Chapter

Development of Androgenesis Studies on Eggplant (*Solanum melongena* L.) in Turkey from Past to Present

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Abstract

Eggplant is one of the most widely cultivated vegetable species in the world and Turkey. The breeding of eggplant with high yields and quality is one of the important efforts in the seed sector today. Traditional breeding activities cannot respond quickly to market mobility. With the integration of dihaploidization methods into the breeding cycles, breeding programs have gained significant momentum. The most used haploidy technique in eggplant is the anther culture based on androgenesis, and its use in public and private sectors has become widespread in recent years. To date, the use of the isolated microspore culture technique as another androgenesis technique is limited; however, the studies are in progress in particular for indirect microspore embryogenesis. Genotype effect is one of the most decisive factors determining the success of androgenesis in eggplant. Also, the other factors such as nutrient medium content, types and concentrations of plant growth regulators, age and growing conditions of donor plants, determination of the appropriate microspore developmental stages, different pre-treatments, temperature shocks and incubation conditions are also effective on androgenesis success. In this review, it is aimed to provide information about the in vitro eggplant androgenesis studies, which have been carried out and are currently being conducted in Turkey.

Keywords: anther culture, aubergine, haploid, doubled haploid, microspore culture, microspore embryogenesis

1. Introduction

The eggplant also known as *Solanum melongena* L. (2n = 2x = 24) from Solanaceae family is one of the important vegetable species in the world and in Turkey, and thus, great importance is given to the breeding studies of this plant. Although it is a perennial plant in tropical climates and annual plant in cool climates, its economic production is done annually. The primary gene center of eggplant is considered to be the Indo-Burmese region of Asia and while India and

China are the secondary gene centers [1]. It was first cultivated in Asia [2, 3]. While the wild forms of the species were small and prickly, and with bitter fruits, current forms of the plant have been achieved through the selections, during the period of cultivation, in the direction of large, thornless and tasty fruits [2, 4, 5]. Of the 51.3 million tons of eggplants produced in the world, 93% is grown in Asia and only 2.5% (about 875.000 tons) across Europe. As for Turkey, with 854.049 tons of eggplant production, it performs almost as much as the eggplant production of all European countries. In addition, Turkey ranks fourth in the eggplant production of the world after China, India and Egypt [6].

Plants are affected by the positive or negative conditions of their environments and try to adapt to those regions. In this adaptation process, non-adaptable plants perish, and adapters hide or develop some properties in order to survive, and in this way, different variations occur in their regions. The different climatic features in Turkey have contributed to the formation of our local cultivars, which have been cultivated by the people living in Anatolia for centuries, and have caused Turkey to be the second diversity region of eggplant. Different researchers have classified the eggplants according to their being early-late, adaptation to the environmental conditions, origin, shape, color and other characteristics [7]. Zhukovsky collected eggplant samples while traveling in Turkey between the years 1925 and 1926 and identified five different eggplant varieties on the Anatolian soils over those samples [8]. However, *S. melongena* L., which is cultivated as a culture plant in different countries, is generally considered to have four subspecies. These are (1) spp. *esculentum*, (2) spp. *insanum*, (3) spp. *serpentium*, and (4) spp. *depressum*, and they differ in color, shape, habitus, efficiency, usage area, etc. [9].

There are many different uses of eggplant in Turkish cuisine. Besides being consumed as fresh, dried and frozen, it is used in jams, pickles, sauces and salads. In addition, it has recently attracted attention with its use in diet lists due to its fibrous structure and low-calorie value. Eggplant, in contrast to general belief, is rich in vitamins and minerals. It has important nutritional value and contains very valuable antioxidants and phenolic substances for human health. For this reason, it has been used frequently in drug production and alternative medicine in the world since ancient times. While the fruit and leaves of eggplant have a lowering effect on cholesterol levels in the blood, eggplant extract is used in different treatments such as diabetes, asthma, bronchitis and digestive disorders [10, 11].

Eggplant cultivation in Turkey was being conducted in the field conditions before. Then, with the development of greenhouse and seed technologies, eggplant production was started in greenhouse and became one of the vegetables produced throughout the year. The yield of the cultivars cultivated in greenhouses has increased by means of the F_1 hybrid cultivars, because they have higher yield values than the standard cultivars and do not show phenotypic variations. Thus, hybrid cultivars have entered the market with great speed. This has led to a rapid decrease in the standard cultivars with phenotypic variations at high rate and even caused some local species to become near extinct. Since the 1970s, genetic erosion has started all over the world, and it is spreading rapidly in Turkey, too. It is compulsory to take serious steps toward the preservation of resources against this genetic erosion and to protect the local populations. It is possible to produce new eggplant hybrids from the local genotypes in the characteristics desired by the market by taking them into breeding programs, selfing and crossing them with the other parent lines. For this purpose, the combined use of classical and modern breeding methods will lead to speed up the progress. The use of haploidy techniques among modern breeding techniques provides tremendous advantages for breeders, especially since it allows the production of 100% homozygous pure lines only in one generation.

2. Haploidization and doubled haploid (DH) technique

If the number of chromosomes in the somatic cells of the plant species is as much as the number of chromosomes found in their generative cells, these plants are called haploid (with n chromosome, single chromosome set), and the process of obtaining haploid plants is called haploidization. The implementation of chromosome doubling of haploid plants with spontaneous doubling or certain antimitotic chemicals brings the chromosome numbers to the normal chromosome numbers and 100% homozygous plants are produced. This stage is called doubled haploidy (DH) or dihaploidization [12]. With the use of DH lines, providing the desired properties, as a male or female parent in hybrid (F_1) seed technology, the importance of DH technology has become even better understood, and its use has become increasingly widespread.

Haploidy studies, which began with the discovery of spontaneous haploid plants in 1922 [13], gained momentum in the laboratory conditions after the 1960s, and they are becoming increasingly popular nowadays. As far as it is known, spontaneous parthenogenetic haploid plant formation in eggplant has not been encountered so far. In laboratory conditions, the first study to obtain this type of haploid plant is on anther culture and reported by Raina and Iyer [14]. It was later reported by the Chinese Haploid Research Group that developed first healthy haploid and doubled haploid (DH) eggplant plants [15]. In other anther culture studies [16–21] following them, the production of haploid and DH eggplant plants has been successfully carried out.

In the eggplant haploidy studies to date, anther culture among the androgenesis techniques has been mostly used. Protocols used in eggplant anther cultures are based on different versions of the protocol used by Dumas de Vaulx et al. [22] for pepper anther culture. Dumas de Vaulx and Chambonet [17] developed a protocol similar to pepper anther culture and started using it in eggplant and reported successful results. The success of this protocol is based on the treatment of anthers with high-temperature (+35 ^{0}C) when they are first introduced into the medium. The positive effects of high-temperature applications on haploid embryogenesis have also been reported in the studies conducted on Brassicaceae family [23, 24].

In eggplant microspore culture studies, the anthers are either cultured after being pre-treated and isolated [25], or different pre-treatments were applied to the isolated microspores [11, 26, 27]. Although the use of microspore culture was quite limited in eggplant breeding until today, the studies on this subject have been continued because of its different advantages compared to anther culture. In particular, the practical microspore culture protocols to be developed for direct embryogenesis have the potential to give great momentum to eggplant breeding studies.

While DH technique is used commercially in vegetable breeding for certain species (pepper, eggplant, melon, cucumber, squash) in Turkey, it is in the process of being improved to an effective level for a variety of vegetables (onion, leek, gherkin, tomato, watermelon, cabbage, carrot, spinach). The use of DH technique is restricted due to the response of haploid plant formation varying on the species. This is because haploid response is under the effect of various factors. For instance, each of the different genotypes within a species reacts differently to the technique used, or several genotypes respond positively, while some genotypes do not respond at all. The basis of these differences is due to genotype. In addition to the genotype, the growing conditions of plants used as donor parents, climatic effect, season, temperature, light intensity, the age of the plant, irrigation and fertilization regimes, type of stress factors and severity of exposure, and the chemicals used for

plant protection have also a great effect on success. The healthier the donor plant is grown, the greater the chance of success in obtaining haploid plants. Another important issue is the nutrient medium consisting of macro- and micro-elements, vitamins, plant growth regulators (PGRs), carbohydrate sources and other unidentified substances. On the other hand, the correct determination of the period of taking the buds and the culture of the microspores at the appropriate stage also directly affects the success. Although different staining techniques are used to determine the bud stage, the most practical method used today is the 4′-6-diamidino-2-phenylindole (DAPI) staining method. As the pretreatments prior to culture, different pre-temperature applications (such as 4, 10, 28, 35°C) are applied to the anthers or buds. However, incubation in the dark at +35°C for 8 days is generally used successfully in eggplant anther cultures. To date, a number of factors affecting microspore embryogenesis have been investigated, and their effects have been demonstrated in several eggplant haploidy studies [11, 17–19, 28–34] conducted in the world and Turkey; however, this article will mainly focus on studies in Turkey.

3. Eggplant androgenesis studies conducted in Turkey

When the public and private sectors' vegetable breeding studies in Turkey were examined, it can be seen that the use of biotechnology, and in particular the use of DH technology based on embryo formation from male gamete cells, namely, androgenesis, is not going far back. The first DH study in vegetables was made in the 1980s in pepper, and then, studies on other species followed. The haploidy studies in eggplant consist of anther, isolated microspore or shed-microspore culture applications in the world. The first haploidization study in eggplant began in 1991 in Turkey with Karakullukçu's [19] anther culture study. This was followed by other anther culture [20, 35–46], isolated microspore culture [32, 34, 47] and shed-microspore culture [19] studies. Although the number of studies on this subject is small at the beginning, the efforts to obtain DH plants through anther culture have been accelerated in recent years thanks to the increase in the state-supported projects and the engagement of private sector. Androgenesis studies on eggplant have a history of 45 years worldwide and 30 years in Turkey.

4. Factors affecting success of eggplant androgenesis studies conducted in Turkey

4.1 Genotype

'Genotype' as being one of the most important factors affecting androgenesis in eggplant was also revealed with the studies conducted in Turkey [19, 20, 31, 33, 38, 43, 48, 49].

Karakullukçu [19], in various anther culture trials, reported the differences between genotypes in regard to embryo and haploid plant formations. The anthers of 13 genotypes of eggplant were cultured in the nutrient medium containing 5 mg L^{-1} kinetin and 5 mg L^{-1} 2–4 D, but only four genotypes have shown androgenesis response. While only embryoid occurred in Kemer and Prelane F_1, both embryo and haploid plants were achieved from Halep Karası and Baluroi F_1 genotypes. In this study, no embryos could be obtained from the other nine genotypes (Dourga, Pala, Şeytan, Birecik Yerlisi, Adana Topağı, Fabina F_1, Galine F_1, Black Beauty, Marfa F_1).

In the anther cultures of Alpsoy [38], conducted with 15 genotypes consisting of Pala, Kemer, Topan, Aydın Siyahı, Manisa, Adana, Urfa Yerlisi, Munica,

Baluroi, Mileda, Ancha, Leila, Barbentane, Bellissima and Purpurea, haploid embryo and plants were obtained from Kemer, Urfa Yerlisi, Adana, Barbentane and Leila genotypes and he reported that the genotype influenced the success of anther cultures.

In the microspore culture study of Özdemir [34], Faselis, Amadeo and Aydın Siyahı were used as the genotypes. Microspores were subjected to the same pretreatment and cultivated in the same nutrient medium. In Aydın Siyahı, no multinucleated structure happened, and only symmetric nucleus divisions were observed; however, symmetrical nucleus divisions and also multinucleated structures formed in Phaselis and Amadeo genotypes. It was stated that these differences are caused by genotype. It has also been reported in previous anther culture studies [38, 41, 43] that the genotype Aydın Siyahı has the low ability to form embryos.

Using the anthers of Yamula, Karabaş F_1, Malkara F_1, Çantalı F_1, and Tatlıcan F_1 genotypes, as explants, Doksöz [20] obtained 136 and 25 embryos and 77 and 15 plants only from Yamula (9.4%) and Karabaş F_1 (1.73%) genotypes, respectively.

According to the literature, there are big differences in embryo formation and haploid plant yield responses of genotypes, even though all the procedures starting from the growing conditions of donor plants until obtaining haploid plants are the same. The basis of genotype-related differences was associated with different internal amino acid contents of different genotypes by Dunwell [50]. As a result of different androgenesis studies, it has been demonstrated that the genotype effect is related to the genetics of the plant and that it is not possible to alter the androgenetic response caused by the genotype even if the conditions of donor plant growth, medium, culture and other factors affecting androgenesis were optimized [12, 51].

Thanks to genetic studies conducted in different plants, it is known that haploid formation is under the effects of genes, and in some species, certain genes initiating haploid formation were identified. For example, in some in vivo studies, *ig* gene in the corn [52] and *hap* gene in the barley [53] were defined as the genes responsible for haploid formation [54]. Thus, in vitro androgenesis is also under genetic control, and this property can be transferred to F_1 progeny by crossing androgenic genotypes with non-androgenic ones. In this respect, Tuberosa et al. [55] cultured the anthers of 8 different eggplant cultivars collected from different countries and 16 hybrid genotypes obtained from their crosses. While parents formed embryos at 17.3%, hybrids generated 42% embryo formation. A similar study was conducted by Başay and Ellialtioglu [44] in Turkey. The researchers examined the androgenesis response of the F_1 hybrid plants, which were obtained from the crosses among responsive genotypes Topan and Halep Karası and unresponsive three different genotypes. From the crosses, haploid plants were obtained from Topan × Vd-1, while embryo and haploid plant occurred from Topan × Teorem F_1 and Teorem F_1 × Topan crosses. These and similar studies show that the success of androgenesis in eggplant is highly dependent on genotype.

One of the most commonly used genotypes as the donor plant in eggplant androgenesis studies in Turkey is Aydın Siyahı. The first embryo formation from this genotype (1.25%) was reported by Başay et al. [41]. However, in other studies [34, 38, 43], the androgenic response of Aydin Siyahı is reported to be lower.

From the anther culture studies of Yücel [56] on *Solanum torvum*, only callus was obtained from Aydın Siyahı and Kemer cultivars in 2012. Haploid plants were produced by using DDV protocol at 4.6% from Aydın Siyahı in 2014. The highest haploid plant yield (36.4%) was obtained in the fall season of 2015 from the anthers of Aydın Siyahı cultured in the medium containing 1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin. This yield was followed by Kemer cultivar (33.8%)

cultured in the medium supplemented with 5 mg L^{-1} 2,4-D + 5 mg L^{-1} kinetin. Of the 79 plants obtained from above anther cultures and examined in terms of ploidy level, 60 were identified as haploid, 13 were diploid, 4 were triploid, 1 was tetraploid and 1 was mixoploid.

In the most recent study, Vural [46] used A117 F_1, Anamur F_1 and Darko F_1 in the anther culture studies. The most embryo response was obtained from A117 F_1 up to 320 embryos/100 anthers.

4.2 Growing conditions of donor plants

Even though the androgenic response of the donor genotype is high, if the conditions of the growing environment are not suitable, the chance of success decreases. The climatic conditions applied during the in vitro haploid culture of a plant should generally be consistent with the environmental conditions required for the cultivation of that plant, except for special temperature shocks. Dunwell [57] stated that the growing conditions of donor plants affect the development of microspores and hence the embryo yield and that successful results will only be achieved when the appropriate temperature, light intensity and lighting period for the plant are optimized. Therefore, although the optimum environmental conditions in donor plant growth vary depending on plant species, various parameters such as temperature in the growth season, amount of daily lighting and light intensity reaching to plant, amount of CO_2 in the environment, fertilization, irrigation and other cultural practices must be met at the right time and in the right amount [12]. Suitable environmental conditions for eggplant cultivation are stated as places where the temperature is 15–20°C at night and 21–30°C at daytime and where the lighting time and light intensity are high [58]. It should also be considered that artificial lighting is not as efficient as sunlight [59]. As another important issue, pesticide applications of donor plants should be ceased at least 3-4 days before the culture to prevent microspores from getting stressed during their development [38, 60].

The effect of growing season of the donor plants is important for the androgenic response of anthers. In the last anther culture study conducted in Turkey, Vural [46] compared the spring- and autumn-season anther cultures in her thesis. Interestingly, the performances of anthers grown in the autumn gave much more successful results. Furthermore, the same genotype may show different responses under different conditions. The best example of this is the eggplant genotype called 'Dourga'. Although this genotype showed high success in forming embryos and haploid plants in the study of Dumas de Vaulx and Chambonnet [17], it showed low success in embryo formation in studies of Tuberosa et al. [55] and Rotino et al. [61]. Also, Karakullukçu [19] reported that it did not generate embryos at all. This study with Dourga and other genotypes grown at two different locations (Adana and Ankara) revealed once again the importance of the growth conditions of donor plants and climate differences. Different results were obtained from this study even though the anthers were cultured in the same laboratory conditions by the same person and the same practices were carried out. Karakullukçu [19] also reported that the anthers taken from the buds of plants grown in short days and low-temperature conditions (greenhouse conditions in winter, especially in December and January) did not produce embryos.

Alpsoy [38] could not receive any androgenesis response from the plants cultivated in the greenhouse in 1994 and 1995. However, the embryo and haploid plants were able to be obtained from the plants grown in field conditions in 1996 and 1998. The researcher obtained embryo and haploid plants in 5 genotypes (Kemer, Urfa Yerlisi, Adana, Barbentane, Leila) out of 15 genotypes by optimizing cultivation

conditions with different applications in Bursa and Ankara locations. This study emphasizes the importance of cultivation conditions of the donor plant and the need to optimize conditions.

The first embryo formation in Aydın Siyahı (1.25%), which is one of the most focused genotypes in eggplant androgenesis studies in Turkey, was reported by Basay et al. [41] who cultured the anthers of the donor plants grown in humid and temperate greenhouse conditions in Yalova province. With the same genotype, no success was obtained in Ankara and Yalova locations at the same time, which shows again that donor plant growth is affected by the environmental growing period conditions. In the same study in 2015, it was shown that androgenetic embryos at 36.4% ratio were obtained in warm and humid Adana province conditions from genotype of Aydın Siyahı.

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The ratio of androgenesis response of the donor plants grown in natural growth conditions is higher than that of the plants grown in artificial conditions such as climatic chambers or aquacultures. However, compared to the plants grown in natural conditions in open fields, higher anther culture success is obtained from the plants grown in the soil in greenhouses under optimized conditions [12].

In the microspore and anther culture studies conducted by Özdemir Çelik [47] in two F1 cultivars (A117 and Amadeo F1), DH plants and lines were produced. In this first successful microspore culture study conducted in Turkey, appropriate culture season has been determined, and haploid plants were produced indirectly from callus regeneration. Significant differences were found among the genotypes in androgenic response not only in anther culture but also in microspore culture. These developments would allow for new prospects for the future eggplant androgenesis studies.

4.3 Developmental stages of buds and microspores

The other important factor affecting the androgenesis success is determination of the suitable microspore development stage for the culture. There will be no embryogenic induction from the microspores unless they are cultured in appropriate development stage. In different eggplant androgenesis studies, it has been reported that no progress took place in microspores cultured in the early or late stages, and also necrosis formed starting from the first days of culture [18, 19].

The suitable microspore stage for androgenesis studies varies to species, genotype, growing conditions of donor plants and the androgenesis technique used [62], which requires a cytological examination step prior to culture on the basis of each species and even genotypes within each species. In this step, one of the classic staining methods such as acetocarmine [60], Feulgen [63] or taking sections with paraffin [64, 65] can be used. However, if the laboratory infrastructure is appropriate, the easiest, fastest and most reliable staining method using DAPI (4′,6-diamidino-2-phenylindole) [66] specific to DNA should be preferred [67]. In various studies carried out in different species, it has been shown that the appropriate microspore covers the time period beginning from the formation of the tetrad's after meiosis to the starch accumulation after the first mitotic division [68, 69].

In order to detect the appropriate microspore stage and bud morphology of four different eggplant genotypes, Karakullukçu [19] primarily divided buds into eight different groups according to their morphological characteristics and, then, determined the microspore development stages of these buds with paraffin and

acetocarmine methods. Four eggplant genotypes (Baluroi F_1, Prelane F_1, Pala and Kemer) were used in this study where anthers of the suitable bud groups are cultured on equal conditions and pre-treated at 35°C for 8 days in dark conditions. While all anthers in the first, second, third, seventh and eight groups among the anther groups turned to dark color and did not show any development, the anthers in groups 5 and 6 were found to be in the correct stage for anther culture. The morphological appearance of buds in these stages was as follows: The sepals and petals were equal for the fifth group, while the sepals were slightly opened, and the petals were extended for 1–2 mm for the sixth group [35]. The appropriate microspore stages in these groups were determined as the uninucleate microspores before the first pollen mitosis or binucleated microspores at the beginning of cytokinesis.

In addition to these indicators in eggplant, anther color can also be a determining feature for the buds in the appropriate stage. Considering the fact that bud selection based on only bud morphologies may be inaccurate with the aging of the plants, it is also recommended to look at the color of the anthers. It is stated that the color of the anthers at the right stage for eggplant anther culture is greenish yellow, while yellow and dark yellow anthers are in late stage, and yellowish green anthers are in the early stage [43]. These criteria and phenotypic markers determined by Karakullukçu [19] constitute the basis for the bud selection phase of all androgenesis studies made in eggplant in Turkey.

In Şeker's study [70], in which DAPI staining technique was used to determine the appropriate microspore stage, DAPI stain and phosphate-buffered saline (PBS) buffer solutions were prepared, and PBS buffer solution was autoclaved. Anthers of each of different sized buds were crushed with PBS buffer in the petri dish. After the second addition of PBS buffer together with DAPI stain onto the crushed anthers, they were incubated in dark conditions for 5 min. Then, 1–2 drops of incubated microspores were placed on a slide and monitored under a fluorescent microscope. As the result of the study, the most appropriate microspore stage for anther culture was determined to be the late uninucleate microspore development stage. Bud morphology of this stage was confirmed as that the calyx and the corolla in the flower buds should be in the same length, or the corolla may be a little longer.

4.4 The nutrient media and additives

In the culture media prepared for androgenesis studies, several components in different proportions are added to induce microspores to divide and form embryos. These components cover macro- and microelements, vitamins, amino acids, carbohydrates as the energy source, PGRs, solidifying agents and substances called unidentified substances such as activated carbon, coconut milk, etc. In addition, other factors like how to prepare the nutrient medium (solid, biphasic or liquid) and the pH of the medium should be handled with care. In the eggplant androgenesis studies in Turkey, C and R media of Dumas de Valux et al. [22] were used as the basal nutrient media in all anther cultures. Differently, Özzambak and Atasayar [71] used MS [72] and NN [73] media, while Alpsoy [38] and Doksöz [20] investigated the effect of MS medium as well as DDV-C and DDV-R media. In the microspore culture studies [32, 34, 47], NLN [74] medium was used.

It is mandatory to use PGRs to convert gametophytic development to sporophytic development in eggplant microspore embryogenesis. For this purpose, initially an auxin (such as 2,4-D, NAA, IAA, IBA, etc.) is needed, while the cytokinin (such as kinetin, BAP, zeatin, etc.)-type PGR is required in the regeneration phase. In eggplant androgenesis studies, sucrose was generally preferred as the

carbon source, whereas maltose and glucose were used in some studies. In the initial stage of culture, adding of sucrose at 12% encourages the formation of embryos and haploid plantlets in eggplant [17, 19].

In the first eggplant anther culture study in Turkey, Karakullukçu [19] examined the effects of sucrose and glucose, different types and concentrations of PGRs and activated charcoal in different ratios on 13 genotypes. Among the different medium trials, the nutrient medium consisting of 120 g L^{-1} sucrose, 5 mg L^{-1} 2–4 D and 5 mg L^{-1} kinetin was determined as the most successful as in the original DDV medium. In this, embryos were obtained at a rate of 12.1% from Balouri F_1, 3.8% from Halep Karası and 1.5% from Kemer. No positive response was received from trials with activated charcoal. In this study [19] a total of 22 embryos and 13 haploid plants were achieved from all genotypes.

Özzambak and Atasayar [71] investigated the effects of BAP (0.1, 4, 8, 10, 15 mg L^{-1}), kinetin (1, 2, 4 mg L^{-1}), 2,4-D (2 mg L^{-1}) and NAA (0.1 and 2 mg L^{-1}) added to the MS and NN basic nutrient media on callus formation in an anther culture study. The highest callus ratio (8%) was obtained from the medium consisting of NN + NAA 2 mg L^{-1} + kinetin 1 mg L^{-1} + 40 g L^{-1} sucrose.

Alpsoy [38] tested different combinations of NAA (0.3, 1, 2, 4 mg L^{-1}), BA (0.7, 1, 3 mg L^{-1}), kinetin (0.1, 1, 5 mg L^{-1}) and 2,4-D (5 mg L^{-1}) as PGR in his anther culture study in which MS and DDV-C were used as the basic nutrient medium. As the result, DDV-C medium with 5 mg L^{-1} 2,4-D and 5 mg L^{-1} kinetin was found to be the most successful medium for haploid plant production as in Karakullukçu's study [19], which was followed by MS medium with 4 mg L^{-1} NAA and 1 mg L^{-1} kinetin.

Ellialtioglu et al. [75] used a total of 36 eggplant genotypes consisting of local accessions and commercial F_1 hybrid cultivars in an anther culture to increase haploid plant yield to be used as parents in the hybrid breeding. The protocol developed by Dumas de Vaulx and Chambonnet [17] was implemented in this study which is carried out in the Serene Laboratories of Dikmen Agriculture Co. According to the DDV protocol. The anthers containing uninucleate microspores were first cultured in C medium and kept in darkness at +35°C for 8 days and in photoperiod at +25°C for 4 days and then transferred to R medium. Haploid embryos were seen after 30–50 days and transferred to MS medium. The haploid embryo formation rates ranged from 0 to 45% depending on the genotypes. There was no embryo development in the genotypes with foreign origin, whereas more or fewer haploid embryos were formed in local genotypes.

In the anther culture study of Doksöz [20], MS and DDV-C media with vitamin B12 (0.03 mg L^{-1}) were compared. 2,4-D (0.01 mg L–1) + kinetin (0.01 mg L^{-1}) was used in the DDV-C medium, while only kinetin (0.1 mg L^{-1}) was added in the DDV-R medium. In the result, DDV-C medium was found to be more successful in terms of embryo yield than MS medium.

Ellialtioglu et al. [76] cultured the anthers of Tombak, Malkara and Mabel eggplant cultivars on DDV-C medium. Maltose and sucrose were used as the carbon source, and different concentrations of kinetin and 2,4-D or NAA and BAP combinations were tested as PGRs. The ratio of haploid embryo formation ranging between 0 and 59.6% differed according to the 'cultivar × carbon source × PGR applications' basis. The highest haploid embryo formation frequency occurred in Mabel cultivar, cultured in C medium with 120 g L^{-1} sucrose and 5 mg L^{-1} 2,4-D and 5 mg L^{-1} kinetin.

Geboloğlu et al. [45] compared the effects of different types of carbohydrate sources and PGR concentrations on anthers cultured in DDV-C medium supplemented with 0.03 mg L^{-1} vitamin B12. Different combinations and concentrations

of sucrose (30, 60, 90, 120 and 150 g L^{-1}), honey (30, 60, 90, 120 and 150 g L^{-1}), kinetin and 2,4-D (1, 3 and 5 mg L^{-1}) were tested. Then, anthers were transferred to DDV-R medium supplemented with 30 g L^{-1} sucrose and 0.1 mg L^{-1} kinetin and subcultured at the fourth week. The highest embryo yield was obtained from the application of 120 g L^{-1} sucrose +1 mg L^{-1} kinetin +3 mg L^{-1} 2,4-D in Yamula genotype (10.7 embryos/10 anthers). In this study, the effect of 'honey' was investigated for the first time in eggplant androgenesis in the world. Although the results obtained from the honey are lower than the sucrose applications, it has been reported that the honey concentrations can be optimized and the protocol can be improved.

In the studies of Ellialtioglu et al. [77] in which anthers of 12 eggplant genotypes are cultured in different laboratories in Ankara, Antalya and Tokat, the anthers with uninucleate microspores, determined by DAPI, are placed on DDV-C medium supplemented with different PGR concentrations. The cultures were first subjected to the heat shock at 35°C in dark conditions for 8 days and then moved to photoperiod conditions at 25°C for 4 days. At the end of fourth day, cultures were transferred to DDV-R medium. In the result, the highest embryo formation (38.4%) was determined in the anthers of Anamur F1 cultured in DDV-C medium containing 120 g L^{-1} sucrose, 1 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin. Also, an interaction was found between the most suitable hormone combinations in compliance with the genotypes.

Vural [46] compared the effect of several culture media modified from DDV-C and DDV-R medium with the addition of certain carbohydrate sources and unidentified substances.

As to the media used in the microspore cultures conducted in Turkey, Bal et al. [32] who made the first study modified a protocol used for tobacco microspore culture and tested this protocol on Bambino eggplant cultivar. According to the modified protocol, microspores were pre-cultured in the B medium and then transferred to the AT3 medium containing 0.25 M maltose. No embryo was formed, but symmetrical nucleus divisions and multinucleated structures were detected in the study.

In the ovary co-cultured microspore culture study of Özdemir [34], the effects of different concentrations of 2,4-D, kinetin, NAA and BAP in NLN medium were investigated on microspores of three different genotypes (Phaselis, Amadeo and Aydın Siyahı) cultured together with wheat ovaries. As for the pretreatment, eggplant anthers were incubated in 0.3 M mannitol solution under dark conditions at +35°C for 8 days, and then microspores were isolated from the anthers at the end of the eighth day and cultured in NLN medium supplemented with 5 mg L^{-1} 2,4-D + 5 mg L^{-1} kinetin or 5 mg L^{-1} NAA + 5 mg L^{-1} BAP for embryogenic stimulation. After that, microspores were co-cultured with wheat ovaries in NLN medium containing 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin or 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP. In the study, a combination of kinetin and 2,4-D was observed to be more effective in inducing eggplant androgenesis. Multinucleated structures were obtained only in the medium containing 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin + ovary, while no embryo and plant were formed.

4.5 Pretreatment shocks applied to cultures

In order to encourage embryo formation in androgenesis studies, the buds prior to culturing or the anthers or microspores after their transfer to the nutrient medium are subjected to different pre-temperature shocks. In addition to the commonly used temperature shocks, keeping under dark–light conditions at different times, using different rates of different PGRs, starvation applications, high osmotic

pressure, centrifugation, ethanol treatment, low atmospheric pressure, the use of radiation sources such as UV and Co_{60} and the use of various chemicals are among the other applications. The most common shock application used in eggplant androgenesis is the incubation of cultured anthers in high temperatures (35°C) in the first days.

Dumas de Valux et al. [22] who observed the positive effects of 35°C temperature applied in the first days of pepper anther cultures established a similar experiment in eggplant according to their previous results. The anthers cultivated at 35°C in dark conditions for the first 8 days of culture gave higher success rate than the control anthers cultivated at 25°C [17].

In the first eggplant anther culture study in Turkey, after the application of 12, 24 or 48 h of cold shock at 4°C to the buds, the dissected anthers were transferred to the culture medium and subjected to heat shock at 35°C for 8 days [19]. All of the cold-pretreated anthers turned to black and could not further develop. In another experiment, the anthers were exposed to different heat shock applications at 25, 30 and 35°C for 4 and 8 days after culture in DDV-C medium, and then they were kept in the climate room with 16-h light/8-h dark photoperiod regime at 25°C for 12 days without cold application to the buds. After 12 days, the cultures were transferred to DDV-R medium. In the trials, there were regular increases in the rate of embryogenesis with the increase in temperature. Based on these results, it was reported that cold pretreatment was not suitable for eggplant anther cultures, and the application of 35°C in 8 days under dark conditions was more successful to encourage embryogenic development than other applications.

Alpsoy [38] could not obtain any embryos from the anthers cultivated without shock pretreatment, during 1994–1995. In the experiments in 1996–1998, he achieved embryo and haploid plant formation from anthers pretreated at 35°C in dark for 8 days and reported that high-temperature application to anther cultures in dark had a direct effect on the success of androgenesis.

Ellialtioglu and Tıpırdamaz [37] applied cold shock to the flower buds of Kemer cultivar at 4°C for 80 h or 9°C for 5 days, besides the control group to which any shock was not applied. In addition, they investigated the effects of activated charcoal, added to the nutrient medium, on the amount of internal abscisic acid (ABA), in the anthers. Cold shocks and activated charcoal decreased the amount of ABA in eggplant anthers but did not have a positive effect on embryo formation. The formation of the embryos was provided by only the pre-treatments of anthers with heat shock at 35°C in dark conditions for 8 days and the control group (7.75%).

In anther culture studies of Doksöz [20], 24 h of cold shock at 4°C were applied to the flower buds as pre-shock application. The post-culture incubation of the anthers was carried out at 9 or 35°C for 8 days in the dark. As in the previous study [37], the anthers coming from control group buds that were not pretreated were found to be more successful. Generally, no result was obtained from the anthers subjected to 9°C for 8 days in the dark. However, the embryo and regenerated plant yield of the anthers pre-shocked at 35°C in dark conditions for 8 days has the highest value consisting of 161 embryos and 88 plants.

In microspore culture study of Bal et al. [32], the isolated microspores of Bambino cultivar were first subjected to 4, 25 or 33°C for 2 days in R medium, then transferred to the AT3 medium and cultured at 25°C in the dark. No embryo was formed, but the symmetrical divisions and the formation of multinucleated structures (19.4%) were observed only in the microspores pretreated at 32°C for 2 days. As the result, it was stated that the modified tobacco protocol was effective and the high-temperature shock as the pre-treatment had an inducing effect for eggplant microspore embryogenesis.

According to the studies conducted in Turkey and the world, the cold shocks applied to the eggplant buds taken at the appropriate stage did not generally resulted in positive response for microspore embryogenesis. On the contrary, it is generally accepted that high-temperature shocks such as 35°C for 8 days at dark conditions have positive effects on induction of microspore embryogenesis and regeneration.

4.6 Culture conditions

Ellialtioglu et al. [77], of which details were mentioned above previously, compared the growth performance of haploid embryos cultured under fluorescent lamps or LED lighting conditions. The ratio of haploid embryo formation ranging between 0 and 38.4% was reported to differ under the interaction of 'cultivar × light source × PGR application'.

After the anthers or microspores are isolated and cultured in a nutrient medium and a heat shock treatment is applied, the culture conditions based on climate data of the growth environment greatly affect the androgenesis success. The temperature and light to be two important variables of environmental conditions should be optimized. In in vitro conditions, light intensity can be used between 300 and 10,000 lux depending on the plant species, explant type, nutrient medium [78] and culture stage. It is recommended that light intensity should be low for anther cultures or even in the dark during the early days of culture. Thus, anthers are usually cultivated in the dark during the first period of culture, and then the 300–1,500 lux light intensity is applied to the cultures for embryo development. The embryos are germinated at 2,000–3,000 lux [12].

As a result of the large number of studies made in both the world and Turkey, the climatic conditions preferred for eggplant anther cultures are:

- Firstly, to exposure the cultures in DDV-C medium to a heat shock pretreatment consisting of 35°C in the dark for 8 days temperature shock

- As additional pretreatment, to keep the cultures in the same nutrient medium for 4 more days but under photoperiod conditions consisting of 16/8 h of light/darkness at 25°C

- At the end of 12 (8 + 4) days, to transfer the cultures to DDV-R medium and culture them under photoperiod conditions consisting of 16/8 h of light/darkness at 25°C

5. Ploidy detection and chromosome doubling

The use of haploid plants, which are very valuable for breeding, depends on chromosome doubling of these plants, thus bringing their chromosome number to the number before haploidization and making them 100% homozygous. Following haploidization procedures, chromosomal set numbers of the regenerated plants are determined by using different ploidy analysis techniques. Although various methods have been used to determine the ploidy in eggplant, flow cytometry analysis is becoming more widespread, since it is more practical and faster.

For chromosome doubling, haploid plants are exposed especially to colchicine or oryzalin, trifluralin and other chemicals with antimitotic effect in either in vitro or in vivo conditions. It has been reported that the lanolin treatment with 0.5%

colchicine for 48 h in darkness to the buds starting from the secondary axillary buds in in vivo conditions resulted in 50–70% doubling [21].

In Turkey, Ellialtioglu et al. [40] compared in vitro and in vivo colchicine treatments. In in vitro treatment, micro shoots were incubated in the colchicine solution containing 0.5 or 1%, for 1 or 2 h. In in vivo treatment, the acclimatized haploid plants transferred to greenhouse were pruned, and then the cotton pieces absorbed with same concentrated colchicine (0.5 or 1%) were placed in their axillary buds for 1 or 2 h. One hundred percent chromosome doubling and dihaploid shoots can be achieved in in vivo by using both 0.5% colchicine treatment for 2 h and 1% colchicine for 1 h from the well-grown haploid plants, of which leaves were pruned after the development of four to five nodes. However, this way takes longer time than in vitro method. Each of the waiting steps in in vivo method consisting of the plant growth in greenhouse, shooting of the buds, formation of the first flower and finally selfing requires some time. In addition, all the procedures may need to be repeated if the doubling did not happen. The in vitro method has been identified as a way that can be an alternative to in vivo and may even be seen as advantageous for saving time. Although there have been a few losses in in vitro method during the applications and acclimatization, these losses will remain unimportant when working with a large number of materials. It has been shown by Ellialtioglu et al. [40] that especially the application of 0.5% colchicine for 2 h in in vitro can be used for dihaploidization of haploid shoots in the eggplant. The most important advantage of this method is that the regenerated plants developed from this application thrive as the complete diploid plants, which offers earlier flowering and thus the possibility of earlier selfing.

In the study conducted by Özdemir Çelik [47], the in vitro method described by Ellialtioglu et al. [40] was used to double the chromosome numbers of haploid plants obtained from anther culture, and chromosome doubling was successfully performed.

6. Microspore embryogenesis efficiency

Among the eggplant androgenesis studies conducted in Turkey so far, anther culture technique has been used predominantly, and feasible androgenetic results are generally derived from these cultures. The highest microspore embryogenesis rate in all the anther culture studies conducted in Turkey was recorded as 320 embryos/100 anthers from A117 F_1 [46] cultivar. This was followed by 59.6% from Mabel cultivar [76], 38.4% from Anamur F_1 [77] and 36.4% [56] from open pollinated cultivar Aydın Siyahı.

As for the efficiency of microspore embryogenesis in eggplant androgenesis studies in the world, as far as we know, the highest embryogenesis rates obtained from anther culture studies were 237.5% (237.5 embryos/100 anthers) from DH36 line developed from Bandera F1, 146.5% from Bandera F1, the parent of DH36 line [79], 60% from Ecavi F1 [33] and 53% from Cristal F1 [80].

In the studies of isolated microspore culture, the first remarkable improvement was achieved by Corral-Martinez et al. [27]. After the refinement studies, the highest success was reported by Rivas-Sendra et al. [81] from the first generation of DH population lines (DHS1 lines) developed from Bandera F1. The callus yields were obtained to be 65.08 callus mL^{-1}, 76.86 callus mL^{-1}, 92 callus mL^{-1}, 149.11 callus mL^{-1} and 267.36 callus mL^{-1} from Bandera F1 (control) and DH15, DH41, DH40 and DH36 lines, respectively. According to the literature, in order to obtain DH eggplant plants via isolated microspore culture nowadays, it is attempted to get callogenesis at first and then to develop regenerated plants

through organogenesis [47, 81]. However, an efficient direct embryogenesis protocol have not yet been developed in the isolated microspore culture studies in eggplant so far.

7. Overview of an efficient anther culture protocol used in eggplant

The stages of an eggplant anther culture protocol (applied in TUBITAK TEYDEB Project No. 68989 conducted by Antalya Tarim Co. R&D Center) which can be used effectively and practically in breeding studies were briefly summarized in **Figures 1–9**.

Figure 1.
Donor plant growing under biotic and abiotic stress-free conditions.

Figure 2.
Suitable bud morphology for eggplant anther culture.

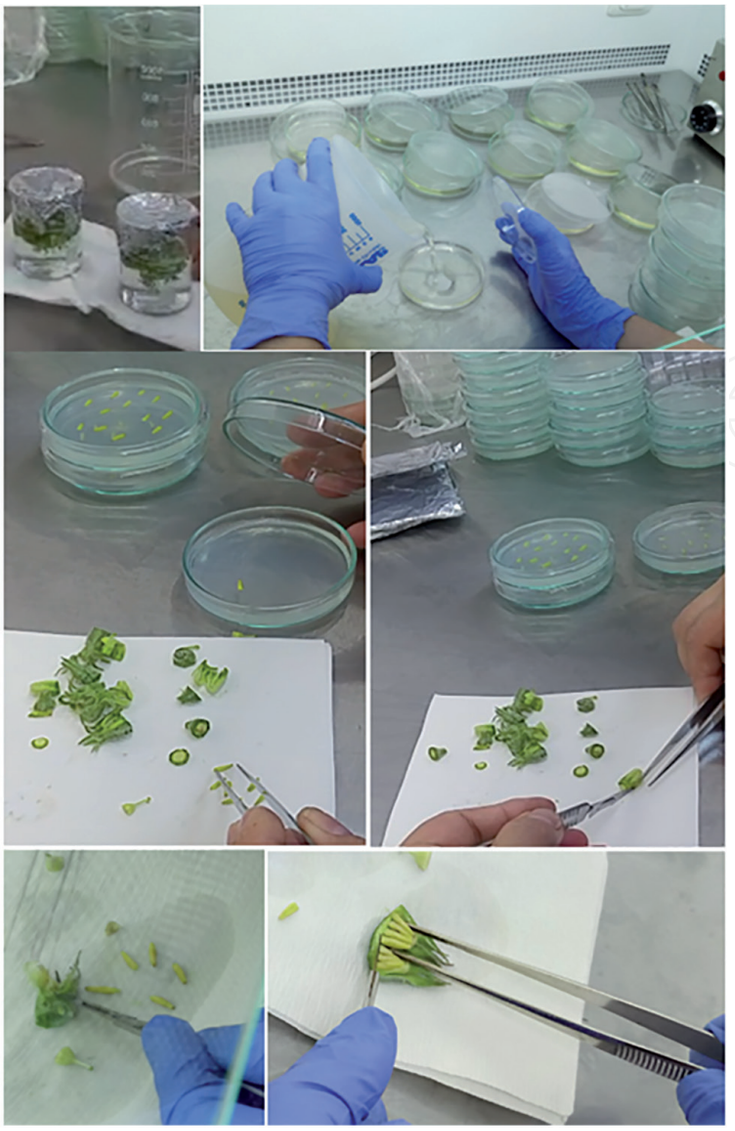

Figure 3.
Culture of anthers under aseptic conditions after appropriate surface sterilization treatment and nutrient medium preparation.

Figure 4.
Pretreatments (temperature applications) and incubation of cultures.

Figure 5.
Direct androgenesis and haploid embryo formation.

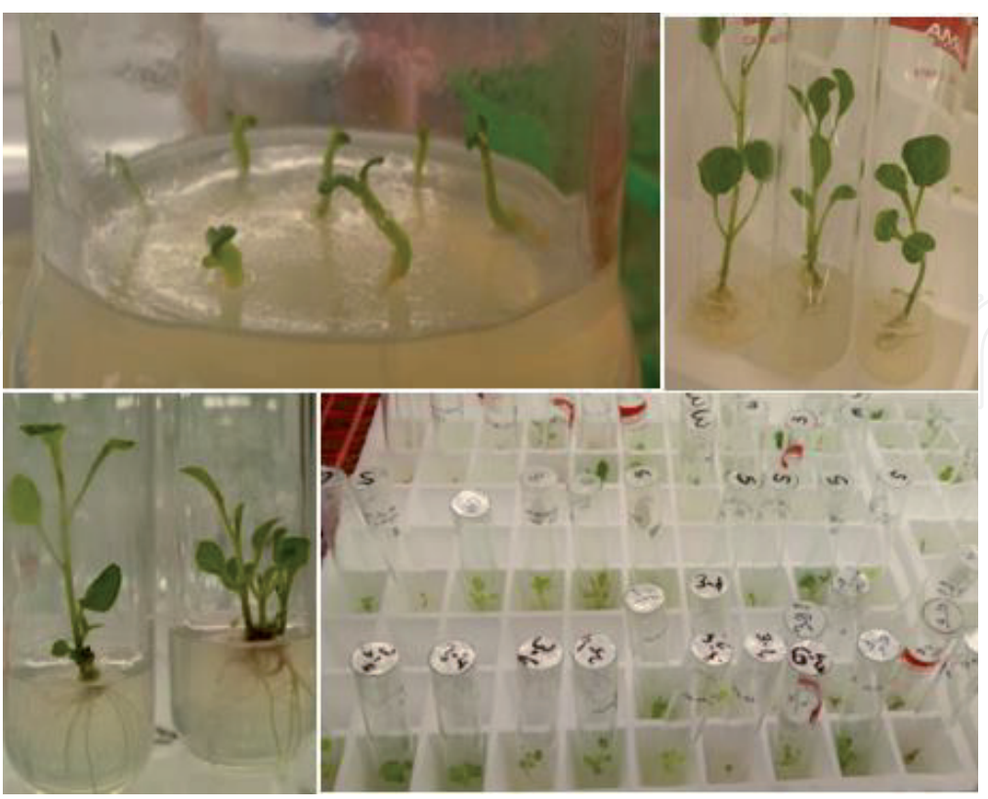

Figure 6.
In vitro regeneration and development of haploid plantlets.

Figure 7.
Acclimatization of in vitro regenerated plantlets.

Figure 8.
Plants obtained from anther culture at the different ploidy levels. Application of chromosome doubling in the greenhouse.

Figure 9.
Colchicine treatment with lanolin. Fruits on the DH branches.

8. Conclusion and recommendations

Each of the androgenesis methods including anther, shed-microspore and isolated microspore culture has advantages or disadvantages from each other. Among the eggplant androgenesis studies in the world, there are applicable protocols for anther culture, the most commonly used method, while the use of shed-microspore culture is not widespread and has not been given enough attention. As for the isolated microspore culture, the studies on indirect microspore embryogenesis have been tried to develop for a long time, and successful protocols have been improved in recent years. However, a practical direct embryogenesis protocol in isolated microspore culture is still missing.

When the recent eggplant androgenesis studies were evaluated, in the latest anther culture study, Vural informed the highest embryo yield to be 320 embryos/100 anthers [46]. Another striking improvement was performed by a Spanish research group who recently made major advances in eggplant microspore culture, that the microspore embryogenesis response is generally caused by genotype rather than by the culture protocol. This group has developed a superior DH eggplant line with very high androgenic response from a DH population improved from a hybrid cultivar with high androgenic response and their inbred lines. The haploidy performance of this superior line has folded the performance of its hybrid parent 1.6 times in anther culture and 4.1 times in microspore culture. These important improvements have shown the significance of population development and the use of DH lines to obtain haploid plant in eggplant, which is still considered to be recalcitrant between tomato and tobacco in Solanaceae [82].

It has long been known that hybrids and DH lines exhibit higher haploid performance than their parents. Therefore, at the beginning of the recommendations to increase the efficacy of microspore embryogenesis in eggplant, the development of populations, carrying the genes responsible from high androgenic performance,

comes first by means of the crosses between the high androgenic hybrids, genotypes and in particular DH lines and non-androgenic elite genotypes. Other suggestions are to focus more on the isolated microspore culture technique which has a much higher embryo or callus yield potential than the anther culture and to improve more practical protocols in particular for direct embryogenesis. Therefore, it may be dwelt on the development of shed-microspore culture protocols since it has an application between anther culture and isolated microspore culture. However, shed-microspore culture is more practical than isolated microspore culture and has the potential for higher embryo yield than anther culture. In addition, the culture media and also the culture conditions can be optimized to increase embryo yield, embryo quality and plant regeneration rate in eggplant androgenesis by different chemical, biochemical, PGR or especially phytohormones that were tested and proved in other plant species.

Finally, it is considered beneficial to increase in vitro androgenesis studies also in wild *Solanum* species to develop rootstock.

Icons and abbreviations

Icons

%	percent
°C	degrees Celsius
Co_{60}	cobalt 60
CO_2	carbon dioxide
g L^{-1}	gram/liter
M	molar
mg L^{-1}	milligrams/liter
L	liter
UV	ultraviolet

Abbreviations

ABA	abscisic acid
AT3 medium	consisted of 13 mM KNO_3, 8.6 mM $(NH_4)_2$ SO_4, 2.9 mM KH_2 PO_4, 1.1 mM $CaCl_2$.$2H_2O$, 0.7 mM $MgSO_4$.$7H_2O$, 10 mM MES buffer, 8.6 mM glutamine, 0.25 M maltose and Fe-EDTA, vitamins and microelements according to Murashige and Skoog [72]
$AgNO_3$	silver nitrate
AVG	aminoethoxy-vinylglycine
BA	N6-benzyladenine
BAP	6-benzylaminopurine
B medium	Kyo and Harada (1986) nutrient medium (consisted of KCl, 1.49 g L^{-1}; $MgSO_4$.$7H_2O$, 0.25 g L^{-1}; $CaCl_2$, 0.11 g L^{-1} and Mannitol (0.3 M) 54.63 g L^{-1} and 1 mM phosphate buffer of pH 7) [83]
DAPI	4′-6-diamidino-2-phenylindole
DDV-C	Dumas de Vaulx—C medium [17]
DDV-R	Dumas de Vaulx—R medium [17]
DH	doubled haploid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
LED	light-emitting diode
NAA	α-naphthalene acetic acid
MS	Murashige and Skoog [72] nutrient medium

NLN	The nutrient medium, whose origin is from Nitsch and Nitsch [73] medium, modified by Lichter (1982) [74]
NN	Nitsch and Nitsch [73] nutrient medium
PBS buffer	phosphate-buffered saline
PGRs	plant growth regulators
2,4-D	2,4-dichlorophenoxyacetic acid

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