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MOLECULAR MARKER ASSISTED SELECTION FOR *Phytophthora capsici* Leon. RESISTANCE LINES IN PEPPER (*Capsicum annuum* L.)

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ABSTRACT

The most important diseases that cause loss of productivity in all areas of pepper production in the world and the limitation of cultivation are Phytophthora crown blight of pepper (*Phytophthora capsici* Leon.). The disease is a soil borne pathogen, and its challenge is difficult. Selection of varieties resistant to diseases of soil-borne plant pathogens is the most efficient, economical and sustainable method. In this study, the resistance of Phytophthora crown blight (*Phytophthora capsici* Leon.) disease on 95 pepper genotype in terms of yield and some quality characteristics was investigated by using SCAR marker (OpD04.717). In the study, pepper lines in 7 pepper genotype F2 population showed durability band at 717 bp of agarose gel, while 88 pepper genotype were found to be sensitive. As a result of molecular studies, 7 pepper genotype resistant to *Phytophthora capsici* Leon. were also confirmed by classical test using seedling immersion method. In the study, as a result of molecular and classical tests, *Phytophthora capsici* Leon. is determined to be resistant to pepper lines in the hybridization programs to obtain new varieties and will be used as a parent or father will contribute to improvement studies.

Key words: pepper, *Phytophthora capsici*, PCR, resistance

INTRODUCTION

Pepper (*Capsicum annuum* L.) is among the most consumed vegetables in the world as in Turkey. Pepper is known to have important vitamins and minerals for human health, all kinds of food can be made, salads, sauces, spices and pickles, as well as increasing the possibilities of different uses, such as paint and bitterness capsaicin in the production of pharmaceutical industry has encouraged to increase the use of pepper production. China takes the first place in pepper production in the world, followed by India, USA and Turkey respectively. According to 2017 data in Turkey, total vegetable production is 30.825.569 tons, and the highest production of pepper is 2.608.172 tons and the highest pepper production is in the Mediterranean region of Turkey [TUİK 2017]. Pepper production in Turkey's ecological conditions can be grown both open and greenhouse.

In the pepper cultivation in the World and Turkey, the most important plant diseases limited pepper production are the root rot and crown blight caused by *Phytophthora capsici* Leon. (*P. capsici*), a soil borne disease. The disease was first described in New Mexico in 1918 [Leonian 1922]. The disease agent is naturally present in the soil and it is able to infect plants in all stages of plant development during high soil moisture and temperature and humid weather conditions. The disease agent produces spores called sporangia on infected plants, and under favorable hu-

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midity conditions, sporongia produces zoospores that are active in water. These zoospores germinate infect pepper plants and cause the plant to dry out from the root. Water plays an important role in the proliferation of the disease and the transport of spores to healthy plants. The disease usually occurs repeatedly during the rainy season, where there is no good drainage system in the production area [Demirci and Dolar 2006, Kousik et al. 2012, Di Dato et al. 2015, Aguilar et al. 2015]. Disease spores are also airborne, transported to long distances with the help of wind and may spread over a large area. Apart from pepper, it makes many vegetables and wild plants sick like eggplant, tomato, squash, cucumber and melon and has a wide range of hosts [Tyler 2002, Zhang et al. 2013].

Phytophthora capsici Leon., which causes crown blight and root rot in pepper that in many countries such as Italy, France, Spain, Bulgaria, Yugoslavia, Netherlands, Korea and Japan and in Turkey, significant yield losses are caused and both damages in open production and greenhouse cultivation has been reported [Hwang and Kim 1995, Tamietti and Valentino 2001, Lee et al. 2001, Demirci and Dolar 2006, Foster and Hausbeck 2010a, Di Dato et al. 2015, Dunn and Smart 2015]. Due to sudden plant deaths in pepper cultivation in Turkey, the study was carried out in Central Anatolia in 1974 revealed the presence of the disease pathogen [Karahan and Maden 1974]. In the surveys conducted to determine the prevalence of the disease in Turkey, it was reported that *P. capsici* disease causes harm in 40% of pepper yield [Yıldız and Delen 1979, Çınar and Biçici 1977, Sağır and Yıldız 1988, Soylu and Kurt 2001, Arıcı and Basım 2001, Öztürk and Çolak 2017].

In the pepper cultivation field, many chemicals are widely used in the control against for this disease [Kaygusuz and Biçici 2007, Hwang and Kim 1995, Ristaino and Johnston 1999, Foster and Hausbeck 2010b]. Although some chemicals have been developed against control disease, no chemical control has been achieved economically because the pathogen is a soil-borne disease, sexual reproduction, and resistance to chemicals due to the inoculum density of the disease [Bowers and Mitchell 1991, Parra and Ristaino 1998, Walker and Bosland 1999, Kim and Hwang 1992, Hwang and Kim 1995]. In parallel with the studies in the world, alternative approaches including organic and integrated agriculture systems have come to the fore in Turkey to reduce the negative effects of chemical control [Aksoy 1999]. Among the alternative agricultural systems, cultural prevention takes the first place in the control against plant diseases. Therefore, the use of resistant varieties in the control against the most effective, most economical, environmental and sustainable method.

Today's breeding programs are primarily aimed at developing resistant varieties. The development of resistant varieties is obtained because of long breeding studies. Disease resistant varieties are mostly produced because of resistant wild species and crosses with existing varieties in terms of quality characteristics such as good taste, shape and color as well as resistance to a certain agent [Scott 2005]. Morphological markers used in conventional breeding programs but will help to distinguish between genotypes can be affected by environmental conditions. If any of these characters are recessive, homozygous and heterozygous individuals cannot be detected. Therefore, in recent years, advances in the use of molecular markers in the field of biotechnology in disease resistance have rapidly gained importance in the use of plant breeding. Thus, the selection of resistant varieties against plant diseases, providing savings in time and place, hundreds of plants at the same time, reliable and can be selected quickly [Sambrook et al. 1989, Darling and Brickell 1994, Summerel et al. 2001, Barone et al. 2005]. For this purpose, Quirin et al. [2005] in their study, using the OpD04.717 primer, obtained a single band in *C. annuum* and *C. chinense* species showing resistant to disease pathogen. They cloned this band and sequenced it to form a marker. They found that the marker they obtained was very close to Phyto 5.2, which is one of the six QTLs that provide resistance, and is located on fifth chromosome, and can be used to discriminate resistant individuals. The disease resistance is a character controlled by multiple genes. However, important QTLs can be effective in making the control against disease sustainable. One of the QTL providing resistance to *Phytophthora capsici* mapped and presented to the use of PCR-based markers translated into breeding programs [Quirin et al. 2005].

The main aim of pepper breeding was to develop high quality and disease resistant hybrid pepper varieties. There is a need to develop a variety of

P. capsici resistant to soil borne pathogens, which restrict production significantly in the countries where pepper cultivation is intense. In this context; the aim of our project is to 95 peppers genotypes that have come to the fore in terms of yield and some quality criteria. It was aimed to determine the resistance of *Phytopthora caspsici* Leon., which causes Phytophthora crown blight in pepper cultivation by using molecular marker assisted selection and classical testing.

MATERIALS AND METHODS

Detection of *P. capsici* resistant pepper lines by molecular marker. In the experiment, 95 pepper genotypes (in F2 generation), which came to the fore in terms of yield and some quality characteristics, were used in breeding studies. In order to determine the resistance of pepper genotypes to *P. capisici*, positive control materials were used in molecular and classical tests. Alata Horticultural Research Institute/Turkey used in pepper breeding program, used disease-resistant CM334 (Criollo de Morelos 334) as positive control and Ph7-9, Ph7-1 coded pepper lines are used as sensitive control.

Genomic DNA Isolation. In this project where the resistance of pepper lines was investigated against *P. capsici*, DNA Prufication Mini Kit (Thermo Scientific GeneJET Plant-KO792) was used in total genomic DNA isolation. For this purpose, 100 mg was taken from the young leaves which were taken in the 2–4-leaf period from the pepper lines where controls and durability were investigated. The obtained DNAs were adjusted to 40 ng/µl by spectrophotometer, controls were made on 0.8% agarose gel and stored at –20°C [Quirin et al. 2005].

PCR conditions. In the study, the dominant SCAR marker (OpD04.717: (F) 5'-CCA TAA GGG TTG GTA AAT TTA CAA AG-3'/(R) 5'-TCG AGA GAT AAT TCA GAT AGT ATA ATC-3'), which was developed in conjunction with the Phyto 5.2 found on the fifth chromosome, was used to determine the resistance to *P. capsici* [Quirin et al. 2005]. PCR reaction (25 µL); 22 µL DreamTaq Green PCR Master Mix (ThermoScientific) (0.5U Taq polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP each containing 0.4 mM and 4 mM $MgCl_2$), 1 µL F + 1 µL R primer (10 pmol/µl) and 2 µL DNA (10 ng/µl) were used. PCR conditions have been modified according to Quirin et al. [2005]; 3 minutes pre-denaturation at 95°C, followed by 1 min at 94°C, 45 s at 49.3°C, 35 cycles at 72°C for 70 s and 1 cycle of final elongation at 72°C for 10 min was programmed in a GeneAmp 9700 thermocycler (Thermo Fisher Scientific). The reactions were prepared under sterile conditions and on ice. All PCR reactions were repeated twice. All The products obtained as a result of PCR were electrophoresed in 2% agarose gel, the gel was put into a solution containing Ethidium Bromide in 0.5 µg/ml and stained by UV light and the results were recorded. Agarose gel images were evaluated according to the size of the band [Quirin et al. 2005]. The lines which were determined as durable by PCR were subjected to classical tests.

Classical test studies

Classical Test of Resistance against *P. capsici.* As a result of molecular studies, validation studies were carried out by classical testing on pepper genotypes which were found to be resistant to *P. capsici.* For this purpose; pepper seedlings with 3–4 weekly were used. *P. capsici* isolate to be used in the experiment was obtained from pepper production areas in the region [Stamps et al. 1990]. The isolate for *P. capsici* inoculation was grown on PDA (Potato Dextrose Agar) medium (Fig. 1). One piece of the fungus isolate grown on PDA medium was taken into discs and placed in containers having PDA and put into an incubator set at 24 ±1°C, the fungus was developed for 10 days. [Andres et al. 2005, Çolak et al. 2018a, b, Colak et. al. 2019]. To the seedlings, 5 ml (2×10^5 spores/ml) spore suspension containing *Phytopthora capsici* inoculation was applied to each plant root. The experiment was set up according to the completely randomized design in Adana Biological Control Research Institute, Turkey. The experiment was set up in a climate chamber where controlled conditions were achieved with five replicates of 3 plants per sterile peat : perlite (1 : 1) medium. 20–35 days after the experiment, evaluations were made according to the 0–3 scale. In the evaluation of the experiment, the resistance of the pepper genotypes of resistant (0: non-symptomatic plants) and sensitive (≥1: symptomatic plants) was made by looking at the symptoms in the plants [Islam et al. 2004, Çolak et al. 2019].

Fig. 1. *Phytophthora capsici* Leon. symptoms of pepper plant disease, mycelial colony and sporangia

RESULTS AND DISCUSSION

One of the most important soil-borne fungal diseases limited the cultivation of pepper was determined as a result of molecular studies on 95 pepper genotypes (F2 generation) which are resistant to Phytophthora crown blight of pepper (*Phytophthora capsici* Leon.). For this purpose, pepper genotypes were tested with the dominant SCAR marker (OpD04.717) developed in connection with Phyto 5.2 which is one of the QTL and found on the 5th chromosome in determining the resistance to *P. caspici* [Quirin et al. 2005]. As a result of PCR studies, 7 pepper genotypes (coded B8, B21, B24, B50, B53, B62, B75) were found to be resistant to *P. capsici* (717 bp) and 88 pepper genotypes were sensitive (Fig. 2, 3).

In breeding programs, molecular markers allow researchers to identify disease-resistant lines. Molecular markers provide time saving and reliability through rapid screening and selection of hundreds of desired genotypes in a single day [Barone et al. 2005, Staniaszek et al. 2007]. Marker-assisted selection provides several advantages in plant breeding programs conducted for polygenetic characteristics. Such a case is considered as a promising tool for quantitative resistance breeding [Thabuis et al. 2004].

The resistance studies were first started to *Phytophthora capsici* which is the Phytophthora crown blight in peppers in 1960s [Kimble and Grogan 1960]. In the early studies on the inheritance of disease resistance, it was reported that the inheritance was provided by two dominant genes independent of each other, whereas the resistance of some lines was determined by one dominant gene or two dominant genes [Smith et al. 1967]. Abak et al. [1982] hybridized PM217 genotype resistant to root-crown rot disease and sensitive Yolo Wonder cultivar and developed 27 dihaploid lines. Researchers also investigated whether the resistance was controlled by different genes of the roots or stems and pointed out that resistance was relatively specific to different organs. Pochard and Daubèze [1982] compared PM 217 and Phyto 636 genotypes with Mexico-originated two genotypes (L29 and SCM). In their study using highly aggressive isolates, PM 217 genotype was reported to be a source of disease resistance. Sotirova and Daskalov [1983] indicated that wild pepper forms had resistance to root-crown rot disease agent but transfer of resistance to culture cultivars may took time. Thus, researchers used mutation breeding and were able to develop 10 resistant lines.

Several researchers reported that PI123469, PI201232, PI201234, AC2258 (Mexican pepper Line 29) and CM334 (Criollo de Morales 334) genotypes could be used as the source of resistant to *P. capsici* Leon. disease [Kimble and Grogan 1960, Pochard and Daubèze 1982, Gil Ortega et al. 1990, 1991, Bosland and Lindsey 1991]. Lefebvre and Palloix [1996] in their QTL study, explained that 13 gene regions are associated with resistance. Thabuis et al. [2003] showed that sensitive parents have resistance genes and resistance can be transferred from the third, fifth and eleventh chromosomes. Therefore, genotypes which has marker in different sizes resistance should

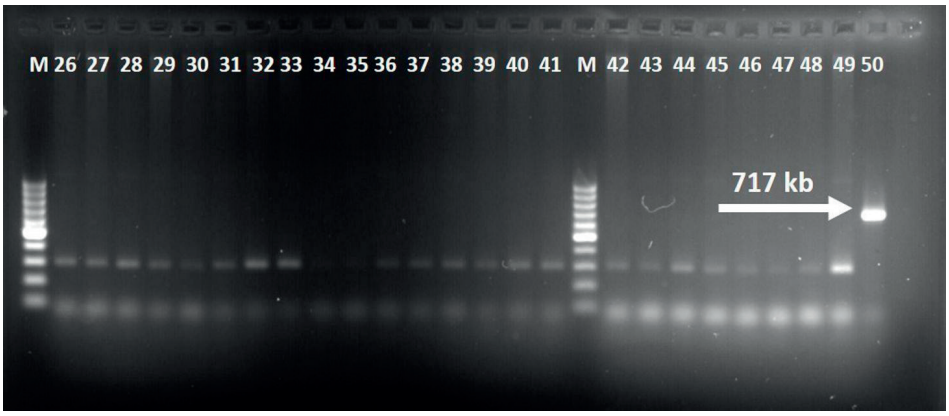

Fig. 2. Agarose gel image of *Phytophthora capsici* Leon. resistant SCAR marker (OpD04.717) in pepper lines. (M: Thermo DNA ladder (100 bp), 50. pepper genotype: resistant and others: sensitive)

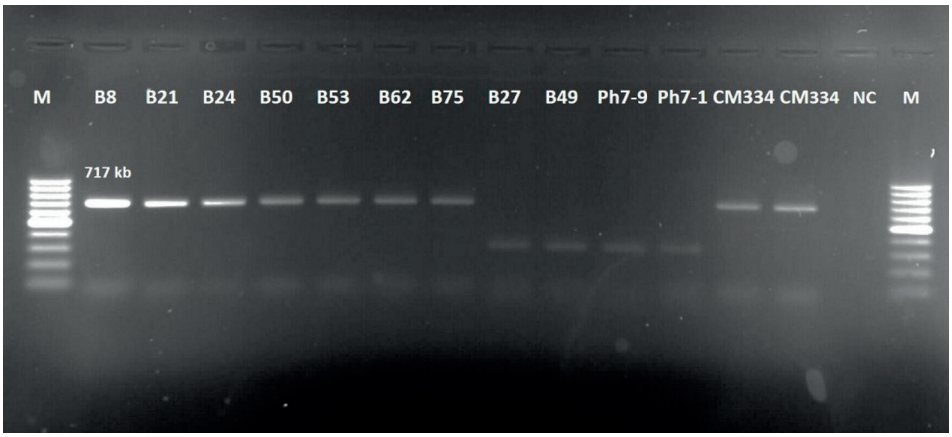

Fig. 3. Agarose gel image of 7 pepper lines resistant to *Phytophthora capsici* Leon. M: thermo DNA ladder (100 bp); B8, B21, B24, B50, B53, B62, B75: resistant pepper genotypes; B27, B49: sensitive pepper genotypes; CM334: resistant positive control for *P. capsici*; Ph7-9 and Ph7-1: sensitive positive control for *P. capsici*

be verified with together the resistance with the root and stem inoculation and root rot index tests. Thabuis et al. [2004] used ASC 012 and ASC 014 markers and repetitive selection method and identified the QLT (Phyto 6.1) on 6th chromosome in populations developed from Yolo Wonder and CM334 genotypes. Ogundiwin et al. [2005] identified that 16 chromosome regions of RIL mapping including single QTL or resistance QTLs were resistant to root-crown rot and leaf blight. A complex set including resistance to *P. capsici* was also revealed. On the F2 map, 5 QTLs were determined which were effective against root rot resistance.

Ri Criollo de Morelos 334'(CM334) is one of the sources that are said to be resistant to *Phytophtora capsici* which is effective in pepper [Hartman and Wang 1992, Hwangsung et al. 2014]. Quirin et al. [2005] used OpD04 primer and obtained a single band in *C. annuum* and *C. chinense* species resistant to disease agent. Researchers then cloned and

sequenced this band and generated a marker. Resultant marker was quite close to Phyto 5.2 located on 5th chromosome and one of 6 QLTs providing resistance to the disease and stated that this marker could be used for separation of resistant individuals. The resistance to the relevant disease is a characteristic controlled by more than one gene. However, significant QTLs may be efficient in making disease control sustainable. One of QTLs supplying resistance to *Phytophthora capsici* was mapped and converted into PCR-based marker and used in breeding programs [Quirin et al. 2005].

The SCAR marker (OpD04.717) developed with this study was considered to be beneficial in breeding programs with MAS selection to identify resistance to *P. capsici* Leon. causing crown blight and root rot disease. In breeding programs, primers the closest to the gene are preferred and such a case increase the reliability of the outcomes. In present study, pepper genotypes were tested with dominant SCAR marker developed as related to Phyto 5.2 located on 5th chromosome of the resistance gene. Use of SCAR markers in breeding programs has some advantages over the other SRAP [Budak et al. 2004], RGA [Mutlu et al. 2006], RAPD [Toppino et al. 2004, Boyacı and Abak 2008] markers. They are quite close to the gene in homozygote or heterozygote identification in F2 and F3 populations and they can reliably and easily be run in agarose gel [Mutlu et al. 2008]. It was one more time proved with the outcomes of the present study that selection of proper genotypes with MAS selections in breeding programs provided significant time and cost savings and allowed the lines to be commercialized rapidly.

Determination of resistance to many diseases based on molecular marker selection in breeding studies; It is emphasized that it is important to conduct validation studies with classical testing at every stage of hybridization programs. Thusly, Mutlu et al. [2008] conducted a study about resistance to Fusarium wilt in eggplants and used SCAR426 and SCAR347

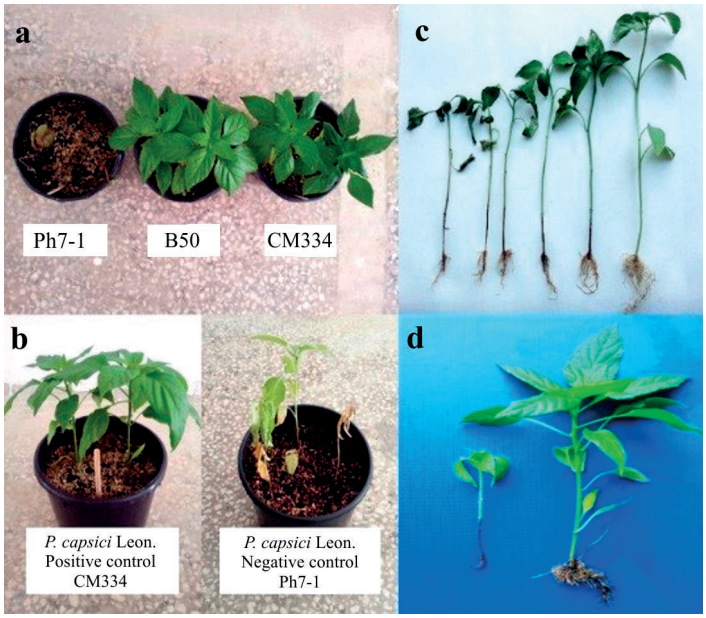

Fig. 4. *Phytopthora capsici* Leon. disease, symptoms of resistant and sensitive pepper genotypes; a: Ph7-1 – sensitive control for *P. capsici*, B50 – resistant pepper genotype and CM334 – resistant control for *P. capsici*; b: positive and negative control plants CM334, Ph7-1; c, d: symptoms of disease caused by *P. capsici* including stem rot and root rot

markers with quite low recombination ratios and quite close the gen and indicated that breeding materials with identified resistance through markers should regularly be proven by classical tests [Scott et al. 2015]. In present study, 7 pepper genotypes (B8, B21, B24, B50, B53, B62, B75) identified as resistant to pepper root-crown rot disease caused by *P. capsici* Leon. with the aid of SCAR marker and OpD04.717 primer were also proven by classical tests. In classical tests, *P. capsici* Leon. symptoms were not observed in roots evaluation (Fig. 4).

Aguilar et al. [2015] studied yield losses of peppers (*Capsicum* spp.) infected with *Phytophthora capsici* Leon. in Mexico and reported 100% loses in some regions of Bajio and Puebla, thus investigated the resistance to disease agent in Serrano pepper collection. Researchers used SCAR marker of OpD04.717 to define the sources of resistance to the pathogen at molecular level and accepted as resistant to *P. capsici* when amplified at 717 bp band. Also under *in vitro* conditions, plants were subjected to reaction tests to *P. capsici* with PCT-17 isolate. That study was composed of 142 Serrano pepper collection and controls (SCM334, resistant and MIRASOL, sensitive). However, resistance to the pathogen was not able to be identified with molecular studies and 11 resistant Serrano peppers were identified through classical tests. Since the resistance to that pathogen is polygenic [Pochard and Daubèze 1980, Ogundiwin et al. 2005, Lefebvre and Palloix 1996, Zhang et al. 2013, Toğaç et al. 2019], it is not sufficient to put forth the resistance to the disease with molecular studies, test results should also be confirmed by classical tests. In present study, resistance of B8, B21, B24, B50, B53, B62, B75 pepper genotypes to *P. capsici* Leon. was confirmed by classical tests. With the identification of pepper genotypes resistant to *P. capsici* Leon., contributions will be provided for transfer of resistance gene to commercial pepper cultivars and significant contributions will also provide to further hybridization programs of breeders.

CONCLUSIONS

The aim of this study was to determine the individuals with *Phytophora capsici* Leon. resistance gene in 95 pepper genotypes which came to the forefront in terms of yield and some quality criteria. As a result of molecular and classical tests, 7 genotypes resistant to *P. capsici* Leon., B8, B21, B24, B50, B53, B62, B75 coded pepper genotypes, were found to be resistant to disease. In order to increase the effectiveness in the control against soil borne pathogens, besides the selection of resistant varieties, it is necessary to consider the system as a whole for protection of environmental resistance and to combine applications in the control. In this context; classical breeding methods, a variety resistant to a single pathogen, ın terms of productivity and quality, it is not sufficient for pepper plant which has high number of diseases and in areas which are intensively cultivated such as pepper. In today's conditions, resistance to at least three or more pest and disease pathogen is needed depending on the production area and time. As the number of resistances required increases, breeding time is prolonged and is not even possible.

In recent years, marker of resistance against *Phtytopthora caps*ici Leon., which is one of the most important diseases restricting cultivation with increasing pepper production, has been developed and resistant pepper genotypes are determined rapidly. However, there is no such study so far for *Fusarium solani* and *Fusarium oxysporum*, which cause wilt and root rot, among other important soil borne diseases that can cause economic damage to pepper. As a result of this study, the disease resistant and sensitive pepper genotypes and multiple disease resistant genotypes were determined to contribute to the infrastructure for the development of markers. Our study results; with the determination of resistance to important biotic factors, it has saved the line owner time for commercialization of candidate cultivars resistant to *P. capsici* Leon. In addition, by identifying the parents who will be resistant to *P. capsici* Leon. as a parent or father, it has also contributed to the establishment of alternative projects in terms of obtaining the new varieties through the fruit quality criterion demands which are at the forefront in the world.

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