Uluslararası Fen Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Science Research 2025, Vol. 9 (4), 98-110
https://doi.org/10.29329/ijiasr.2025.1386.3

Original article

Development of PMMoV (L3)–Resistant Breeding Lines in Pepper (*Capsicum annuum* L.) through the Combination of Haploidy and Molecular Marker Technologies

Ahmet Naci Onus *

Department of Garden Plants, Faculty of Agriculture, Akdeniz University, Antalya, Türkiye

Abstract

This study addressed the increasing need for effective and rapid breeding strategies against Pepper mild mottle virus (PMMoV), a major constraint in pepper (Capsicum annuum L.) production due to the lack of chemical control options for viral diseases. The research aimed to develop PMMoV-resistant pepper lines representing different fruit types demanded by the market by integrating SNP-based Kompetitive Allele Specific PCR (KASP) genotyping with phenotypic biotest assays and subsequently using selected resistant plants as donor material for doubled haploid (DH) line production. Five F_2 genotypes (B05, B06, B07, B08, and B10) obtained from a private sector breeding program were evaluated under controlled conditions. Seedlings were subjected to mechanical PMMoV inoculation and scored for resistance on a 1–10 scale, while KASP analysis was conducted in parallel to confirm resistance at the marker level. Across genotypes, 113–146 plants per population were biotested, and 69–108 individuals per genotype were initially classified as resistant. Combined evaluation of biotest and KASP results ultimately confirmed 23–32 resistant plants per genotype, which were transplanted and cultivated under soilless greenhouse conditions to provide flower buds for androgenesis. Anther culture was performed on MS-based induction medium, and embryo formation was monitored. Embryos were obtained from four genotypes, whereas no embryo induction occurred in B05. A total of 6, 1, 2, and 21 embryos were produced from B06, B07, B08, and B10, respectively, and 16 regenerated plants developed into spontaneous doubled haploids and successfully produced DH0 seed. Overall, the findings demonstrated that combining KASP-based marker validation with DH technology enabled the rapid development of PMMoV-resistant, fully homozygous breeding lines, supporting the advancement of domestic hybrid cultivar development and strengthening the competitiveness of local seed companies.

Keywords: *Capsicum Annuum*, Modern Breeding, Speed Breeding, F_1 Hybrid Breeding

Received: 17 December 2025 * **Accepted:** 30 December 2025 * **DOI:** https://doi.org/10.29329/ijiasr.2025.1386.3

* **Corresponding author:**

Ahmet Naci Onus, Prof. Dr., Department of Garden Plants, Faculty of Agriculture, Akdeniz University, Antalya, Türkiye.
Email: onus@akdeniz.edu.tr

INTRODUCTION

Pepper (*Capsicum annuum* L.), which is included in the genus *Capsicum* of the *Solanaceae* family, is an important vegetable, spice, and industrial crop that can be cultivated in subtropical and tropical climatic regions. Pepper has great importance in Turkish agriculture both due to its nutritional and mineral content and in terms of its production area and quantity. According to archaeological evidence, pepper is one of the earliest domesticated plant species, with a history dating back at least to 7000 BC in South and Central America (Tripodi and Kumar, 2019).

There are 27 species included in the genus *Capsicum*. Among these, *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. pubescens* R. & P. are cultivated. The majority of the remaining 22 uncultivated wild species are endemic to the tropical regions of the Americas. Among the cultivated species, *C. chinense* is known to originate from the Amazon region; *C. baccatum* and *C. pubescens* from Peru and Bolivia; and *C. annuum* and *C. frutescens* from Mexico and Central America (Pickersgill, 1997). Most scientific studies have focused on *C. annuum* L., which has major commercial importance.

Pepper production and consumption have been steadily increasing both worldwide and in Turkey. According to FAO statistics for 2023, the global cultivated area of pepper is 3.7 million hectares, of which 2.0 million hectares are devoted to fresh pepper and 1.7 million hectares to dried red pepper for spice production. Over the last ten years (2009–2019), the cultivated area has increased by 6%. Global pepper production has shown a similar steady upward trend. Over the past decade, production increased by approximately 35% for fresh peppers and 43% for dried peppers; fresh pepper production rose from 28 million tons to 38 million tons, while dried red pepper increased from 3.0 million tons to 4.3 million tons. Two-thirds of total production is carried out in Asia (67% for fresh pepper and 70% for dried pepper). In fresh pepper production, the Americas rank second and Africa third (23% and 10%, respectively), while Europe ranks last. For dried red pepper, Africa ranks second (21%), followed by the Americas and Europe (6% and 3%, respectively). The most important reason for these increases is undoubtedly the growing recognition of the health benefits of pepper, particularly its richness in minerals, vitamins, and antioxidants.

In terms of country-level production, China ranks first in fresh pepper production (18.9 million tons), followed by Mexico (3.3 million tons), Turkey (2.7 million tons), Indonesia (2.5 million tons), Spain (1.3 million tons), and the United States (963 thousand tons). In dried pepper production, India ranks first (2.1 million tons), followed by Thailand (350 thousand tons), China (314 thousand tons), Pakistan (148 thousand tons), Bangladesh (137 thousand tons), and Myanmar (131 thousand tons) (FAOSTAT, 2023).

Pepper, which is an indispensable component of Turkish cuisine, has also shown a steady increase in production and consumption in Turkey. In recent years, fresh pepper production has increased from 1.8 million tons to 2.7 million tons, representing a 43% increase. Per capita annual fresh pepper production has risen from 25.1 kg to 33.9 kg, an increase of 35%. In production, kapya-type peppers—also used for paste production—rank first with a share of 44%, followed by long green peppers with 36%, stuffed peppers with 16%, and charleston-type peppers with 4%.

Although fresh pepper is produced in all regions and provinces of Turkey, the highest levels of cultivation are observed for kapya or paste-type peppers in Çanakkale (30%), Manisa (11%), and Adana (9%); and for long green peppers in Mersin (30%), Antalya (20%), and Bursa (10%). Production of dried red pepper for spice purposes does not vary significantly by year. Dried pepper production, which has stabilized at around 225 thousand tons, is mainly carried out in Southeastern Anatolia (Anonymous 2024).

Throughout the twentieth century, significant breeding efforts have been carried out in pepper focusing on fruit quality, resistance to diseases, earliness, yield potential, tolerance to abiotic stresses, and plant morphology. In this context, breeding is defined as the purposeful modification of the genetic structure of a population in order to make it more productive or more resilient, and it is inherently a time-consuming process. In recent years, plant biotechnology has become one of the most important tools used to shorten the duration of breeding programs.

One of the primary driving forces behind the development of new vegetable cultivars is resistance to diseases. In particular, because chemical control options for viral diseases are not available, the development of resistant cultivars is of critical importance. In recent years, PMMoV has become increasingly prevalent in pepper cultivation and has begun to cause significantly greater damage compared to other viral diseases affecting pepper production (Tsuda et al., 2007; Parisi et al., 2020).

In conventional breeding programs, the selection of genotypes and the subsequent fixation (purification) of these selected genotypes require many years. Moreover, tolerance to abiotic conditions such as low and high temperatures in pepper is controlled by multiple genes, which further complicates the transfer of resistance to subsequent generations. As a result of experimental studies, the fixation of heterozygous individuals identified as resistant or tolerant to a given stress factor can be achieved more easily through haploidization techniques without losing the desired traits.

The production of doubled haploid (DH) lines is carried out in order to shorten the time required to develop pure lines and to obtain lines that are homozygous for all alleles. In pepper, haploid plants can be obtained through polyembryony and *in vitro* androgenesis. *In vitro* induction of haploid embryos in pepper is achieved via embryogenesis in male gametes. Anther culture involves the excision of anthers

containing immature pollen grains from flower buds and their transfer to artificial nutrient media under *in vitro* conditions, where haploid embryos are obtained from immature pollen.

Through anther culture, pollen grains that are normally at the binucleate stage are redirected toward a somatic developmental pathway, resulting in androgenesis. *In vitro* androgenesis is one of the most widely used techniques for obtaining haploid plants, employing either anther or microspore culture. Fundamentally, this process involves arresting the development of pollen cells that would normally form male gametes at a specific stage and forcing them to undergo direct embryogenesis, similar to somatic cells.

Numerous studies on androgenesis in pepper have been conducted by many researchers using the MS (Murashige and Skoog, 1962) basal medium with various medium combinations. The general conclusion of these studies is that, compared to other species, haploid plant production in pepper is highly genotype-dependent (Dumas et al., 1981; Çömlekçioğlu et al., 2001; Çağlar et al., 2004; Ercan et al., 2006; Ercan and Şensoy, 2011; Çömlekçioğlu and Ellialtıoğlu, 2018; Niklas-Nowak et al. 2012).

The most effective approach for disease management is the development of cultivars that are resistant to this disease. In recent years, some foreign pepper cultivars reported to be tolerant to PMMoV have entered the market. However, the number of domestically developed pepper cultivars reported to be resistant to this disease is limited, and there is a substantial need for such varieties.

The aim of this study is to develop pepper lines with different fruit types that meet current market demands and possess PMMoV resistance, by integrating a molecular approach—namely the KASP method—with biotest assays. Achieving these objectives will ultimately contribute to the development of domestic, PMMoV-resistant hybrid cultivars, which constitutes the primary goal of this research.

MATERIALS and METHODS

Plant Material

The plant material to be used in this study consists of five different pepper genotypes at the F_2 generation belonging to a private sector company (B05, B06, B07, B08, and B10).

Methods

Plant Growth Conditions

Seed Sowing

Seeds of the genotypes used in the study were sown in seedling trays containing a suitable growing medium (a peat + perlite mixture). The seeds were covered with vermiculite and irrigated. The trays were then placed in germination chambers set at 25 °C and a minimum relative humidity of 85% to allow germination. Following seed germination and emergence, appropriate irrigation and fertilization

practices were applied, and necessary plant protection treatments were carried out in accordance with standard cultivation procedures.

Biotest and Molecular Marker (KASP) Analyses

Lines derived from the selected high-quality genotypes were sown and raised as seedlings following standard seedling production practices. When the seedlings reached the two–true-leaf stage, inoculum was applied to the true leaves, and after a defined period, the lines were evaluated and scored as resistant or susceptible. Resistance and susceptibility were determined using a scoring scale ranging from 1 to 10.

Molecular analyses based on the SNP marker–based KASP method were conducted in parallel with the biotest. The objective was to identify resistant lines through both marker assays and biotesting. The primers used in the molecular analyses were developed by the supplier company. After verifying whether the results of the marker test and the conventional disease assay were consistent and supportive of each other, the lines identified as resistant were grown as seedlings to be used as donor plants.

For biotesting at the cotyledon stage, the protocol described below was applied.

Biotesting protocol:

Required Materials

- Virus-infected plant leaves
- Beaker
- Sponge
- Mortar or blender
- Distilled water
- Gloves and ice

Materials Required for Phosphate Buffer

- KH_2PO_4 (potassium dihydrogen phosphate)
- Na_2HPO_4 (disodium hydrogen phosphate)
- β-mercaptoethanol

Mechanical Inoculation:

When virus-infected plants were available, leaves from PMMoV-infected plants were collected. The leaves were placed in a blender or mortar, phosphate buffer was added, and plant sap was extracted. The homogenate was then filtered through gauze to prevent plant debris from passing through. To

maintain virulence and preserve the cold chain, ice was placed in a container and the beaker containing the extract was positioned on the ice. Throughout the inoculation process, care was taken to keep the solution continuously cold. Inoculation was performed using a sponge on plants whose cotyledons had reached the appropriate stage for inoculation.

Five to ten minutes after inoculation, the leaves were rinsed with water. A second inoculation was carried out two days after the first. Under appropriate temperature and humidity conditions, symptoms were observed within 7–10 days and disease severity were scored. For the PMMoV disease assay, the temperature conditions were as follows: daytime 23–28 °C and nighttime 20–23 °C.

KASP protocol:

FTA Purification

- DNA samples were collected using FTA cards.
- Discs were punched from the FTA cards and transferred into PCR tubes using a punch tool.
- Fifty microliters (50 µL) of FTA purification solution were added to the discs, and the tubes were gently tapped to mix.
- The samples were incubated at room temperature for 5 minutes, after which the buffer was aspirated.
- Fifty microliters (50 µL) of TE buffer were added to the discs, and the tubes were gently tapped to mix.
- The samples were incubated at room temperature for 5 minutes, and the buffer was then aspirated.
- Fifty microliters (50 µL) of TE buffer were added again to the discs, and the tubes were gently tapped to mix.
- The samples were incubated at room temperature for 5 minutes, and the buffer was aspirated.
- Finally, the discs were incubated at 65 °C for 10 minutes.

Pre-amplification

- 10 µL DreamTaq Green PCR Master Mix
- 10 µL ddH_2O
- 1 µL STA primer
- 1 µL C1 primer

Total reaction volume per sample: 22 µL

Pre-amplification PCR Cycling Conditions:

- 94 °C …… 1 min
- 94 °C …… 30 s
- 57 °C …… 1 min
- 72 °C …… 30 s
- 72 °C …… 7 min
- 12 °C …… 5 min

Dilution: One hundred fifty microliters (150 µL) of dH_2O were added to the pre-PCR product and mixed thoroughly.

Final volume: 22 µL pre-PCR product + 150 µL dH_2O = 172 µL

Preparation of the KASP Primer Mix

- A1 primer (100 µM): 12 µL
- A2 primer (100 µM): 12 µL
- C1 primer (100 µM): 30 µL
- TE-1 buffer: 46 µL

Total volume: 100 µL

KASP Genotyping Mix

- 2× PACE Master Mix: 2.5 µL
- KASP primer mix: 0.07 µL
- Pre-PCR product: 2.5 µL

Total reaction volume per sample: 5.07 µL

All reaction mixes were dispensed into a 384-well plate and mixed thoroughly.

KASP PCR Cycling Conditions:

- 94 °C …… 15 min (hot-start activation)
- 94 °C …… 20 s
- 65–57 °C …… 1 min (touchdown annealing)

- 94 °C …… 20 s
- 57 °C …… 1 min
- 12 °C …… ∞ (hold)

After PCR, the plate was gently mixed and briefly centrifuged.

Plate Reading and Evaluation of Analysis Results

The data were transferred to an Excel file and evaluated.

Cultivation of PMMoV-Resistant DH Donor Plants

Based on the test results, plants identified as resistant were transplanted. The plants were grown under soilless culture conditions using cocopeat as the growing substrate. Prior to planting, the cocopeat was prepared to be suitable for cultivation. Plot numbers were assigned for each cultivar, and each line was planted separately. During the growing period, the following cultural practices were applied:

Training and Twining

Elongating shoots were supported with strings and trained by twining around the strings.

Pruning

Plants were trained to three main stems. Shoots developing outside these three stems were removed.

Irrigation and Fertilization

An appropriate greenhouse environment was maintained according to seasonal conditions. The irrigation regime was applied and monitored accordingly. Fertilization programs were determined and implemented based on plant observations and nutritional requirements.

Plant Protection

Plants were regularly monitored for diseases and pests. Based on observations, the necessary plant protection measures were applied according to a scheduled program.

Collection of flower buds commenced one month after transplanting.

Collection of Flower Buds

For the anthers to be used in the experiment, microspores were required to be at the uninucleate stage. Therefore, prior to culturing, the microspore developmental stage was determined for each genotype using microscopic examination.

Flower buds were surface-sterilized by immersion in a 2% commercial sodium hypochlorite solution containing one to two drops of Tween-20 per 100 mL for 15 minutes, followed by three rinses with sterile distilled water, each lasting one minute.

Preparation of Culture Media

The culture medium used in the experiment was prepared as follows:

Anther Culture Medium: MS medium supplemented with 4 mg L^{-1} NAA, 1 mg L^{-1} BAP, 0.25% activated charcoal, 30 g L^{-1} sucrose, 15 mg L^{-1} $AgNO_3$, and 7% agar.

After all chemicals were added, the pH of the medium was adjusted to 5.8. The prepared media were sterilized in an autoclave at 121 °C and 1.2 kg cm^{-2} pressure for 20 minutes.

Placement of Anthers on Culture Media

Anthers were carefully excised intact from the flower buds without causing damage and placed onto the prepared culture media.

Incubation of Anthers

Following disinfection of the flower buds and excision of the anthers, the anthers were cultured on the media. Petri dishes were incubated at 35 °C under dark conditions for two days. Subsequently, they were transferred to growth chambers maintained at 25 °C with a photoperiod of 18 hours light and 6 hours dark.

Embryo Formation, Germination, and Development

Once embryo formation was observed, embryos were transferred to hormone-free MS medium for germination.

Development of n-Chromosome Plants, Acclimatization, Ploidy Determination, and Chromosome Doubling

Haploid plants with well-developed lateral roots and 4–6 true leaves were transferred to an acclimatization greenhouse to adapt to greenhouse conditions. After approximately 10 days, the plants were moved to the main greenhouse, and their growth was monitored.

Ploidy levels of the regenerated plants were determined by stomatal counts to identify haploid or spontaneously doubled haploid plants.

Seed Production from DH0 Plants

DH_0 plants were self-pollinated under greenhouse conditions to ensure fruit set. Upon fruit maturity, fruits were harvested, and seeds were extracted. Each seed lot was assigned a unique pedigree number and packaged for use in subsequent breeding programs.

RESULTS and DISCUSSION

In this study, a total of 113 plants from genotype B5, 124 from genotype B6, 146 from genotype B07, 141 from genotype B08, and 99 from genotype B10 were subjected to biotesting. According to the biotest results, 82 plants from genotype B5, 83 from genotype B6, 108 from genotype B07, 84 from genotype B08, and 69 from genotype B10 were identified as resistant. All plants classified as resistant were subsequently analyzed using the KASP method. In this way, plants showing resistance in both the biotest and the KASP assay were identified.

Based on the combined evaluation of biotest and KASP results, 32 plants from genotype B5, 25 from genotype B6, 31 from genotype B07, 27 from genotype B08, and 23 from genotype B10 were ultimately confirmed as resistant.

Table 3. Number of plants subjected to biotest and KASP analysis

Genotype	Number of plants tested	Number of L3 resistant plants after biotest	Number of L3 resistant plants after KASP molecular analysis
B05	113	82	32
B06	124	83	25
B07	146	108	31
B08	141	84	27
B10	113	82	32

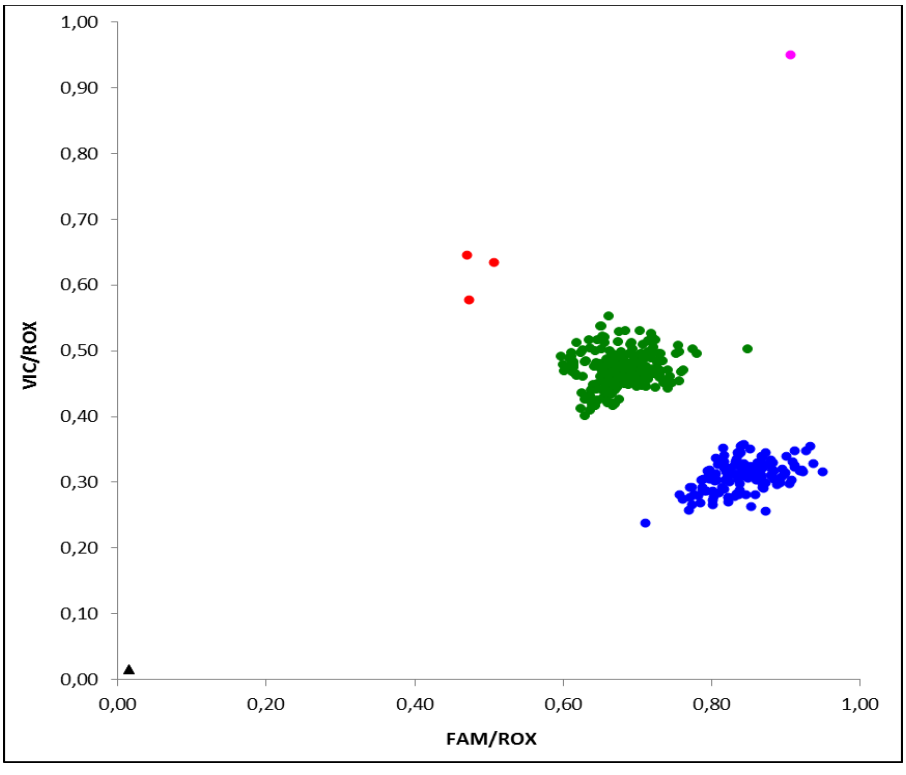

Figure 1. KASP analyses results

Plant materials identified as resistant in both tests were grown as donor plants for use in doubled haploid (DH) studies, and DH experiments were initiated approximately one month after transplanting these plants.

A total of 400 anthers from genotype B5, 400 from genotype B6, 265 from genotype B07, 180 from genotype B08, and 255 from genotype B10 were cultured on the induction medium. Embryo formation was first observed 30–40 days after culture initiation.

Table 2. Anter culture results based on genotype

Genotype	Number of buds	Number of anthers	Number of embryos	Number of embryos/bud	Number of DH plants	Number of DH plants/anther
B05	80	400	0	0,00	0	0,00
B06	80	400	6	0,08	4	0,05
B07	53	265	1	0,02	1	0,02
B08	36	180	2	0,06	1	0,03
B10	51	255	21	0,41	10	0,20

No embryo formation was obtained from genotype B5. In contrast, 6 embryos were produced from genotype B6, 1 from genotype B07, 2 from genotype B08, and 21 from genotype B10. Among these embryos, 4 from genotype B6, 1 from genotype B07, 1 from genotype B08, and 10 from genotype B10 exhibited healthy development and were identified as spontaneous doubled haploids.

Seed production was successfully carried out from a total of 16 DH0 plants.

Conclusion

No doubt that there is a substantial need for virus resistance research in pepper, particularly studies focusing on resistance to PMMoV. Although various international studies have investigated the molecular identification of PMMoV resistance, the practical utilization of these results in the form of breeding lines and commercial cultivars remains limited. In order to achieve sustainable development and enhance competitiveness in both domestic and international markets, it is essential for domestic seed breeding companies to recognize the necessity of incorporating biotechnological tools such as molecular markers into their breeding programs. In other words, in order to remain competitive with multinational companies, it is essential for domestic seed companies to utilize dihaploidization techniques to obtain parental lines for hybrid breeding from selfed and hybrid genotypes derived through conventional breeding programs. In this research, pure lines were rapidly developed from pepper genotypes possessing various superior traits using the DH method. Their resistance was subsequently confirmed through SNP-based KASP molecular marker analysis, and these lines were rendered suitable for use in subsequent stages of breeding programs.

Author Contributions

In this study, theoretical framework of the study was created by the author, the data collection, analysis process and writing were also carried out by the author.

Funding

This study was not funded by any institution or organization.

Responsible Artificial Intelligence Statement

In this study, artificial intelligence tools were used in language editing, and to correct language errors.

Conflicts of Interest

The author declare that there are no conflicts of interest related to the publication of this study.

Ethics Approval

This study does not require ethics committee approval as it does not involve any direct application on human or animal subjects.

REFERENCES

Anonim. (2024). *Tarımsal ürünler istatistiği.* İstatistiklerle Türkiye. Türkiye İstatistik Kurumu. https://www.tuik.gov.tr

Çömlekçioğlu, N., & Ellialtıoğlu, Ş. Ş. (2018). Review on the research carried out on *in vitro* androgenesis of peppers (*Capsicum annuum* L.) in Turkey. *Research Journal of Biotechnology*, 13(6), 75–84.

Çömlekcioğlu, N., Büyükalaca, S., & Abak, K. (2001). Effect of silver nitrate on haploid embryo induction by anther culture in pepper (*Capsicum annuum* L.). In K. Abak, S. Büyükalaca & Y. Daşgan (Ed.), *Proceedings of the XIth EUCARPIA Meeting on Genetics and Breeding of Capsicum & Eggplant* (ss. 133–136). Antalya, Türkiye.

Çağlar, Ç., Aras, V., & Bayram, A. (2004). Kurutmalık kırmızı biberlerde androgenesis yoluyla *in vitro* haploid embriyo uyartımı. *Akdeniz Üniversitesi Ziraat Fakültesi Dergisi*, 17(1), 87–94.

Dumas de Vaulx, R., Chambonnet, D., & Pochard, E. (1981). *In vitro* anther culture in red pepper (*Capsicum annuum* L.): Improvement of the rate of plant production in different genotypes by treatments at 35 °C. *Agronomie*, 1, 859–864.

Ercan, N., Ayar Şensoy, F., & Şensoy, A. S. (2006). Influence of growing season and donor plant age on anther culture response of some pepper cultivars (*Capsicum annuum* L.). *Scientia Horticulturae*, 110(1), 16–20.

Ercan, N., & Ayar Şensoy, F. (2011). Androgenic responses of different *Capsicum annuum* L. cultivars. *Biyoloji Bilimleri Araştırma Dergisi*, 4(2), 59–61.

Food and Agriculture Organization of the United Nations. (2023). *FAOSTAT.* http://www.fao.org/faostat/en/#data/QC

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473–497.

Niklas-Nowak, A., Olszewska, D., Kisiala, A., & Nowaczyk, P. (2012). Study of individual plant responsiveness in anther cultures of selected pepper (*Capsicum* spp.) genotypes. *Folia Horticulturae*, 24(2), 141–146.

Parisi, M., Alioto, D., & Tripodi, P. (2020). Overview of biotic stresses in pepper (*Capsicum* spp.): Sources of genetic resistance, molecular breeding and genomics. *International Journal of Molecular Sciences*, 21, 2587. https://doi.org/10.3390/ijms21072587

Pickersgill, B. (1997). Genetic resources and breeding of *Capsicum* spp. *Euphytica*, 96, 129–133. https://doi.org/10.1023/A:1002913228101

Tripodi, P., & Kumar, S. (2019). The *Capsicum* crop: An introduction. In N. Ramchiary & C. Kole (Ed.), *The Capsicum genome* (Compendium of Plant Genomes). Springer. https://doi.org/10.1007/978-3-319-97217-6_1

Tsuda, S., Kubota, K., Kanda, A., Ohki, T., & Meshi, T. (2007). Pathogenicity of *Pepper mild mottle virus* is controlled by the RNA silencing suppression activity of its replication protein but not the viral accumulation. *Phytopathology*, 97, 412–420.