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Accelerated Pepper Breeding Using Molecular Markers and Doubled Haploidy Technique

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ABSTRACT

Hatay pepper is preferred in terms of quality characteristics e.g. hot, fresh and suitable for dried consumption, but it does not contain any disease resistance genes. Within the scope, we established the study is aiming to develop resistant hybrid varieties to TSWV is causing high yield and quality losses in pepper production areas. For this purpose, studies are carried out to obtain inbreed resistant lines in a short time, a Tsw gene found in *Capsicum chinense*, which provides resistance to TSWV, was combined with Hatay pepper lines by crossbreeding. First of all, 20 of the Hatay pepper lines from the gene pool were selected according to weighted grading criteria, then the number was reduced to 4. In *C. chinense* lines, molecular screening was performed with SCAC568 primers and individuals with Tsw gene were determined from donor parents and crosses. After then, backcrosses to Hatay peppers were conducted and BC_1F_1 was generated. Their seed samples were sown from them and brought until the first true leaves during the transition from seed to seedling stage. Meanwhile, molecular analyses were applied to find resistant individuals with Tsw gene. The homozygous and heterozygous plants were planted in a greenhouse and used in anther culture study. Whole study takes 22 months from the initiation of hybridization to the emergence of androgenic embryos and acclimatization to external conditions and development of DH seeds. Here, these findings are presented as a case study in biotechnology and the combined techniques are an indispensable part of accelerated breeding processes.

Keywords: Anther culture, *Capsicum* spp., Tsw gene, Hatay pepper, molecular marker

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Introduction

Annual pepper production in the world is around 37 million tons (FAOSTAT 2020). Pepper, which is in the same family as tomato and eggplant, ranks second after tomato in terms of production value among Solanaceae vegetables in our country and has a total production amount of 2.6 million tons in an area of 792,617 ha (Anonymous 2020). Pepper is one of the vegetables with a wide distribution of types. Blocky bell peppers, Cubanella, Long sweet/hot, Poblano types of peppers are widely grown in our country. Pepper originated in South America and entered Anatolia in the 16th century and is cultivated. Anatolia, which has different ecologies and is on migration routes, has had a very rich diversity in terms of genetic resources in pepper over the years. One of them is our local variety, which is grown in and around Hatay, also known as Hatay pepper or Samandağ pepper, which is preferred in its smooth pointed form,

and which is also consumed as fresh and dried chili peppers. However, crop losses have been experienced in recent years due to Tomato Spotted Wilt Virus (TSWV), which is one of the leading viral diseases that negatively affect yield and quality in open pepper cultivation. TSWV; causes diseases and losses in important agricultural products such as tomatoes, peppers, eggplant, lettuce, beans, artichokes, celery and tobacco (Şevik 2014). It is estimated that the TSWV causes more than 1 billion dollars of damage to agricultural products every year (Griep et al., 2000). TSWV viral infection causes yield losses of 30-100% (German et al., 1992). The use of resistant cultivars against the disease factor has become mandatory. Resistance is not available in the current native cultivars. Commercial foreign hybrid varieties have this resistance. Although the producer does not want to give up traditional pepper varieties, he necessarily turns to foreign varieties from which he can buy products. If resistance to TSWV factor is not transferred to the native material, it does not seem possible to maintain our genetic resources and existing native varieties in the market. It takes many years to develop varieties with traditional breeding methods, and although the cost is high, its effectiveness remains low. DH technologies especially anther culture in pepper has advantages to short breeding time. On the other hand, complex genotypes are fixed by completely homozygous plants obtained through dihaploidization of haploids (Heberle-Bors 1985, Vagera 1990). Haploids and diploids can be successfully applied to study pepper resistance to viruses (Dumas De Vaulx et al., 1982, Pochard et al., 1983) and transmission of resistance to *Phytophthora capsici* (Abak et al., 1982). It has been determined that the conditions found to be successful in pepper with sufficient androgenesis frequency in previous studies also gave positive results in the Hatay pepper population in the preliminary trials (Nar et al., 2022). The inclusion of folded haploid techniques and molecular marker technologies in breeding programs is a basic need in today's conditions. In this study, the determination of the TSWV resistance gene (Tsw) with a molecular marker and its transfer to selected Hatay pepper breeding lines constituted the first stage of the study. The development of haploid pepper pure lines folded by the androgenesis technique, which is one of the genotypes with resistance in the first backcross generation, is the subject that is aimed to be done in the second stage.

Materials and Methods

In this study, which is aimed at breeding Hatay pepper varieties resistant to TSWV; Two basic biotechnological methods were used to accelerate the classical breeding methods: a. Determining the presence of disease resistance gene with a molecular marker, b. Obtaining homozygous pure lines using the folded haploid technique by androgenesis. To create a pepper gene pool, foreign hybrid cultivars, genitors, and local populations were collected from all over our country, some of which were interbred and some of them were inbred up to the F_4-F_7 stage.

In the first stage, morphological characterization (according to the selected UPOV criteria) and observation of agronomic traits were performed during a growing period in 20 local Hatay pepper breeding lines from the gene pool. Some features selected from the UPOV criteria were examined (UPOV 2021). For example; plant features: Maturation, height; (1 very small-5 large), aspect; (1 spreading-5 upright), leaf length diameter ratio (1<, 1, >1), leaf color (1 light green-5 dark green), fruit set (1 weak -5 good); and fruit features: Length (cm), diameter (cm), Cross-sectional shape, immature fruit color, ripe fruit color, flesh thickness (1 thin-5 thick), stance position (1 up - 5 down), number of lobes, flavor (1 bitter - 5 sweet), Stem gap (absent / present), tip shape, curling at the tip (absent/present), wrinkling (absent/present). Hatay pepper local populations have different fruit shapes (Öntürk and Çürük, 2019). 20 lines used in this study have more or less different characteristics from each other. Following the recording of the observations obtained from this stage, the selection was made with the weighted grading method to determine the 4 lines from the gene pool of AG Seed Co., that are preferred to market primarily (Sönmez et al., 2015).

Determination of the main (repetitive) parent lines by weighted grading method: In 20 local Hatay pepper breeding lines selected Weighed Grading Criteria (Table 1) such as plant structure, plant vigor, yield per plant, leaf cover, bitterness, fruit color and brightness were determined from Hatay type pepper populations, which is one of our local gene resources, and 4 different pepper lines with the highest scores were selected accordingly.

Used plant material and its preparation for speed breeding technologies

Sowing seeds from selected Hatay pepper and TSWV resistant lines in the nursery (greenhouse). Main parent (Hatay pepper lines) and paternal parent (lines carrying the resistance gene) plants and seeds were planted in seedling trays filled with a mixture of peat perlite (3:1) and in a fully controlled seedling greenhouse. They are grown until they have 5 true leaves.

Greenhouses are arranged with materials suitable for growing plants and ventilation system in a way

that will not cause disease and insect damage. To grow the plants in an environment free from diseases and thrips, aphid whitefly pests, applications such as 40 mesh insect netting, yellow and blue sticky traps, chemical control etc. were applied to the greenhouse ventilation openings. When the seedlings reach the stage with 4-5 true leaves, they were transferred to 1000 m^2 polyethylene covered greenhouses with 50x50 row spacing.

When the plants of 4 lines selected from Hatay peppers and 3-4 paternal parents that would give the resistance gene reached the flowering stage, crossing procedure was performed. Pollen was first collected from fully opened paternal flowers into tubes, petri dishes or other suitable material with the help of a vibrator. Then, the fully developed but not opened buds in the main parent, and the anthers that have not yet burst, were selected and the petals and anthers were removed. The stigmas of the emasculated buds were pollinated by dipping into previously collected pollen, and the process was completed by attaching a label to the flower stem. Seeds from the fruits developed as a result of hybridization were harvested during the red maturity period, dried and replanted. In the second half of 2021, at this stage in the autumn, backcrossing was done to Hatay pepper types and their seeds were taken in a healthy way.

In crosses made in 4 different Hatay pepper lines, pollen taken from 4 paternal parents was mixed and used in the pollination process with the mixed pollen technique. This method has a positive effect on obtaining hybrids with high adaptability and seed set rate. When the fruits formed were reddened, the seeds were removed, cleaned and dried. The seedlings were obtained from the seeds and they planted in AG Seed Co.'s greenhouses on August 16, 2021. Hybridizations were started in the bud development period. Thus, backcrossings were started in F_1 plants and each line was crossed with its parent and BC_1F_1 A, B, C and D seeds were reached during the harvest period of the fruits. Seeds of these fruits were used as donor plants in anther culture studies in the 2022 spring period.

Molecular marker resistance tests

Our lines developed from Chile peppers were tested for the presence of the Tsw gene. At this stage, 4 different pepper materials were selected as the parental plants. The pollen of these parental parents was mixed and used in crosses with 4 Hatay pepper lines. The lines formed as a result of hybridizations were tested with the help of molecular markers in the molecular genetics laboratory established within the AG Seed Co. During the seedling period, 100 mg of leaves were taken from the true leaf samples of the pepper plant, and the CTAB protocol (Doyle and Doyle 1987) was applied and DNA was isolated (Figure 1). DNA samples were run on agarose gel and after quality control and concentration equalization, Moury et al., (2000) PCR reaction was carried out using the CAPS marker (SCAC568). The protocol used as follows: SCAC568 primers specific to the Tsw gene were used forward (5'GTGCCAGAGGAGGATTTAT 3') and reverse (5'GCGAGGTGACACTGATACT 3'). The PCR reaction is completed to a final volume of 50 µl with EcoTaq 2x PCR Master Mix 25 µl, Forward primer 10 µM 2 µl, Reverse primer 10 µM 2 µl, genomic DNA 10 pg-500 µg and ddH_2O. Cycle conditions 98 ºC for 1 minute; 94 ºC for 30 seconds, 57 ºC for 30 seconds, 72 ºC for 30 seconds 36 cycles and final elongation at 72 ºC for 1 minute. The mixture prepared as 10 µl of the obtained PCR products, 1 µl of Thermo Scientific FastDigest XbaI enzyme, 2 µl of buffer and 17 µl of ddH_2O was cut at 37 ºC for 5 minutes and incubated at 65 ºC for 15 minutes. Laboratory processes were completed with gel readings (Nar et al., 2022).

The PCR result of Scac 568 primer is given in Figure 2 and the gel image after cutting with XBAI enzyme is given in Figure 3.

Androgenesis studies

In anther cultures, buds in the morphological development stage must be collected, subjected to surface sterilization in the laboratory and then cultured. It has been determined in previous studies that the buds in the suitable microspore period in pepper are the period when the petals and sepals reach the same level or pass 1-2 mm (Çömlekçioğlu and Ellialtıoğlu 2018). The blue-purple color transformation at the tips of the anthers is the key feature.

The protocol, which includes combinations of MS nutrient medium, activated charcoal, $AgNO_3$, NAA and BAP in pepper, gives very successful results (Alremi et al., 2014). Using the same method with some modifications is also recommended by Keleş et al., (2015). They used MS medium containing 4 mg/L NAA, 1.0 mg/L BAP, 0.25% activated charcoal, 30 g/L sucrose, 7 g/L agar and 15 mg/L silver nitrate was used as nutrient medium. In this study, process flow in anther cultures Bat et al., (2020) according to the method described (Figure 4). After the prepared nutrient media were adjusted to pH 5.8, they were sterilized in an autoclave at 121 ºC for 20 minutes. The media removed from the autoclave was poured into petri dishes with a diameter of 60 mm in a sterile cabinet in equal amounts and left to solidify.

The cultures were incubated in a growth cabinet at a temperature of 25±1 ºC, with 16 hours of light and 8 hours of darkness (Vural et al., 2019). After the anthers

were cultured, embryo emergence was observed in the 30-70 days of the incubation period in the climate chamber and the emergence was determined. A few days after the embryos were seen, they were first arranged in contact with the same environment, then germinated in hormone-free MS medium.

Plantlets that formed fringe roots and 4-6 true leaves were planted in mini pots filled with autoclaved ½ perlite + ½ peat mixture, giving life water. Developed plants were transferred to the greenhouse.

Results and Discussion

Identification of suitable lines from the gene pool

Weighed grading has been used for many years as a statistical and consistent selection method used in the selection of starting material suitable for breeding purposes from the gene pool or in highlighting the candidate variety among the variants obtained after the breeding program (Sönmez et al., 2015). It was also used effectively in the selection of breeding material for our study. Figure 5 shows the 4 Hatay pepper genotypes selected to be used as replicates in the study.

To transfer the Tsw gene to the sensitive Hatay peppers, the chile peppers at the F_1-F_3 stage found in our gene pool were tested with the molecular marker technique, and the genitors whose Tsw gene presence was confirmed and similar to the structure of the Hatay type pepper were selected.

Crossbreeding, generation advancement

Crosses were made with 4 selected Hatay-type pepper lines and Tsw-resistant chile peppers with mixed pollen technique. Harvesting and seed extraction of fruits that are at the stage of red ripening in the greenhouse was performed (Figure 6). Backcrossing was carried out on the plants grown from the seeds obtained. Backcrossings in the autumn season were made towards Hatay pepper types and their seeds were taken in a healthy way. Obtained individuals were subjected to molecular marker testing and it was examined whether they contain the Tsw gene. Those containing the resistance gene were used as donor plants for androgenesis studies.

After the backcrossing, molecular marker tests were carried out in BC_1F_1 generation plants of 4 different Hatay lines using SCAC568 primers specific to the Tsw gene. In this context, the lines whose seeds were taken as a result of crosses in the previous season were grown and samples were taken from the real leaves while they were still in the seedling stage, and PCR tests were carried out after total nucleic acid isolation. Approximately 376 plants were tested during the season (Table 2). As a result, individuals with homozygous or heterozygous resistance were separated from individuals with homozygous recessive disease susceptibility characteristics in both alleles. Plants found to contain the resistance gene were transferred to the donor plant growing greenhouse to be used in the process of obtaining DH-lines by androgenesis method, and anther culture was made from them at the stage of flower bud formation.

From androgenesis studies, haploid embryo formation was obtained successfully (Figure 7). The process of obtaining haploid plants, chromosome doubling and obtaining DH seeds are completed. DH seeds harvested and stored at the cool conditions (+7 °C) in the paper bags.

In Table 3, anther planting was made in the spring of 2022 and the embryo and plant numbers taken from them are given. Embryo emergence is continuing. Embryo formation frequencies close to each other were obtained from anthers taken from backcross donor plants in lines A, B, C and D, which have genetically similar characteristics. An average embryo formation frequency of 2.05% was obtained. This number within the embryo formation frequency range compatible with many previous studies (Çömlekçioğlu and Ellialtıoğlu 2018; Atasoy et al., 2021). With the increase in the performance of the working system and personal over time in the laboratory, it can be predicted that this ratio will also improve somewhat. However, since the frequency of embryo formation is a genetic feature, it is thought that the average success will still be in the same range.

Conclusions

This article has been prepared from a presentation made to provide an overview of the phases carried out in a project that lasted 3 years, and to share the outputs. For this reason, it is designed to explain the flow of the breeding program rather than giving the details and procedure of a single study. The study mainly consisted of the following stages and was performed successfully:

a. To identify 4 prominent genotypes from the Hatay pepper gene pool;

b. Identifying parental parents with the Tsw resistance gene from the chile pepper gene pool using molecular markers;

c. Crossing and backcrossing;

d. Performing anther cultures from BC_1F_1 plants and obtaining Tsw resistant DH lines.

In order to develop hybrid varieties in pepper, obtaining parent lines requires 6 generations of selfing, and breeding takes a long time. Pepper androgenesis, which was first initiated in the 1980s in Turkey, can be used successfully (Çömlekçioğlu and Ellialtıoğlu 2018). Until recently, almost all of the F_1 hybrid

varieties grown in our country were imported from abroad. In recent years, domestic hybrid varieties have started to take place in the market in some of the vegetable species, thanks to the establishment of their R&D enterprises by local seed companies, projects and incentives received with from government facilities. With the subject of trade in pepper and pepper products, an economic activity of approximately 1 billion dollars occurs in Turkey. In 2016, around 40,000 k of open-pollinated and 350 k of hybrid pepper seeds were produced, and some of the hybrid pepper seeds, all of which were purchased from abroad, could be met by domestic companies. The number of hybrids is less because Turkish consumer habits have peculiar characteristics in pepper, these demands cannot be met with varieties produced abroad, and most of the varieties grown in the open are local populations. Significant developments are expected in the seed sector in Turkey as well. Firms need to constantly increase their competitiveness.

Since tissue culture and MAS technological infrastructure will contribute to product development in new areas, it will bring important innovations in the breeding process and shorten the development period of the variety. Obtaining pure lines in a short time, dihaploidization technique and anther culture can be practically integrated into breeding studies. When MAS technology is combined with selection and backcrossing programs, both faster and more reliable and easy results are obtained.

With the use of both techniques, it is possible to complete a 10-12 year breeding program in a short period of 3 years. Short-term breeding efforts and the rapid introduction of new varieties will generally improve the seed potential of Turkey. Thanks to the transfer of TSWV resistance trait with MAS, which is one of the accelerator breeding techniques, and the acquisition of DH plants through androgenesis, qualified genotypes were obtained in about 2 years. With this ongoing study, inbreed lines in which the TSWV disease resistance gene is transferred to Hatay-type peppers, which is one of the genetic resources of Turkey, are successfully obtained. This material will be used as a parent in hybrid cultivar breeding and thus disease resistant local pepper varieties will be developed. In this way, a chance to compete in the seed sector can be provided.

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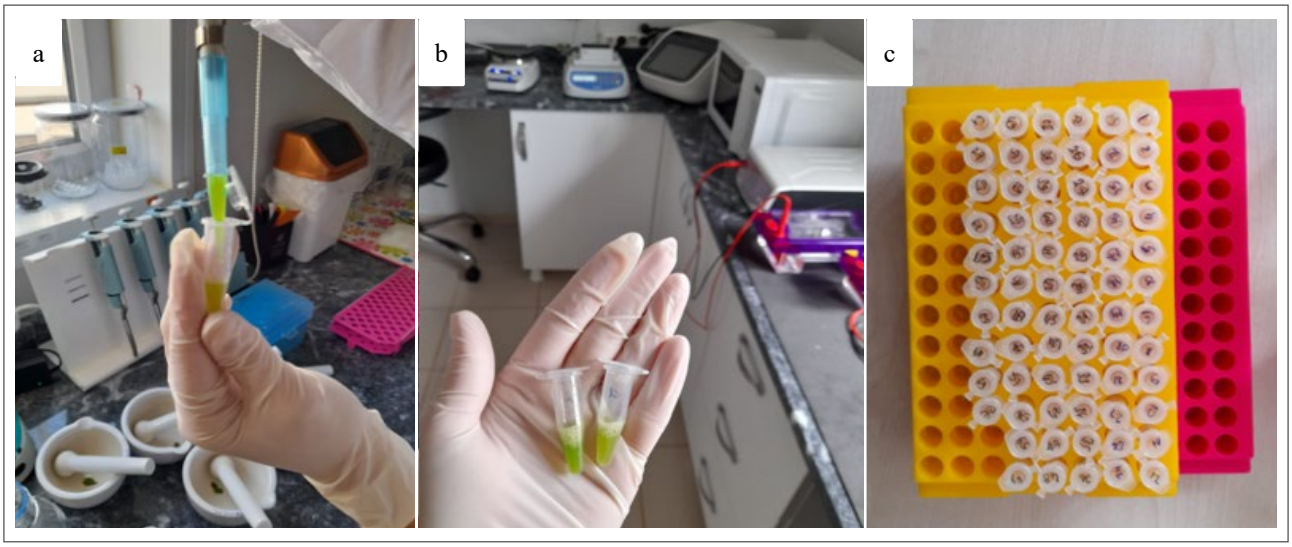

Figure 1. Examples of DNA isolation steps. a. Crushing the leaf samples with CTAB solution in a mortar, b. Transferring the crushed samples to 1.5 microtubes, c. Arrangement of the crushed samples. (Original)

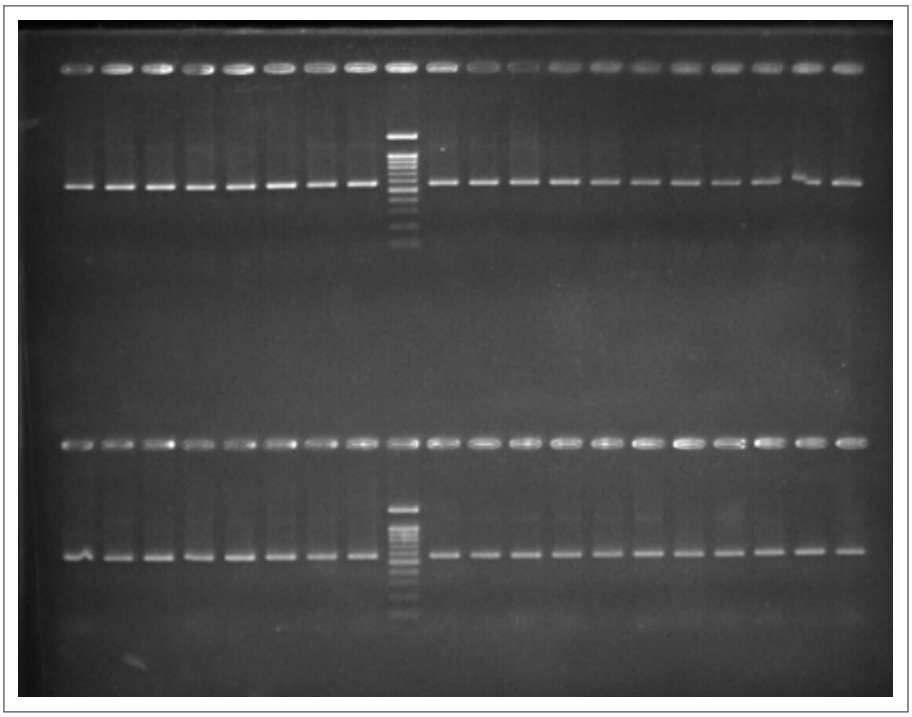

Figure 2. The PCR result of SCAC 568 primer.

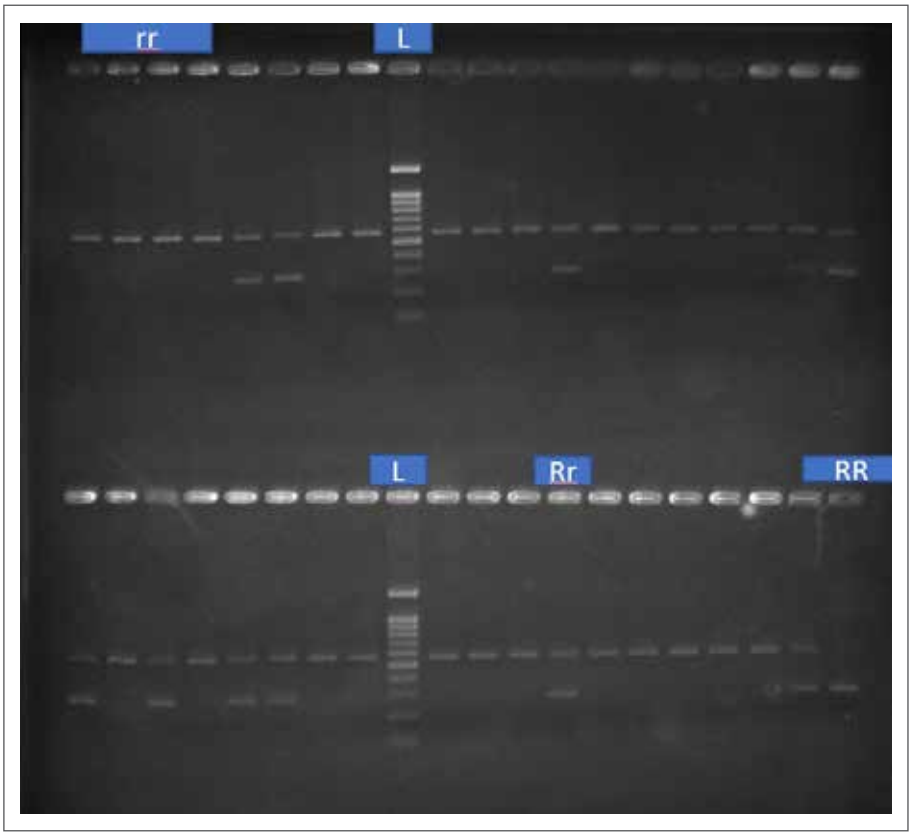

Figure 3. The gel image after cutting with Xbal enzyme (280bp:RR, 280bp, 568bp:Rr, 568bp: rr, L: 100bp ladder)

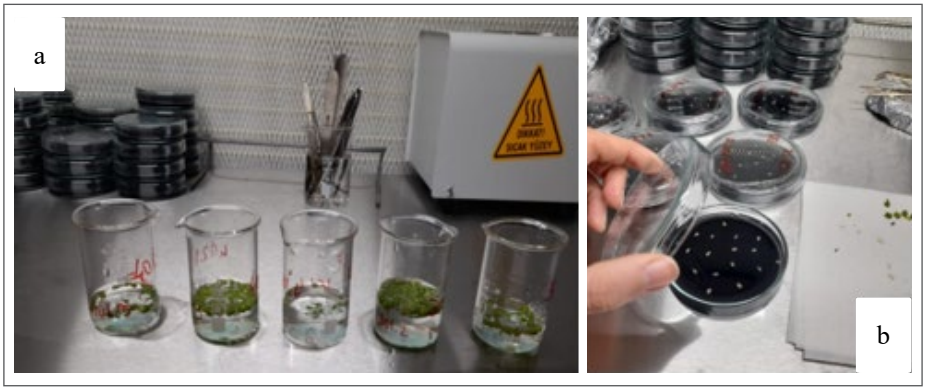

Figure 4. Anther culture stages. a. Disinfection of buds in 15% sodium hypochlorite for 12 minutes, b. Culturing of anthers in nutrient media. (Original)

Figure 5. Plant habitus and fruit appearance of selected Hatay type peppers (a-b Hatay 1, c-d Hatay 2, d-e Hatay 3, f-g Hatay 4). (Original)

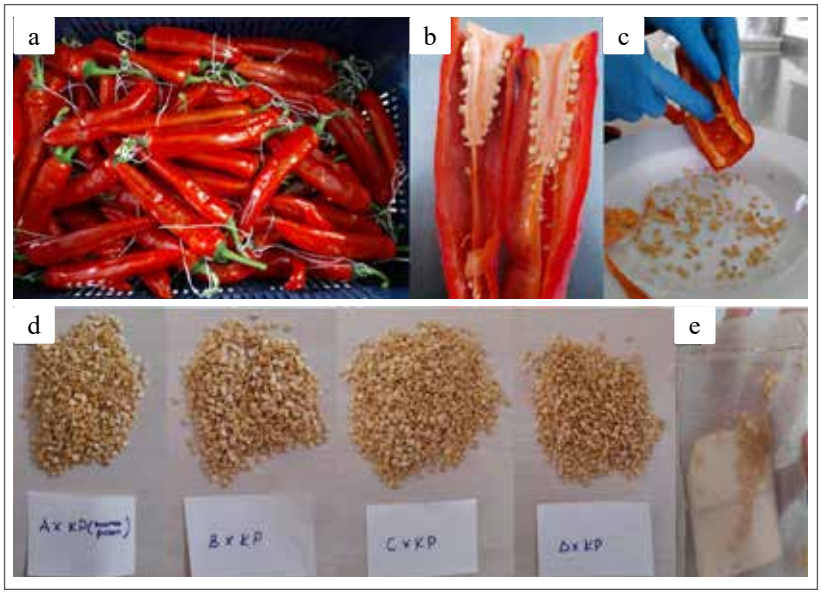

Figure 6. Cleaning and packaging of the fruits and seeds obtained as a result of the hybridization of Hatay pepper lines with Tsw-resistant peppers. (Original)

Figure 7. Development of pepper embryos from microspores and their transformation into plants (above); Growing haploid plantlets *in vitro*, transferring to soil and acclimatization to external conditions (below). (Original)

Table 1. Weighed grading observation criteria.

Observation Criteria	Weight in the Selection (%)
Bitterness	30
Yield per plant	20
Plant power	10
Plant habitus (Closed)	10
Fruit shape	10
Fruit color (dark green)	10
Brilliance in fruit	10

Table 2. Molecular marker (Tsw) test results.

Number of Plants Tested	Homozygous (RR)	Heterozygous (Rr)	Susceptible (rr)	No Tape
94	17	67	6	4
94	13	52	19	10
94	6	70	14	4
94	13	56	17	8
Total=376	**49**	**245**	**56**	**26**

Table 3. Anther numbers from plants from four different backcross (BC) combinations, their ratios with the number of embryos and plantlets obtained from them.

Genotype	Number of Anthers Cultured	Formed Embryo Number	The Number of Plantlets Obtained	Embryo Formation Rate (%)	Plant Formation Rate (%)
BC_1F_1 A	4000	82	73	2.05	1.83
BC_1F_1 B	3500	71	50	2.02	1.42
BC_1F_1 C	3500	96	81	2.40	2.31
BC_1F_1 D	3000	52	38	1.73	1.26
Total	**13500**	**301**	**242**	**2.05**	**1.71**

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