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SHORT COMMUNICATION

Microsporogenesis in faba bean (*Vicia faba* L.) grown in Mersin, Turkey

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Abstract

Legumes are an alternative to animal foods due to their high protein content. Like other legumes, *Vicia faba* (faba bean) has high protein content. However, faba bean breeding with classical methods is challenging due to inbreeding depression, self-incompatibility, and abortive embryo formation. The inadequacy of classical breeding methods due to the problems in the fertilization biology of the plant limits the production of new varieties. Therefore, the importance of using *in vitro* haploidization technique in legume breeding is increasing. Anther culture is a widely preferred tissue culture technique for obtaining haploid plants. The initial and most crucial stage in anther culture is the identification of anthers containing microspores in the appropriate developmental stage. In the study, anther samples with single and triple-nucleated microspores were cultured in MS nutrient medium containing 2,4-D (0.5 mg L^{-1}) and Kinetin (2.5 mg L^{-1}). In the first month of culture, embryo and embryoid-like structures were obtained at the heart stage from anthers containing microspores in the mononuclear stage, while anther samples containing microspores in the trinucleated stage were observed to darken and did not develop.

Keywords: *Vicia faba*, anther culture, haploidization, microspore

Authors' contributions: Aslı Küçükrecep and Dilek Tekdal conceived and designed the experiments, performed the experiments, and wrote the paper. Dilek Tekdal supervised the study.

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Introduction

Leguminosae is the third largest family of dicotyledons. Legumes have an essential economic place in human and animal nutrition with their high protein content. At the same time, they are significant plants because they are used as raw materials in the industry (Duke, 1981; Selva et al., 1989).

In addition to their high nutritional value, legumes, which can bind the free nitrogen of the air to the soil, provide the enrichment of the soil they are planted by attaching the free nitrogen of the air to the soil, thanks to the symbiotic relationship they establish with the Rhizobium bacteria. They have the capacity to fix about 5 to 20 kg da-1 of nitrogen per year. This amount varies according to the type of plant and environmental conditions (Şehirali et al., 2010).

While the consumption of legumes was 28 million tons in 2000, it increased to 52 million tons (82 % increase) in 2017 due to the increase in the human population. Worldwide,

in 2017, it decreased by 78% (64 million tons) compared to 2005, and the usage increased by 75% (51 million tons) (TAGEM, 2019). Most chickpeas, lentils, beans, and faba beans are produced commercially in Turkey, followed by cowpea and peas. The share of agricultural lands belonging to edible legumes increased to 3% in 1980 and 11.2% in 1990 (Dogan et al., 2020).

Vicia faba L., which has the most robust growth feature and nitrogen fixation capacity among the edible legumes, is known worldwide as faba bean, brad bean, horse bean, Windsor bean, tick bean, and fava bean. Faba beans have medicinal properties. It has long been known to be used as a medicine to treat kidney, liver, and ocular sensory diseases (Sathya Prabhu & Devi Rajeswari, 2018). Faba bean is the most protein-containing legume, with a protein content of approximately 21%. In addition to its high protein content, it is ahead of other legumes with its capacity to bind the free nitrogen of the air to the soil. The chemical content of faba beans is 51–68% carbohydrates, 20–41% protein (79% globulin, 7% albumin, and 7% gluten), 2.3–3.9% fat, folic acid, niacin vitamins, and mineral elements (Sathya Prabhu & Devi Rajeswari, 2018). Despite the increase in demand for legumes in developed and developing countries, a decrease was observed in the cultivation areas of legumes, as the cultivation of products such as sugar cane and corn increased to obtain raw materials in production areas such as biofuels (Gülümser, 2016).

As a result of the legume breeding studies that started in Turkey in 1965, three faba bean varieties registered in the National Cultivars List were developed and presented to the producers (TAGEM, 2019). As a result of the studies, it has been determined that the production amount of faba beans was 13.8% (5.9 thousand tons), 7.5% (5.5 thousand tons), and 8.8% (5 thousand tons) in 2018, 2019, and 2020, respectively (TAGEM, 2019).

With all these features, the faba bean should be considered an alternative to meet the current and predicted increase in protein needs and enrich the soils where more than one crop is obtained and impoverished in organic matter. However, the faba bean is susceptible to environmental conditions and biotic and abiotic stresses, and progress is slow in improving crop varieties due to difficult pollination control (Bond, 1987). Therefore, developing new varieties tolerant to environmental conditions and various stress factors is crucial. It takes 10 to 14 years to develop new varieties with classical breeding studies (Singh et al., 2013; Gülümser, 2016). In tissue culture practices and breeding studies, homozygous individuals can be obtained in a short time, mainly thanks to anther culture, and the period of obtaining new varieties is shortened (Singh, 1997).

The microspore development stage is one of the most important stages affecting success in anther culture (Grewal et al., 2009). This stage is defined as the 'mononuclear microspore stage' in many species and refers to the time from the formation of tetrads after meiosis to mitosis (Smýkal et al., 2015). In the studies, it has been stated that the androgenic capacity of plants with fewer flowering days and small bud structures is more intense; therefore, it has been reported that collecting and working the buds in the early stages of development affects the success (Smýkal, 2000; Smýkal et al., 2015).

This study aimed to investigate the effect of appropriate bud selection and mononuclear microspore stage on callus induction by using buds at different developmental stages.

Material and methods

Plant material

In this study, faba bean seeds were obtained from local growers in Adana (Turkey). Seeds were sown in plastic pots mixed with 1:1:1 garden soil, decayed sheep manure, and turf in the field in Mersin, Turkey. The soil was first cleared of weeds, and a drip irrigation system was installed. The soil has been dug up, and the soil has been sprayed against the possibility of worms and different organisms eating the plant roots. The soil was well watered. Ten seeds were planted directly on the land soil. Since the optimum temperature value for the germination of the seeds is 18–20 °C, and these temperature values are felt in March in Mersin, the seeds were sown in February.

Microscopic observations

Since the cultured microspore cells being in the mononuclear or early binuclear

Figure 1. Two-week-old (A) and one-month-old (B) *Vicia faba* grew in soil.

stage increases the success of the culture, the mononuclear stage in anthers was determined by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Flower buds at different developmental stages were collected and grouped according to size. Anthers were isolated from buds of different sizes, and crush preparations were prepared. Preparations were stained with DAPI and examined under a fluorescence microscope Olympus BX51 (Japan).

Surface sterilization and *in vitro* culture conditions

Suitable-sized flower buds determined for anther culture experiment were kept under tap water for 30 minutes to remove soil and/or dust residues. After standing, it was washed several times with sterile distilled water. After standing, it was washed with distilled water. Explants taken into a sterile cabinet were soaked in 70% EtOH for 1 min. Flower buds were placed in 25% NaOCl for 15 min., shaken and kept. Surface sterilization of the samples was completed by washing four-five times with sterile distilled water. The anthers of the sterilized flower bud were isolated under a stereo microscope Olympus SZ61 (Japan) and used as the primary material in the tissue culture study. In order to test the effect of the microspore development stage on the success of anther culture, anthers containing microspores in the late developmental stage (trinucleate stage) were also cultured. MS medium containing 2,4-D (0.5 mg L^{-1}) and Kinetin (Kin; 2.5 mg L^{-1}) was used in anther culture experiments, and MS medium without plant growth regulator was used as the control group. The experiment was carried out with five replications and 20 anthers in each trial.

Results and discussion

Firstly, seeds obtained from local growers were germinated in the field area in Mersin, Turkey. After one week, the seeds germinated, and the plants were kept for a month to reach an appropriate size and developmental period for further analysis (Fig. 1).

Anthers and microspores are used to obtain haploid plants through androgenesis, and success in both techniques depends on the effect of many biotic and abiotic factors (Murovec & Bohanec, 2011). In anther culture study, the success of haploidization is affected by the developmental stages of microspore cells. The most suitable stage for culture is the mononuclear or early binuclear stage of microspore cells. It is stated that this stage can be determined by evaluating the buds in terms of size and shape (Zamir et al., 1980). Therefore, buds of different sizes were collected to determine the stage in which mononuclear microspore cells were cultured and the appropriate flower bud developmental stage to isolate anthers (Fig. 2).

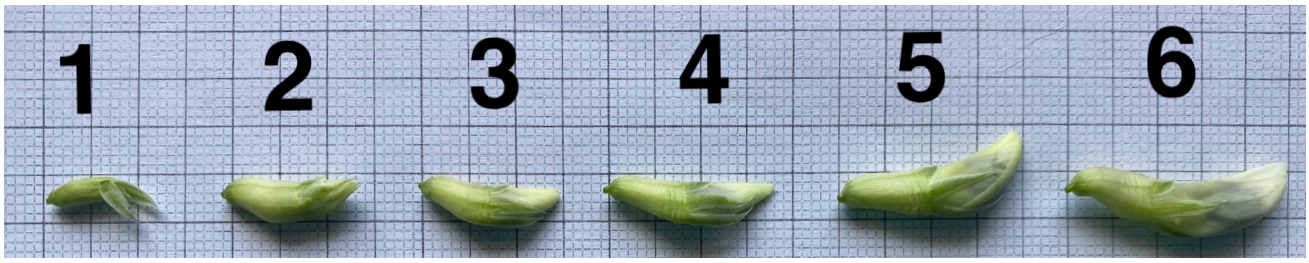

Figure 2. Flower buds of *Vicia faba* at different developmental stages analyzed for the determination of the mononuclear microspore stage.

Bud size and mononuclear stage images were determined for the studied faba bean genotype and are given in Figs. 2 & 3. Accordingly, it was found that the microspore cells of the anthers taken from the buds with a size of about 2–3 mm were in the mononuclear stage (Fig. 3). In the mononuclear stage, nuclei of microspore cells were observed to shift from the center of the cell to the poles of the cell (Fig. 3). As a result of the analysis, the appropriate flower bud stage for anther culture was determined.

Anther culture was performed to determine the high success rate of culture studies with anther containing microspores in the mononuclear stage. For this reason, anthers containing microspores in the mononuclear stage and triple nucleated stage were cultured in nutrient media containing Kin (2.5 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}) growth regulators and not containing these plant growth regulators as a control group. Callus regeneration is the first and most important step in anther culture. It was observed that callus formation started from the second week of culture in anthers cultured in MS medium containing Kin and 2,4-D (Fig. 4), while browning began in anthers with triple nucleated microspores cultured in the same media containing. No improvement was observed in all samples cultured in MS media without Kin and 2,4-D as a control group. In a haploidization study performed with three

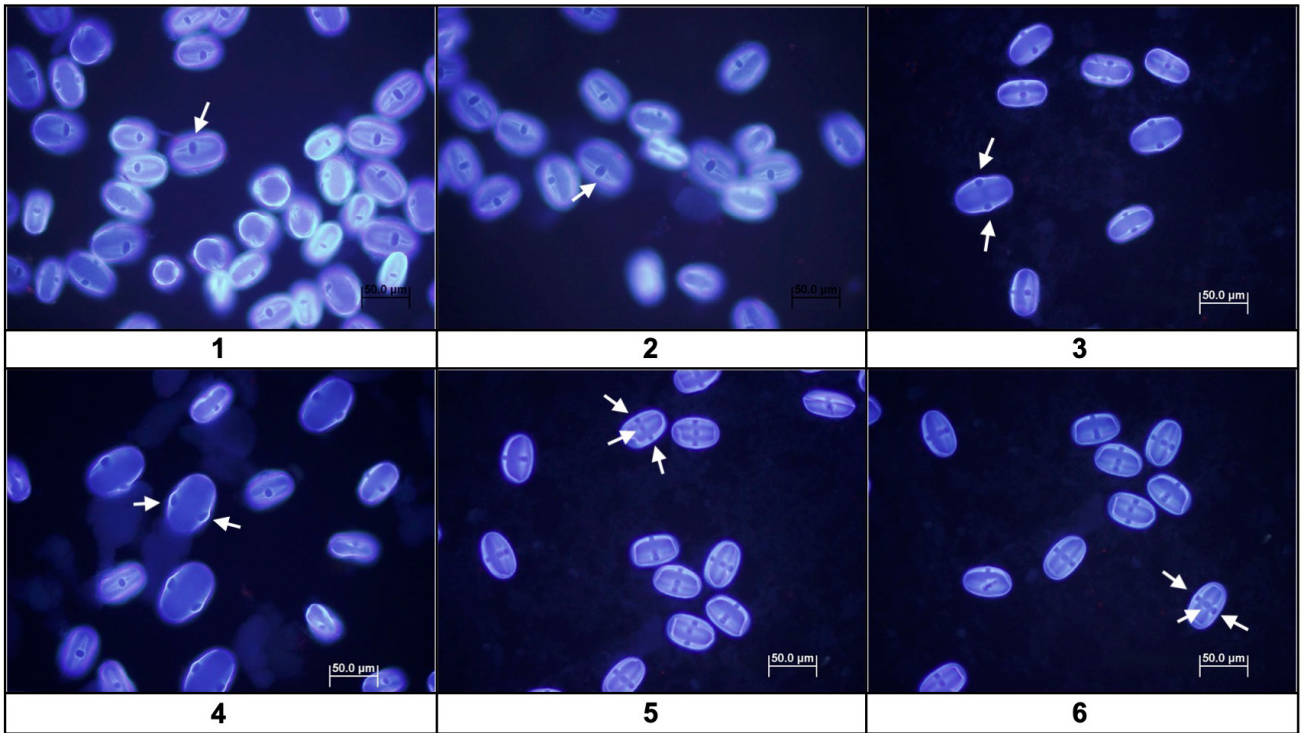

Figure 3. Determination of mononuclear stage in microspores of *Vicia faba* by DAPI staining. Images of flower buds with uni- (**1** and **2**), bi- (**3** and **4**), and trinucleate (**5** and **6**) microspores (filter UMVIBA3). **Numbers** refer to the numbers of the flower buds indicated in Fig. 2. **Arrows** indicate the nuclear number.

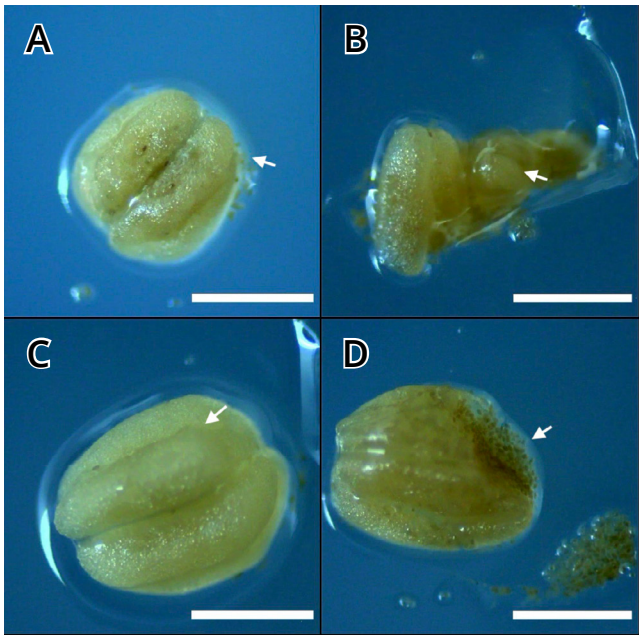

Figure 4. Stereomicroscope images of anthers cultured in MS medium containing Kin (2.5 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}). **Arrows** indicate in **A** – callus initiation, in **B** – globular embryo, in **C** – embryoid like structure, **D** – callus. Scale bars = 200 µm.

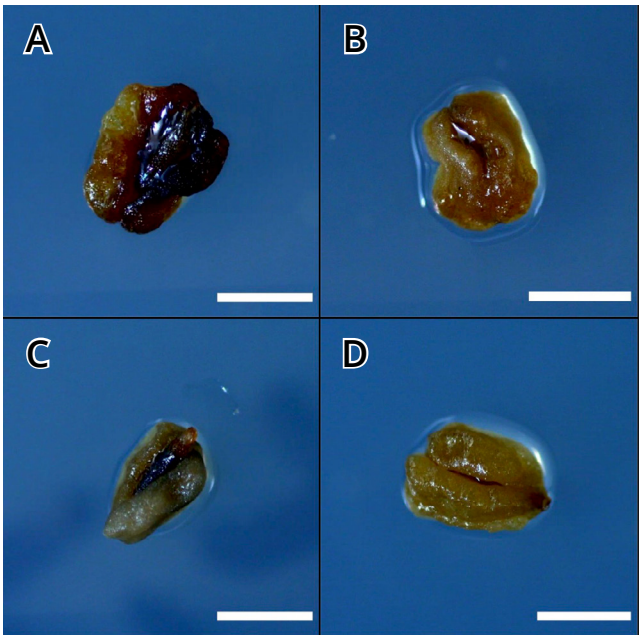

Figure 5. Stereomicroscope images of anthers having trinucleate stage cultured in MS medium including Kin (2.5 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}). Scale bars = 200 µm.

wild-types, *Phaseolus vulgaris* from Colomba and Mexico and one cultivar of *P. coccineus* from Rwanda, it was determined that 2,4-D and Kin growth regulators, as well as the microspore development stage, were important in the success of anther culture (Muñoz et al., 1993).

It was observed that embryoid structures, embryogenic callus tissue, and embryo in the heart stage were formed in the calli of the anther tissue within a month of the culture studies (Fig. 6).

Various problems have been encountered *in vitro* cultures of legumes, including V. *faba* (Fakhrai et al., 1989). *In vitro* culture has not been particularly successful with grain legumes since plant regeneration from callus has been challenging. Previous studies reported that calli secreted a significant amount of phenolic substances and showed poor growth in tissue culture (Fakhrai et al., 1989). Many researchers working with V. *faba* before have reported failure to observe shoot organogenesis in culture (Röper, 1979; Jelaska et al., 1981). In this study, heart shape embryo was obtained successfully from the callus regenerated from anthers with mononucleated microspores.

Conclusions

Anther culture is applied for many purposes, such as propagation and haploid plant production. The developmental stage of microspores in anthers is a key point for the success of the study. This study determined that the culture of anthers at the stage where microspore cells were mononucleated successfully obtained calli. It is thought that the findings from this study are important for the anther culture method to be applied to obtain pure lines from the faba bean. Future steps for getting pure lines include callus culture and plant regeneration.

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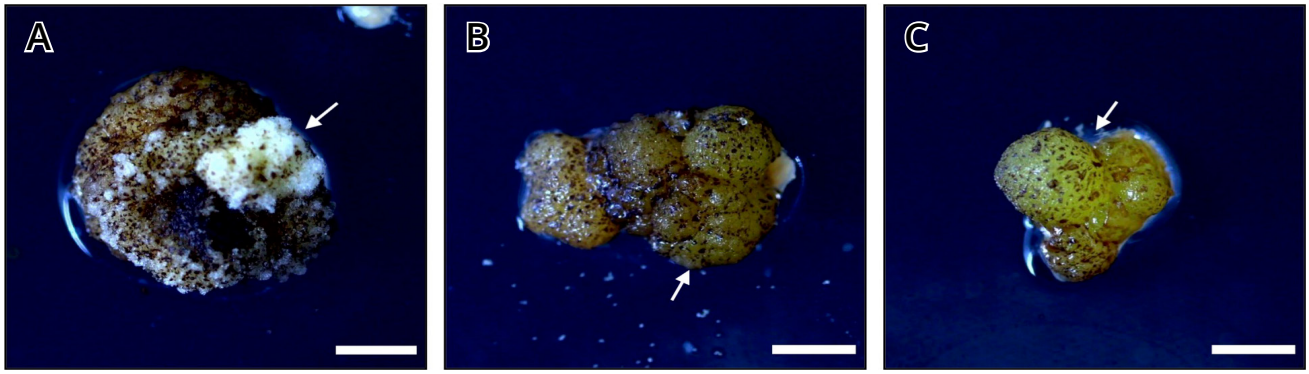

Figure 6. Images of the structures developing from the anther at different developmental stages cultured in the MS medium supplemented with Kin (2.5 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}). **Arrows** indicate in **A** – callus, in **B** – embryoid, and in **C** – heart stage embryo. Scale bars = 200 µm.

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Мікроспорогенез у бобів (*Vicia faba* L.), вирощених у Мерсіні, Туреччина

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Бобові є альтернативою тваринній їжі завдяки високому вмісту білка. Як і інші бобові, *Vicia faba* також відрізняється високим вмістом білка. Однак селекція бобів класичними методами є складною через пригнічення інбридингу, самонесумісність та невдале утворення ембріонів. Неприйнятність класичних методів селекції через проблеми в біології запліднення цих рослини обмежує продукування нових сортів. Тому інтерес до використання техніки гаплоїдизації *in vitro* в селекції бобових зростає. Культивування пиляків є широко застосовуваним методом культивування тканин для отримання гаплоїдних клонів. Початковим і найважливішим етапом культури пиляків є ідентифікація пиляків, що містять мікроспори, у відповідній стадії розвитку. Саме тому під час дослідження зразки пиляків з одно- та триядерними мікроспорами культивували в живильному середовищі MS, що містить 2,4-D (0,5 мг $л^{-1}$) і кінетин (2,5 мг $л^{-1}$). У перший місяць культивування ембріон та ембріоїдоподібні структури на стадії серця були отримані з пиляків, що містять мононуклеарні мікроспори. В той час як пиляки, що містили мікроспори на триядерній стадії, темніли та не давали розвитку новим тканинам.

Ключові слова: *Vicia faba*, культура пиляків, гаплоїдизація, мікроспори