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Original article

Eggplant Anther Culture: Association Between Bud/Anther Size and Microspore Developmental Stage in Different Eggplant Genotypes

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Abstract

Anther culture is a valuable method for obtaining haploid and doubled haploid (DH) plants from microspores and there are several factors influencing the induction of androgenesis such as genotype and microspore development stage. The aim of the present work was, therefore, to identify the bud stages, with maximum amount of young microspores (YM) and mid microspores (MM), thought to be most responsive to embryogenesis induction in anther cultured, and to investigate the influence of genotype on embryogenesis. In this work, first of all anthers and buds containing the highest percentage of YM and MM in four different F1 eggplant genotypes were identified. Results revealed that a certain bud/ anther size group in each genotype might correspond to different microspore / pollen development stage in different genotypes. After determining the best anther length in order to increase the presence of YM and MM corresponding anthers were collected for four genotypes and cultured. Embryo and regenerated plantlet production were taken into consideration to evaluate the response to anther culture for each genotype. Embryos were obtained in all 4 genotypes with variable percentages, ranging from 3,57% to 40.67 %. As a conclusion, related bud and anther length determined for each genotype could be used as fast and reliable criteria to determine the most responsive microspore/pollen developmental stage, which has maximum amount of YM and MM, to increase the efficiency in eggplant anther culture.

Keywords: Solanum melongena L., Microspore Development Stage, Androgenesis, Doubled Haploids, Genotype.

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INTRODUCTION

Androgenesis is described as an induction of haploid male gamete deviating its development from the gametophytic program towards embryogenesis to generate haploid or DH plants. Androgenesis is likely to remain an essential method to obtain pure lines for accelerate the breeding process and increase plant breeding (Forster et al., 2007; Dunwell, 2010; Germana, 2011). Through androgenesis, DH lines can be produced *in vitro* either with anther culture or isolated microspore culture. Among these techniques to produce doubled haploids, anther culture is by far the most common as in eggplant.

Eggplant (*Solanum melongena* L.) is one of well-known and the most important vegetables among horticultural species. Doubled haploid technology in eggplant is yet to be optimized for breeding programs since eggplant genotypes are reported to have low efficiency as well as the recaltcitrancy (Segui-Simarro et al., 2011). Since the first reports of eggplant anther culture were published by Raina and Iyer (1973), Dumas de Vaulx and Chambonnet (1982) introduced an efficient protocol. In the following period this method have been conducted to enhanced the efficiency of anther culture in different eggplant genotypes (Tuberosa et al., 1987, Rotino et al., 1987; Chambonnet, 1988; Rotino et al., 1990; Matsubara et al., 1992; Rotino, 1996; Alpsoy and Seniz, 2004; Gemes Juhasz et al., 2006; Salas et al., 2011, 2012; Basay et al., 2011; Basay and Ellialtıoğlu, 2013). After all these above stated studies it is possible to say that anther culture is a promising method for production of DH plants and androgenic respond is highly dependent the genotype used.

Previous studies also revealed that one of the important criteria on the efficiency of anther culture is the development stage of the microspores (Reynolds, 1997; Salas et al., 2012). For all species, there is a wide consensus that microspores can be diverted from their gametophytic pathway towards embryogenesis, corresponds to just before and after the first pollen mitosis (Reynolds, 1997, Touraev et al., 2001), although stated stage may vary between species (Raghavan 1986). Furthermore, culture conditions affect the most appropriate stage that well responds to induction and it has been also shown that anther culture requires earlier microspore development stages than isolated microspore culture (Soriano et al., 2013). Regarding eggplant anther culture, in most of the previous studies researches reported that generally uninuclate, late uninuclate (vacuolate microspores) and young bicellular pollen stages have been used (Tuberosa et al., 1987, Salas et al., 2011, Basay and Ellialtıoğlu, 2013). However, Salas et al. (2012) conducted a detailed study on the development stages of microspore/pollen in eggplant and reported that although vacuolate microspores and young bicellular pollen stages were the most responsive for isolated microspore culture, they were not the best choices for anther culture. Because of the unusual thickness of eggplant anthers they suggested to use young anthers, containing mostly young and mid microspores for anther culture.

For an efficient embryo induction, it is essential to precisely identify the buds and anthers containing mostly YM and MM stages at culture initiation. In eggplant, the identifying buds with

convenient anthers have been defined with visual descriptors by different authors and they admitted that these descriptors might show variations among genotypes or even buds of the same plant (Dumas de Vaulx and Chambonnet, 1982; Tuberosa et al., 1987; Rotino, 1996). It is known that plant growing conditions have an effect on morphology of donor plants. Nevertheless it may be still possible to find a relationship between microspore development stage and plants' morphological features (Lauxen et. al., 2003). In order to find out the most accurate morphological marker for determining the right stage of microspore development, Salas et al. (2012) suggested to use bud-anther length since there is a correlation between bud-anther length and microspore/pollen development stage. This report is the only research on the different developmental stages of microspore/pollen which were based on morphological criteria of gametophytic pollen development in eggplant. There is a need to increase the efficiency of androgenesis induction by identifying appropriate stages with visible and measurable characteristics in different genotypes. In present research, therefore, an anther culture protocol was used to increase haploid embryo and plant regeneration by using young and mid microspores in different eggplant genotypes as well as to see the effectiveness of bud and anther classification method.

MATERIALS and METHODS

Four eggplant F1 hybrids, namely 'Faselis', 'Amadeo', 'Anamur' and 'A117' were chosen to carry out the anther culture experiments. The plants were grown in greenhouse conditions. In order to determine the distribution of microspore/pollen developmental stage of buds and anthers of different sizes, 50 flower buds analyzed per genotype. It is assumed that the anthers of the stated bud contain microspores/pollens at the same developmental stage. Therefore, one anther of a given bud was removed, measured, crushed and observed under a light microscope. At the time of the cytological observations, we identified 7 microspore/pollen developmental stages (tetrad, young, mid and late (vacuolate) microspore and young, mid and late, mature pollen) as described by Salas et al. (2012). Percentage of different microspore/pollen development stage was calculated for the anthers at the same length.

Flower buds were collected and surface sterilized. Length of one anther was measured with a caliper to determine the optimal length and these anters were used for anther culture studies. Anthers were cultured according to protocols and media, described by Dumas de Vaulx and Chambonnet (1982) and by Chambonnet (1988)i respectively. Anthers were cultured in C medium for 8 days in darkness at 35°C,then transferred to light at 25°C for 4 more days. At the end of 12 days, anthers in C medium were transferred to R medium and cultured at 25°C. Embryos emerging from anthers were isolated and cultured in Murashige and Skoog (MS) (1962) medium for germination and for further plantlets development.

Flow cytometry analysis was performed to determine the ploidy level of anther derived plantlets. Nuclear DNA content of these plants was determined by flow cytometer using fresh plant materials.

Small pieces of anther-derived plantlets were transferred to Plant Genetics and Cytogenetic Lab of Agricultural Faculty of Namık Kemal University. The materials were kept at 4°C between two moistured filter papers placed into a disposible petri dish until they were analyzed. Absolute 2C DNA contents were determined according to Tuna (2015). After the ploidy level is determined, these plantlets were moved to greenhouse.

RESULTS and DISCUSSION

In present study 50 buds/genotype were used to find out the bud and anther lengths in order to determine the anthers which having the highest amount of YM and MM. Fig. 1 shows the distribution percentages of different microspore and pollen development stages present in different anther lengths as an example of cv. Faselis genotype. Seven different microspore/pollen development stages (tetrads, young, mid, and late (vacuolated) microspores, and young, mid and late, mature pollen), based on findings of Salas et al. (2012), were shown in Fig. 2. Development stages of microspore or pollen showed variation in accordance with the increase on related anther length. In other words, different microspore/pollen development stages were recorded in anthers with different length (Fig. 1).

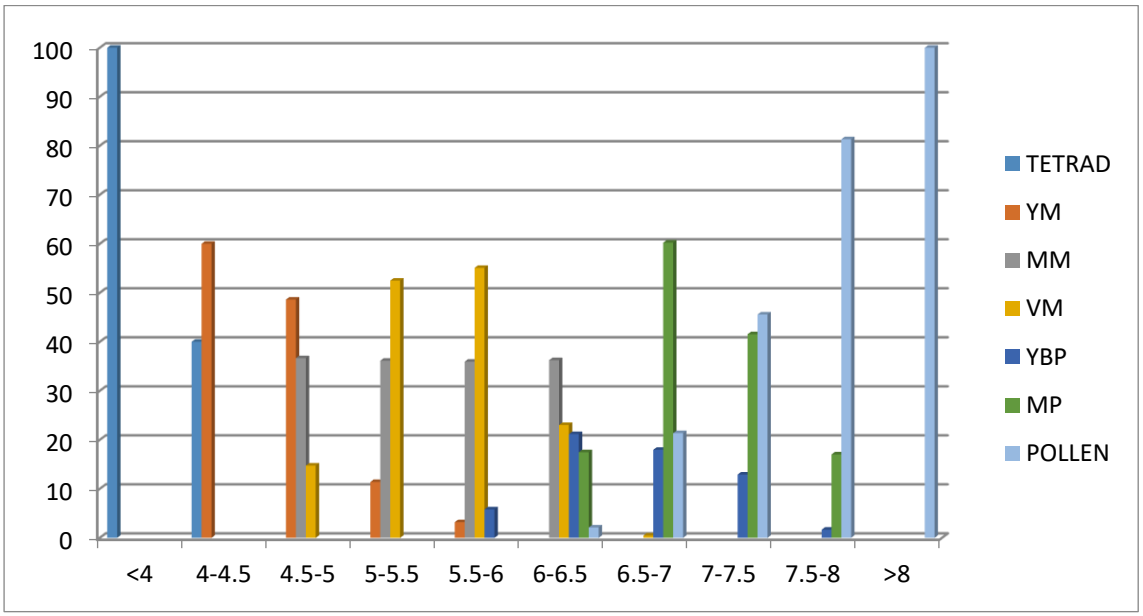
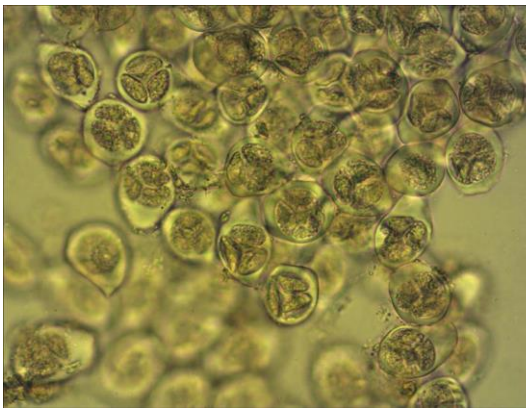


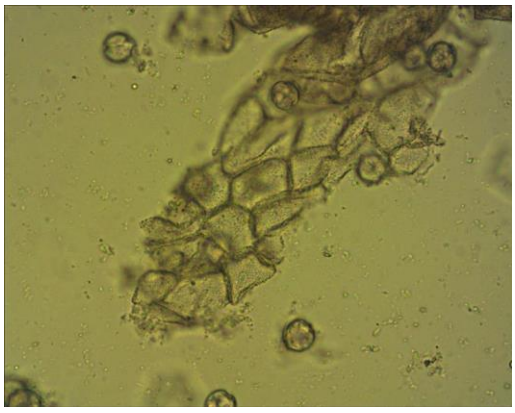
Fig 1. The distribution percentages of different microspore and pollen development stages present in different anther lengths in the eggplant cv. Faselis genotype.

We evaluated bud and anther lengths to determine the anther with the most responsive stage/s to induce androgenesis in different eggplant genotypes. Assuming that the anthers, containing maximum amount of YM and MM, are the most responsive for eggplant anther culture of eggplant, Table 1 shows that bud and anther length which contains maximum average of YM and MM for each genotype. It was recorded that with the exception of A117, the optimal bud length for all three genotypes ranged between 8 and 12.5 mm while it was observed between 9 and 12.5 mm for A117. Anther lengths ranged between

4 and 6.5 mm for all genotypes. All genotypes used in the study showed variations in terms of optimum bud size.

One of the most convenient morphological criteria to determine the buds and anthers at optimum microspore/pollen development stage was found to be length of bud or anther. It is possible to set up a correlation between microspore/pollen development stage and bud-anther length for almost all species (Kasperbauer and Wilson, 1979; Summers et al., 1992; Lauxen et al., 2003; Segui-Simarro and Nuez, 2005; Salas et al., 2012). Since it is possible to determine the most convenient microspore/polen development stage by measuring bud and anther lenght, this criteria may be considered as rapid and reliable way to choose the anthers. In a study by Salas et al. (2012) was the first study to examine the different developmental stage of microspore / pollen using bud and anther length by using 12 eggplant accessions. In all genotypes except S. aethiopicum, they reported the optimum bud length ranged from 8 to 16 mm, while the anther length varied from 4 to 7 mm while bud and anther lengths were around 6 and 3 mm, respectively for S. aethiopicum. Ellialtıoğlu et al. (2012) found that the bud length of the buds in the appropriate period was 15-17 mm in all genotypes they used and the petals reached the sepal segregation level and the anther color was greenish yellow. In our study, bud and anther length was used to determine the appropriate microspore / pollen developmental stage, like in above stated studies, a correlation between microspore/pollen developmental stage and bud and anter length was recorded. In all these studies including present clearly revealed that determined appreciate anther and bud length showed variation among used. Therefore it possible to say that there is an absolute necessity to determined right bud and anther length for each genotype at the beginning of study.





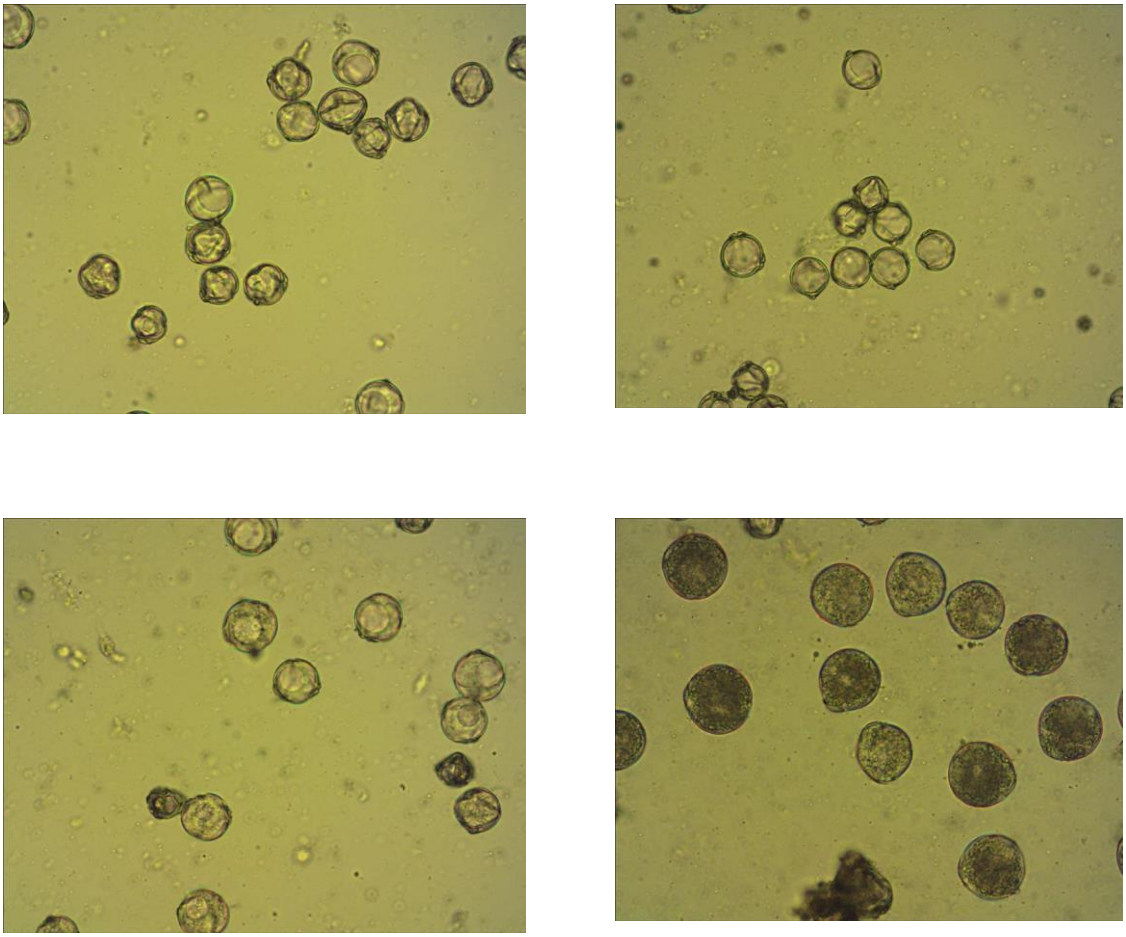


Fig 2. The stages of microspore/pollen developments. A: Tetrads. B: Young microspores. C: Mid-vacuolate microspores. D: Vacuolate microspores. E: Youn bicellular pollen grains. F: Mid-late and mature pollen.

When anthers are excised and cultured in medium, typical gametophytic pollen program is inhibited, and cell division, embryo or callus formation and plantlet regeneration can occur in a portion of the microspores. The efficiency of androgenetic response depends on the stage of anther development and the genotype (Dunwell, 1985). The most responsive microspore/pollen development stage for embryogenic induction in many species, even it is genotypes dependent, is reported to be immediately before or immediately after the first pollen mitosis. (Raghavan, 1986). It is interesting to note that culture conditions (androgenesis method) also influence the optimal stage for induction response. Previous studies reported that anther culture required an earlier microspore development stage than isolated microspore culture. Anther tissues can provide a better environment for microspores at early stages by providing nutrients and protection against stress factors. It has been suggested to use earlier stages of microspore development due to separation of microspores from culture medium with anther wall and late induction (Soriano et al., 2013). Studies showed that the stage of tomato-anther development

influences whether calli or embryos would be obtained (Summers et al., 1992). Salas et al. (2012) cultured the anthers in different lengths to cover all stage of microsporogenesis and microgametogenesis and indicated that embryogenic respond was obtained from anthers with young and mid microspores.

Table 1. Response of the four eggplant genotype to anther culture and optimum anther length with the highest amount of MM and YM for each genotype.

Genotype	Bud lenght (mm)	Anther lenght (mm)	Number of Anthers plated	Embryo formation (%)	Plant formation anther (%) /embriyo (%)
A117	9-12,5	4,5-6,5	1035	40,67	16,81/ 41,33
Faselis	7-12	4-6	846	11,34	11,31/63,53
Amadeo	7,5-12,5	4-6	853	9,96	5,73/57,64
Anamur	7,5-11,5	4,5-6,5	616	3,57	1,78/50

The four genotypes used in present study were evaluated for their androgenic responds by culturing the anthers under the same experimental conditions. After determining the optimum anther length with the highest amount of MM and YM for each genotype, anthers corresponding to these lengths were cultured (Dumas de Vaulx and Chambonnet, 1982; Chambonnet, 1988). The response of each genotype to anther culture in terms of embryo formation and regenerated plant is shown in Table 1. Embryo production was obtained for all genotypes with variable percentages ranging from 3.57% to 40.67%.

When the embryo reached to a few millimetres, embryos of all genotypes, were cultured to obtain plant, and also assessed in terms of germination rates. When cultured alone, embryos of all genotypes germinated with variable germination rates. Embryos obtained from all genotypes further developed into plantlets in different ratios and there was a considerable variation among genotypes in terms of embryo yield (Table 1). Result clearly showed that there was heterogeneity in the embryogenic response and there was no clear correlation between inductive and germinative response. For the cultivars A117, Faselis, Amadeo, Anamur while the percentage plantlet formation per anther was 16.81 , 11.31, 5.73 and 1.78%, respectively; percentage plantlet formation per cultured embryos was 41.33, 63.53, 57.64 and 50% ,respectively. While the most responsive cultivar for embryo formation was A117, the highest percentage of embryo germination was recorded in cultivar Faselis. Cultivar A117 gave the lowest percentage of plant formation from embryos. The vast majority of ungerminated embryos were characterized by the presence of anatomical abnormalities. The normal-looking *in vitro* plantlets of germinated embryos were then acclimatized to in vivo conditions for bringing them to full plant size. In this work microspore-derived embryos are shown in Fig 2. Few weeks later anther culture, microspore-derived embryos were observed from the anther locule (Fig 3a). When the embryos were separated from the anthers and taken to germination medium, the germinated embryos gave rise to roots

and shoots (Fig 3 b,c). DH plants were produced after in vitro growth and after completion of acclimatization phases to external conditions (Fig 3d).

Fig 3. Anther culture of eggplant. A. Embryos emergated from anther. B. Embryo separated from the anther. C. Embryo derived plantlets. D. Acclimatized haploid plant obtained through anther culture

All studies conducted on eggplant anther culture it was concluded that throughout the haploid induction the genotype played an important role. Significant affect of genotype on efficiency of anther culture was also reported by Rotino (1987); Salas et al. (2011); Basay and Ellialtıoğlu (2013). In different eggplant genotypes studied up to now, responsive, low responsive and non-responsive genotypes were reported. More recently Salas et al. (2011) examined the androgenic response of 12 different eggplant genotypes in anther culture. In their study, somatic calli was obtained in 11 genotypes but embryos from microspores were produced in 5 genotypes. They also reported that embryo formation ratio varied between 0.7 to 60.9 embryos/100 anther. Karakullukçu and Abak (1992) conducted an anther culture study of 4 different eggplant genotypes and reported that they obtained 0-7.8% haploid embryos, depending on genotype. Similar results were also reported by Rotino et al. (1987) and Tuberosa et al. (1987). Variation among genotypes to androgenic responds is a common feature not only for eggplant but also for many other species (Dunwell, 2010; Segui-Simarro and Nuez, 2008). Our results are in agreement with most of the previous studies conducted with different eggplant cultivars, in terms of genotypic effects on embryo yield and plant regeneration.

Flow cytometer analyse revealed that, plants of all responding genotypes were initially haploid, and reached to the DH status after chromose doubling. Tuberosa et al. (1987) reported that among the 328 plants obtained through via anther culture were 77 doubled haploid, 250 haploid and one trisomic. Dumas de Vaulx and Chambonnet (1982) also mentioned about haploid and doubled haploid plants.

Rotino et al. (1987) reported that the percentage of haploid and doubled haploid was 65% and 35%, respectively. But in our study, all anther derived plants from each genotype was haploid and we did not observed any spontaneously doubled haploid plant.

Conclusion

A certain bud/ anther length determined for each genotype might correspond to different microspore / pollen development stages in different genotypes. Therefore the right bud /anther lengths should be carefully checked for each individual genotype at the beginning of anther culture studies. As overall conclusion, related anther length determined for each genotype could be used as fast and accurate criteria to determine the most responsive microspore/polen developmental stage which has maximum amount of YM and MM. Since numbers of plants obtained with DH technology in present study are high enough, it is possible to say that DH method in eggplant has great potential to support the development of new cultivars in eggplant breeding.

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