 6-(γ,γ-dimethylallylamino) purine) [65]. Cytokinins other than auxin were used for leaf explants to promote efficient direct organogenesis in *Chrysanthemum* [66][67]. Similarly, BAP is more effective than kinetin (Kin) in strengthening shoot amplification in *Chrysanthemum* and other plant species, as mentioned in [68][69][70]. The utilization of different concentrations of cytokinin and auxin in tissue culture of (Pyrethrum) *Chrysanthemum cinerariaefolium* was examined for the first time by Lindiro [71], which investigated nodal explants in MS medium treated with various amounts of cytokinins, 2-

isopentyl adenine (2iP), benzylamino purine (BAP), kinetin (KIN), thidiazuron (TDZ), and cysteine. According to these findings, BAP was superior in propagating axillary shoots, with 5 M BAP yielding the highest average shoot length and 40 M BAP yielding the highest average number of shoots. The superiority of BAP for inducing axillary buds in *C. morifolium* was demonstrated by Pant et al. [18]. The ability of low BAP levels itself stimulates shoot growth and proliferation, and callus development in the presence of auxin reflects high endogenous hormone levels in the mother plant [18]. The endogenous content of cytokinins found in leaves of *Chrysanthemum* was much lower to stimulate shoot regeneration. One of the biological functions of KIN, a cytokinin, is to inhibit apical dominance, increase lateral shoot growth, and create vegetative shoots [72]. It also helps discover and enlarge blood vessels that carry phloem and xylem, inhibiting chlorophyll breakdown, promoting cell division, and improving nucleic acid production [73][74]. Additionally, ClO2 was investigated as a growth stimulant for chrysanthemum tissue culture without using any other known PGRs in a recent study by Tian et al. [75]. It was observed that 10 $\mu g \cdot L^{-1}$ ClO2 caused *Chrysanthemum* regeneration in a single step. This shows that a microgram-grade concentration of ClO2 may stimulate the accumulation of endogenous auxin in *Chrysanthemum*, further encouraging roots and growth. The regenerates formed in a single step and were transplantable within three weeks of culture. The transplantation success rate was 100%.

5.2. Plant Growth Regulators in the Best Combination

The growth regulators' interaction determines the speed and direction of the development of culture supplemented to the medium and those generated endogenously by plant cells (Gunawan et al. [76]). It has been suggested that two important factors, the chemical base and its side-chain groups, could explain the variations in the effectiveness of plant growth regulators on plant development [77]. Different plant or shoot regeneration responses are produced when different culture medium compositions are used; the developmental stages should be considered when choosing the culture medium with various hormone combinations [78].

5.3. Optimization of Light Conditions

High shoot regeneration was attained during the dark incubation period, possibly due to an accumulation of auxin. Above the optimal concentration, auxin aggregation is more likely to prevent shoot regeneration [63]. The incubation period in the darkness of 10 days produced greater shoot regeneration in most cases compared to other durations (0, 20, 30 days), according to Naing et al. [16]. In addition, explants housed in darkness for a week had the greatest degree of shoot regenerability, followed by explants incubated in light (control) [17]. Meanwhile, darkness lasting longer than seven days exhibited inhibiting effects, and longer periods of darkness (in sequence 4 > 3 > 2 weeks) had greater inhibiting effects [16][17]. Endogenous auxins are supposed to accumulate when explants are incubated in the dark; however, excessive auxin aggregation prevents shoot regeneration [79]. In contrast to earlier research, [63] found that *Chrysanthemum* leaf ex- plants benefited most from a 12- to 18-day dark treatment for optimal shoot induction. Variations in explant types, genotypes, and plant growth regulators could bring these changes. On the contrary, a study by Teixeirada Silva and Kulus [22] found that explants of *Chrysanthemum* cv. "Shuhou-no-Chikara" responded uniformly to various plant growth regulators in light and dark circumstances. This is true for both disc and ray florets.

6. Irradiation Treatment In Vitro

Changing the color of the flowers is one of the most important breeding goals. Classical mutation breeding is viable for commercial plant breeders because it does not require advanced molecular laboratories with high-tech instruments or expert technicians with genetic engineering degrees. It is also ubiquitous and does not need an in-depth comprehension of gene sequences, structures, and functions of genes [80]. A remarkable number of novel chrysanthemum varieties are submitted to the Community Plant Variety Office (CPVO) each year. This European organization is equivalent to the parallel office for protecting breeders' property [81]. Greenhouse tests confirm the nominated varieties' uniqueness, uniformity, and stability (so-called DUS tests). In the CPVO department, cuttings of a given cultivar are grown at the CPVO department in a certain number of cuttings (20 for chrysanthemums) are grown, and their novelty is verified based on the assessment of their external traits [82].

MW is a form of electromagnetic radiation (EM) with frequencies between 300 MHz to 300 GHz and wavelengths between 1 m to 1 mm [83]. Following the use of gamma radiation to induce mutations in a purple-flowering cultivar, *Chrysanthemum grandiflorum* (Ramat./Kitam.) emerged with three new phenotypes: light purple (77B), silver–purple (RHSCC code: 77C) and claret gold (60C).

7. The Acclimatization Stage

Acclimatization under nursery conditions is highly critical for successful micropropagation techniques, wherein the plants are typically kept in high-humidity environments for a few days before transferring them to the greenhouse [32]. During the acclimatization stage, plants are subjected to various hazardous environmental influences, including microbial infections (mostly fungi and bacteria), temperature fluctuations, low humidity, and inadequate nutrition, all of which significantly reduce plant survival rates. However, there is in vitro control of plant growth [84]. In addition, when plant organs are transferred to ex vivo environments, physiological modulations within the organs result in morphological and anatomical defects. Plant stomata do not function correctly; roots are weak, and the epidermal layer is thin [85]. To develop methods that improve plant survival, growth, and development in greenhouses, it is essential to recognize the physiological and biochemical changes that occur in plants during acclimation [33]. To acclimate, the plants must develop leaf cuticles before being removed from tissue culture. The growing environment's humidity should be reduced to enable the plants to develop a sturdy cuticle layer [2].

8. Alternative Light Sources in the Greenhouse

Light-emitting diodes (LEDs) can take the place of standard fluorescent lights to cut down on energy costs. In some studies, LEDs are more suitable fluorescent lights for in vitro and ex vitro study [86]. Two biological research areas of photosynthesis [87][88] and morphogenesis [87] utilize LEDs [88][89]. Light intensity, quality, spectrum, photoperiod, lighting direction, and photoperiod are the factors that affect its response [90]. Greenlight—G (565 nm), blue light—B (450 nm), red light—R (660 nm), and yellow light—Y (590 nm) [91] are all considered alternatives to traditional fluorescence lamps (FL) as light sources for micropropagation [92]. In greenhouses and tissue culture, the light

sources (B) can control light intensity, CO_2 percentage, relative humidity, temperature, chloroplast aggregation, and open stomata to promote plant growth and development [92][93]. In plants, the synthesis of chlorophyll was also aided by blue light [94]. The growth of greenhouse plant *C. grandiflorum* 'Coral Charm' was observed taking blue to red LEDs in various ratios. Plants were stunted in growth when exposed to 40% blue + 60% red light, while plants exposed to 100% red light had the lowest overall biomass. Stomatal conductance was higher in all red + blue LED ratios than in control, even though photosynthesis was unaffected. The levels of flavonoids were lowest when exposed to only red light, but the levels of flavonoids and phenolic acids were higher in treatments that used a high blue light proportion. The morphology of plants may benefit from these discoveries in the future [95].

References

1. Arora, J.S. Introductory Ornamental Horticulture; Kalyani Publishers: New Delhi, India, 1990; p. 48.
2. Spaargaren, J.; Geest, G.V. Chrysanthemum. In Ornamental Crops; Van Huylenbroeck, J., Ed.; Springer: Cham, Switzerland, 2018; Volume 11, pp. 319–348. ISBN 9783319906973.
3. Ryu, J.; Nam, B.; Kim, B.R.; Kim, S.H.; Jo, Y.D.; Ahn, J.W.; Han, A.R.; Kim, J.B.; Jin, C.H.; Han, A.-R. Comparative Analysis of Phytochemical Composition of Gamma-Irradiated Mutant Cultivars of Chrysanthemum Morifolium. Molecules 2019, 24, 3003.
4. Sassi, A.B.; Harzallah-Skhiri, F.; Bourgougnon, N.; Aouni, M. Antimicrobial Activities of Four Tunisian Chrysanthemum Species. Indian J. Med. Res. 2008, 127, 183–192.
5. Marongiu, B.; Piras, A.; Porcedda, S.; Tuveri, E.; Laconi, S.; Deidda, D.; Maxia, A. Chemical and Biological Comparisons on Supercritical Extracts of Tanacetum cinerariifolium (Trevir) Sch. Bip. with Three Related Species of Chrysanthemums of Sardinia (Italy). Nat. Prod. Res. 2009, 23, 190–199.
6. Collins, R.A.; Ng, T.B.; Fong, W.P.; Wan, C.C.; Yeung, H.W. A Comparison of Human Immunodeficiency Virus Type 1 Inhibition by Partially Purified Aqueous Extracts of Chinese Medicinal Herbs. Life Sci. 1997, 60, 345–351.
7. Kumar, R.K.; Singh, K.P.; Raju, D.V.S. Effect of Different Strains of Arbuscular Mycorrhizal Fungi (AMF) on Macro and Micro Nutrient Uptake in Micropropagated Chrysanthemum Plantlets. Vegetos 2015, 28, 47–54.
8. Rout, G.R.; Das, P. Recent Trends in the Biotechnology of Chrysanthemum: A Critical Review. Sci. Hortic. 1997, 69, 239–257.
9. Datta, S.K. Induced Mutations: Technological Advancement for Development of New Ornamental Varieties. Nucleus 2020, 63, 119–129.

10. Miler, N.; Iwona, J.; Jakubowski, S.; Winiecki, J. Ovaries of Chrysanthemum Irradiated with High-Energy Photons and High-Energy Electrons Can Regenerate Plants with Novel Traits. Agronomy 2021, 11, 1111.
11. Datta, S.K. Need Based Tissue Culture in Floriculture: Asuccess Story. J. Plant Sci. Res. 2019, 35, 245–254.
12. Kyte, L.; Kleyn, J.; Scoggins, H.; Bridgen, M. Plants from Test Tubes: An Introduction to Micropropogation, 4th ed.; Timber Press: Portland, OR, USA, 2013; p. 270.
13. Anderson, W.C. Mass Propogation by Tissue-Culture-Principles and Techniques. Sci. Educ. Adm. Publ. 1980, 1–10.
14. Thrope, T.A. Organogenesis in Vitro: Structural, Physiological, and Biochemical Aspects. In Plant Aging. NATO ASI Series; Rodríguez, R., Tamés, R.S., Durzan, D.J., Eds.; Springer: Boston, MA, USA, 1990; Volume 186, pp. 191–197.
15. Lim, K.B.; Kwon, S.J.; Lee, S.I.; Hwang, Y.J.; Naing, A.H. Influence of Genotype, Explant Source, and Gelling Agent on in Vitro Shoot Regeneration of Chrysanthemum. Hortic. Environ. Biotechnol. 2012, 53, 329–335.
16. Naing, A.H.; Jeon, S.M.; Han, J.S.; Lim, S.H.; Lim, K.B.; Kim, C.K. Factors Influencing in Vitro Shoot Regeneration from Leaf Segments of Chrysanthemum. Comptes Rendus-Biol. 2014, 337, 383–390.
17. Naing, A.H.; Il Park, K.; Chung, M.Y.; Lim, K.B.; Kim, C.K. Optimization of Factors Affecting Efficient Shoot Regeneration in Chrysanthemum Cv. Shinma. Rev. Bras. Bot. 2016, 39, 975–984.
18. Pant, M.; Lal, A.; Jain, R. A Simple Cost Effective Method for Mass Propagation of Chrysanthemum Morifoliumi and Antibacterial Activity Assessment of in Vitro Raised Plantlets. J. Appl. Pharm. Sci. 2015, 5, 103–111.
19. Waseem, K.; Jilani, M.; Jaskani, M.; Khan, M.; Kiran, M.; Khan, G. Significance of Different Plant Growth Regulators on the Regeneration of Chrysanthemum Plantlets (Dendranthema Morifolium L.) through Shoot Tip Culture. Pak. J. Bot. 2011, 43, 1843–1848.
20. Lacostales, L.E.; Acedo, V.Z. Single Nodal Cutting Propagation of Tissue Culture-Derived Chrysanthemum (Chrysanthemum Morifolium Ramat.). Philipp. J. Crop Sci. (Philipp.) 2015, 40, 84–85.
21. Barakat, M.N.; AbdelFattah, R.S.; Badr, M.; El-Torky, M.G. In Vitro Culture and Plant Regeneration Derived from Ray Florets of Chrysanthemum Morifolium. Afr. J. Biotechnol. 2010, 9, 1151–1158.
22. TeixeiradaSilva, J.A.; Kulus, D. Chrysanthemum Biotechnology: Discoveries from the Recent Literature. Folia Hortic. 2014, 26, 67–77.

23. Datta, S.K. Indian Floriculture: Role of CSIR; Regency Publications, A Division of Astral International (P) Ltd.: New Delhi, India, 2015; p. 432.
24. Miler, N.; Zalewska, M. Somaclonal Variation of Chrysanthemum Propagated in Vitro from Different Explants Types. Acta Sci. Pol. Hortorum Cultus 2014, 13, 69–82.
25. Kengkarj, P.; Smitamana, P.; Fujime, Y. Assessment of Somaclonal Variation in Chrysan-Themum (Dendranthema Grandiflora Kitam.) Using RAPD and Morphological Analysis. Plant Tissue Cult. Biotechnol. 2008, 18, 139–149.
26. Murashige, T.; Skoog, F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 1962, 15, 473–497.
27. Rahmy, N.; Thomy, Z.; Yunita; Harnelly, E. The Effect of Some of Coconut Water Concentration in Artificial Media to Chrysanthemum Growth (Dendranthema Grandiflora) by in Vitro. J. Nat. 2019, 19, 42–44.
28. Lee, T.; Huang, M.E.E.; Pua, E.-C.C. High Frequency Shoot Regeneration from Leaf Disc Explants of Garland Chrysanthemum (Chrysanthemum Coronarium L.) in vitro. Plant Sci. 1997, 2, 219–226.
29. Tyagi, R.K.; Agrawal, A.; Mahalakshmi, C.; Hussain, Z.; Tyagi, H. Low-Cost Media for in Vitro Conservation of Turmeric (Curcuma Longa L.) and Genetic Stability Assessment Using RAPD Markers. Vitr. Cell. Dev. Biol. 2007, 43, 51–58.
30. Atici, T.; Khawar, K.M.; Ozel, C.A.; Katircioglu, H.; Ates, M.A. Use of Psyllium (Isubgol) Husk as an Alternative Gelling Agent for the Culture of Prokaryotic Microalgae (Cyanobacteria) Chroococcus Limneticus Lemmermann and Eukaryotic Green Microalgae (Chlorophyta) Scenedesmus Quadricauda (Turpin) Brebisson. Afr. J. Biotechnol. 2008, 7, 1163–1167.
31. Rao, S.N.P.; Kumar, A.Y.R. Effects of Antioxidants and Gelling Agents on Regeneration, in Vitro Conservation and Genetic Stability of Bacopa Monnieri (L.) Pennell. Int. J. Ayurvedic Herb. Med. 2011, 1, 51–67.
32. George, E.F.; Hall, M.A.; Klerk, G.-J. De The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and PH Effects, and Support Systems. In Plant Propagation by Tissue Culture; George, E.F., Hall, M.A., Klerk, G.D., Eds.; Springer: Dordrecht, The Netherlands, 2008; pp. 115–173.
33. Tung, H.T.; Nam, N.B.; Huy, N.P.; Luan, V.Q.; Hien, V.T.; Phuong, T.T.B.; Nhut, D.T. A System for Large Scale Production of Chrysanthemum Using Microponics with the Supplement of Silver Nanoparticles under Light-Emitting Diodes. Sci. Hortic. 2018, 232, 153–161.
34. Tung, H.T.; Bao, H.G.; Cuong, D.M.; Ngan, H.T.M.; Hien, V.T.; Luan, V.Q.; Nhut, D.T. Silver Nanoparticles as the Sterilant in Large-Scale Micropropagation of Chrysanthemum. Vitr. Cell. Dev. Biol.-Plant 2021, 57, 897–906.

35. Chen, C. Cost Analysis of Plant Micropropagation of Phalaenopsis. Plant Cell Tissue Organ Cult. 2016, 126, 167–175.
36. Wang, X.-J.; Hsiao, K.-C. Sugar Degradation during Autoclaving: Effects of Duration and Solution Volume on Breakdown of Glucose. Physiol. Plant. 1995, 94, 415–418.
37. Hahn, E.J.; Lee, Y.B.; Ahn, C.H. A New Method on Mass-Production of Micropropagated Chrysanthemum Plants Using Microponic System in Plant Factory. In Proceedings of the International Symposium on Plant Production in Closed Ecosystems, 26–29 August 1996; Kozai, T., Ed.; Acta Horticulture: Narita, Japan; 440, pp. 527–532.
38. Hahn, E.J.; Bae, J.H.; Lee, Y.B. Growth and Leaf-Surface Characteristics of Chrysanthemum Plantlets between Micropropagation and Microponic System. J. Korean Soc. Hortic. Sci. (Korea Repub.) 1998, 39, 838–842.
39. Hahn, E.-J.; Bae, J.-H.; Lee, Y.-B. Growth and Photosynthetic Characteristics of Chrysanthemum Plantlets as Affected by PH and EC of the Nutrient Solution in Microponic Culture. Hortic. Environ. Biotechnol. 2000, 41, 12–15.
40. Deein, W.; Thepsithar, C.; Thongpukdee, A. In Vitro Culture Medium Sterilization by Chemicals and Essential Oils without Autoclaving and Growth of Chrysanthemum Nodes. World Acad. Sci. 2013, 7, 1041–1044.
41. Thepsithar, C.; Thongpukdee, A.; Daorat, A. Sterilisation of in Vitro Culture Medium of Chrysanthemum by Plant Essential Oils without Autoclaving. Int. J. Bioeng. Life Sci. 2013, 7, 802–805.
42. Sarmast, M.; Salehi, H.; Khosh-Khui, M. Nano Silver Treatment Is Effective in Reducing Bacterial Contaminations of Araucaria Excelsa R. Br. Var. Glauca Explants. Acta Biol. Hung. 2011, 62, 477–484.
43. Sarmast, M.K.; Salehi, H. Silver Nanoparticles: An Influential Element in Plant Nanobiotechnology. Mol. Biotechnol. 2016, 58, 441–449.
44. Shukla, P.K.; Misra, P.; Kole, C. Plant Nanotechnology: Principles and Practices; Springer: Berlin/Heidelberg, Germany, 2016; pp. 219–256.
45. Sahu, N.; Soni, D.; Chandrashekhar, B.; Sarangi, B.K.; Satpute, D.; Pandey, R.A. Synthesis and Characterization of Silver Nanoparticles Using Cynodon Dactylon Leaves and Assessment of Their Antibacterial Activity. Bioprocess Biosyst. Eng. 2012, 36, 999–1004.
46. Savithramma, N.; Ankanna, S.; Bhumi, G. Effect of Nanoparticles on Seed Germination and Seedling Growth of Boswellia Ovalifoliolata an Endemic and Endangered Medicinal Tree Taxon. Nano Vis. 2012, 2, 2.

47. Tymoszuk, A.; Miler, N. Silver and Gold Nanoparticles Impact on in Vitro Adventitious Organogenesis in Chrysanthemum, Gerbera and Cape Primrose. Sci. Hortic. 2019, 257, 108766.
48. Dimkpa, C.O.; McLean, J.E.; Britt, D.W.; Anderson, A.J. Bioactivity and Biomodification of Ag, ZnO, and CuO Nanoparticles with Relevance to Plant Performance in Agriculture. Ind. Biotechnol. 2012, 8, 344–357.
49. Tymoszuk, A. Application of Silver and Copper Nanocolloids in Disinfection of Explants in Chrysanthemum In Vitro Cultures. In Book of Abstracts, Proceedings of the NanoPL; Nano PL on-line journal, 2014; Volume 10, Available online: http://science24.com/paper/31231 (accessed on 29 November 2022).
50. Tung, H.T.; Bao, H.G.; Buu, N.Q.; Chau, N.H.; Nhut, D.T. The Use of Silver Nanoparticles as a Disinfectant and Media Additive in Plant Micropropagation. In Plant Tissue Culture: New Techniques and Application in Horticultural Species of Tropical Region; Springer: Singapore, 2022; pp. 287–302.
51. Azcón-Aguilar, C.; Barea, J.M. Arbuscular Mycorrhizas and Biological Control of Soil-Borne Plant Pathogens—An Overview of the Mechanisms Involved. Mycorrhiza 1997, 6, 457–464.
52. Zandavalli, R.B.; Dillenburg, L.R.; de Souza, P.V.D. Growth Responses of Araucaria Angustifolia (Araucariaceae) to Inoculation with the Mycorrhizal Fungus Glomus Clarum. Appl. Soil Ecol. 2004, 25, 245–255.
53. Sohn, B.K.; Kim, K.Y.; Chung, S.J.; Kim, W.S.; Park, S.M.; Kang, J.G.; Rim, Y.S.; Cho, J.S.; Kim, T.H.; Lee, J.H. Effect of the Different Timing of AMF Inoculation on Plant Growth and Flower Quality of Chrysanthemum. Sci. Hortic. 2003, 98, 173–183.
54. Abdalla, M.E.; Abdel-Fattah, G.M. Influence of the Endomycorrhizal Fungus Glomus Mosseae on the Development of Peanut Pod Rot Disease in Egypt. Mycorrhiza 2000, 10, 29–35.
55. Ruiz-Lozano, J.M.; Azcón, R. Hyphal Contribution to Water Uptake in Mycorrhizal Plants as Affected by the Fungal Species and Water Status. Physiol. Plant. 1995, 95, 472–478.
56. Johansson, J.F.; Paul, L.R.; Finlay, R.D. Microbial Interactions in the Mycorrhizosphere and Their Significance for Sustainable Agriculture. FEMS Microbiol. Ecol. 2004, 48, 1–13.
57. Parsons, B.J. Sterilisation Procedures for Tissue Allografts. Stand. Cell Tissue Eng. 2013, 197–211.
58. Nahid, J.S.; Shyamali, S.; Kazumi, H. High Frequency Shoot Regeneration from Petal Explants of Chrysanthemum Morifolium Ramat. in Vitro. Pak. J. Biol. Sci. 2007, 10, 3356–3361.
59. Jevremovic, S.; Subotic, A.; Miljkovic, D.; Trifunovic, M.; Petric, M.; Cingel, A. Clonal Fidelity of Chrysanthemum Cultivars after Long Term Micropropagation by Stem Segment Culture. Acta Hortic. 2011, 961, 211–216.

60. Jahan, M.T.; Islam, M.R.; Islam, S.S.; Das, P.; Islam, M.M.; Kabir, M.H.; Mamun, A.N.K. Clonal Propagation of Chrysanthemum Morifolium Ramat Using Various Explants Obtained from Field Grown Plants. GSC Biol. Pharm. Sci. 2021, 16, 087–093.
61. Waseem, K.; Jilani, M.S.; Khan, M.S. Rapid Plant Regeneration of Chrysanthemum (Chrysanthemum Morifolium L.) through Shoot Tip Culture. Afr. J. Biotechnol. 2009, 8, 1871–1877.
62. Imtiaz, M.; Khattak, A.M.; Ara, N.; Iqbal, A.; Rahman, H.U. Micropropagation of Jartorpha Curcas L. through Shoot Tip Explants Using Different Concentrations of Phytohormones. J. Anim. Plant Sci. 2014, 24, 229–233.
63. Park, S.H.; Kim, G.H.; Jeong, B.R. Adventitious Shoot Regeneration from Cultured Petal Explants of Chrysanthemum. Hortic. Environ. Biotechnol. 2007, 48, 387–392.
64. Janarthanam, B.; Seshadri, S. Plantlet Regeneration from Leaf Derived Callus of Vanilla Planifolia Andr. Vitr. Cell. Dev. Biol. 2008, 44, 84–89.
65. Trigiano, R.N.; Gray, D.J. Plant Development and Biotechnology; CRC Press: Boca Raton, FL, USA, 2004; p. 376.
66. Gao, Y.; Zhao, B.; Ding, G.; Zhang, Q. Shoot Regeneration from Stem and Leaf Explants of Dendrathema Grandiflorum. J. Beijing For. Univ. 2001, 23, 32–33.
67. Kashif, W.; Khan, M.Q.; Jaffar, J.; Khan, M.S. Impact of Different Auxins on the Regeneration of Chrysanthemum (Dendranthema Morifolium) through in Vitro Shoot Tip Culture. Pak. J. Agric. Res. 2007, 20, 51–57.
68. Hoque, M.I.; Fatema, M. In Vitro Multiple Shoot Regeneration in Chrysanthemum Morifolium Ramat. Plant Tissue Cult 1995, 5, 153–162.
69. Hossain, S.N.; Hakim, L.; Islam, M.R.; Munshi, M.K.; Hossain, M. In Vitro Plant Regeneration of Apple (Malus Domestica Borkh). Bangladesh J. Bot. 2002, 31, 61–64.
70. Jahan, M.T.; Islam, M.R.; Khan, R.; Mamun, A.N.K.; Ahmed, G.; Hakim, L. In Vitro Clonal Propagation of Anthurium (Anthurium Andraeanum L.) Using Callus Culture. Plant Tissue Cult. Biotechnol. 2009, 19, 61–69.
71. Lindiro, C.; Kahia, J.; Asiimwe, T.; Mushimiyimana, I.; Waweru, B.; Kouassi, M.; Koffi, E.; Kone, S.; Sallah, P.Y. In Vitro Regeneration of Pyrethrum (Chrysanthemum Cinerariaefolium) Plantlets from Nodal Explants of in Vitro Raised Plantlets. Int. J. Appl. Or Innov. Eng. Manag. 2013, 2, 207–213.
72. Alsoufi, A.S.M.M.; Ahmed, Z.S.; Salim, A.M. The Efficiency of Interaction between Cytokines and Auxins in Micropropagation of Chrysanthemum Plant (Chrysanthemum Indicum L.). IOP Conf. Ser. Earth Environ. Sci. 2021, 735, 12048.

73. Haberer, G.; Kieber, J.J. Cytokinins. New Insights into a Classic Phytohormone. Plant Physiol. 2002, 128, 354–362.
74. Al-Jobouri, A.H. Studying Some The Functional Properties of Tamarind Tamarindus Indica L. Mucilage. Al-Qadisiyah J. Agric. Sci. 2020, 10, 304–307.
75. Tian, C.; Xie, Z.; Zhao, Y.; Zhang, Z.; Xue, T.; Sheng, W.; Zhao, F.; Duan, Y. Microgram-Grade Concentration of Chlorine Dioxide Induces One-Step Plant Regeneration in Chrysanthemum. Vitr. Cell. Dev. Biol.-Plant 2022, 1–7.
76. Gunawan, B.; Braun, S.; Cortés, M.J.; Bergmann, F.; Karl, C.; Füzesi, L. Characterization of a Newly Established Endometrial Stromal Sarcoma Cell Line. Int. J. Cancer 1998, 77, 424–428.
77. Basri, Z. Multiplikasi Empat Varietas Krisan Melalui Teknik Kultur Jaringan. J. Agroland. 2008, 15, 271–277. (In Indonesian)
78. Fu-Yun, L. Tissue Culture and Rapid Propagation of Chrysanthemum Morifolium. Plant Phisiol. Commun. 2010, 12, 314–316.
79. Hitmi, A.; Barthomeuf, C.; Sallanon, H. Rapid Mass Propagation of Chrysanthemum Cinerariaefolium Vis. by Callus Culture and Ability to Synthesise Pyrethrins. Plant Cell Rep. 1999, 19, 156–160.
80. Shelake, R.M.; Pramanik, D.; Kim, J.-Y. Evolution of Plant Mutagenesis Tools: A Shifting Paradigm from Random to Targeted Genome Editing. Plant Biotechnol. Rep. 2019, 13, 423–445.
81. CPVO Varieties Database. Available online: http://Cpvo.Europa.Eu (accessed on 12 April 2017).
82. Protocol for Distinctness, Uniformity and Stability Test for Chrysanthemum × Morifolium (Ramat.), Community Plant Variety Office (CPVO). 2008. Available online: https://cpvo.europa.eu/sites/default/files/documents/chrysanthemum_2.pdf (accessed on 22 May 2021).
83. Halmagyi, A.; Surducan, E.; Surducan, V. The Effect of Low- and High-Power Microwave Irradiation on in Vitro Grown Sequoia Plants and Their Recovery after Cryostorage. J. Biol. Phys. Vol. 2017, 43, 367–379.
84. Valero-Aracama, C.; Kane, M.E.; Wilson, S.B.; Vu, J.C.; Anderson, J.; Philman, N.L. Photosynthetic and Carbohydrate Status of Easy-and Difficult-to-Acclimatize Sea Oats (Uniola Paniculata L.) Genotypes during in Vitro Culture and Ex Vitro Acclimatization. Vitr. Cell. Dev. Biol. 2006, 42, 572–583.
85. Mathur, A.; Mathur, A.K.; Verma, P.; Yadav, S.; Gupta, M.L.; Darokar, M.P. Biological Hardening and Genetic Fidelity Testing of Micro-Cloned Progeny of Chlorophytum Borivilianum Sant. et Fernand. Afr. J. Biotechnol. 2008, 7, 1046–1053.

86. Gupta, S.D.; Jatothu, B. Fundamentals and Applications of Light-Emitting Diodes (LEDs) in in Vitro Plant Growth and Morphogenesis. Plant Biotechnol. Rep. 2013, 7, 211–220.
87. Tripathy, B.C.; Brown, C.S. Root-Shoot Interaction in the Greening of Wheat Seedlings Grown under Red Light. Plant Physiol. 1995, 107, 407–411.
88. Tennessen, D.J.; Singsaas, E.L.; Sharkey, T.D. Light-Emitting Diodes as a Light Source for Photosynthesis Research. Photosynth. Res. 1994, 39, 85–92.
89. Hoenecke, M.E.; Bula, R.J.; Tibbitts, T.W. Importance of Blue'Photon Levels for Lettuce Seedlings Grown under Red-Light-Emitting Diodes. HortScience 1992, 27, 427–430.
90. Taiz, L.; Zeiger, E. Plant Physiology; Benjamin/Cummings Pub Co: San Francisco, CA, USA, 2007; pp. 115–575.
91. Steigerwald, D.A.; Bhat, J.C.; Collins, D.; Fletcher, R.M.; Holcomb, M.O.; Ludowise, M.J.; Martin, P.S.; Rudaz, S.L. Illumination with Solid State Lighting Technology. IEEE J. Sel. Top. Quantum Electron. 2002, 8, 310–320.
92. Bula, R.J.; Morrow, R.C.; Tibbitts, T.W.; Barta, D.J.; Ignatius, R.W.; Martin, T.S. Light-Emitting Diodes as a Radiation Source for Plants. HortScience 1991, 26, 203–205.
93. Kinoshita, T.; Doi, M.; Suetsugu, N.; Kagawa, T.; Wada, M.; Shimazaki, K. Phot1 and Phot2 Mediate Blue Light Regulation of Stomatal Opening. Nature 2001, 414, 656–660.
94. Akoyunoglou, G.; Anni, H. Blue Light Effect on Chloroplast Development in Higher Plants. In Blue Light Effects in Biological Systems; Springer: Berlin/Heidelberg, Germany, 1984; pp. 397–406.
95. Ouzounis, T.; Fretté, X.; Rosenqvist, E.; Ottosen, C.-O. Spectral Effects of Supplementary Lighting on the Secondary Metabolites in Roses, Chrysanthemums, and Campanulas. J. Plant Physiol. 2014, 171, 1491–1499.