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Molecular Diversity and Assessment of Reactions of Pepper Pure Line Germplasm to *Botrytis cinerea*

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Abstract

Polat I., Baysal Ö., Gümrükcü E., Sülü G., Kitapci A., Özalp R., Çelik I., Devran Z., Polat E. (2018): Molecular diversity and assessment of reactions of pepper pure line germplasm to *Botrytis cinerea.* Plant Protect. Sci., 54: 147–152.

The host resistance level of pure line materials was assessed in the genepool for the purpose of breeding. The highest resistance to the pathogen was observed in bell-type pepper. Moreover, genetic diversity of pure lines was investigated using selected inter-simple sequence repeat (ISSR) primers. Generally, genetic markers showed genetic diversity, so that long-type pure lines were separated from the other accessions. This is the first report on host reactions of Turkish pure lines as breeding material. These results provide significant information for future pepper breeding programs.

Keywords: disease severity; genetic diversity; grey mould; ISSR; reaction

Pepper (*Capsicum annuum* L.) is a species of the family Solanaceae, which belongs to the genus *Capsicum* that grows in greenhouse conditions under tropical and subtropical climates (Esbaugh 1970). Turkey ranks the third at the list of pepper production in the world (FAO 2014). *B. cinerea*, the causal agent of grey mould, is an airborne plant pathogen attacking over 200 crop hosts worldwide.

It is difficult to control because it has a variety of the modes of attack, diverse hosts as inoculum sources, and the pathogen can survive as mycelia and/or conidia for a long time (Williamson *et al.* 2007). Chemical fungicides are often used to effectively control the fungus (Rosslenbroich & Stuebler 2000; Sun *et al.* 2010). Unconscious and unnecessary pesticide consumption to combat pathogenic microorganisms may lead to a decrease in sensitivity and causes resistance (Delen *et al.* 1985; Zhang *et al.* 2007; Sun *et al.* 2010; Shao *et al.* 2015). Unfortunately, *B. cinerea* is a classical 'high-risk' pathogen by the Fungicide Resistance Action Committee (Brent & Hollomon 1998; Angelini *et al.* 2012).

Therefore, it is important to improve resistant/tolerant varieties. Most of plants grown under greenhouse conditions comprise hybrid varieties. Resistant/tolerant parental lines are necessary for a breeding program to improve the variety against abiotic and biotic stresses. However, there is no pepper variety showing resistance/tolerance to *B. cinerea* (Williamson *et al.* 2007; Anonymous 2013). Besides, plant varieties/types of reactions against *B. cinerea* could show significant differences. Pathogenicity of the agent and reactions of some common hazelnut cultivars to *B. cinerea* were investigated. It was concluded from the reaction tests that the Çakıldak cultivar with 33.33–61.11% disease severity rate was more tolerant than the other five cultivars examined (Sezer & Dolar 2012).

In this study, reaction levels of four pepper pure lines of different type in the genepool to *B. cinerea*

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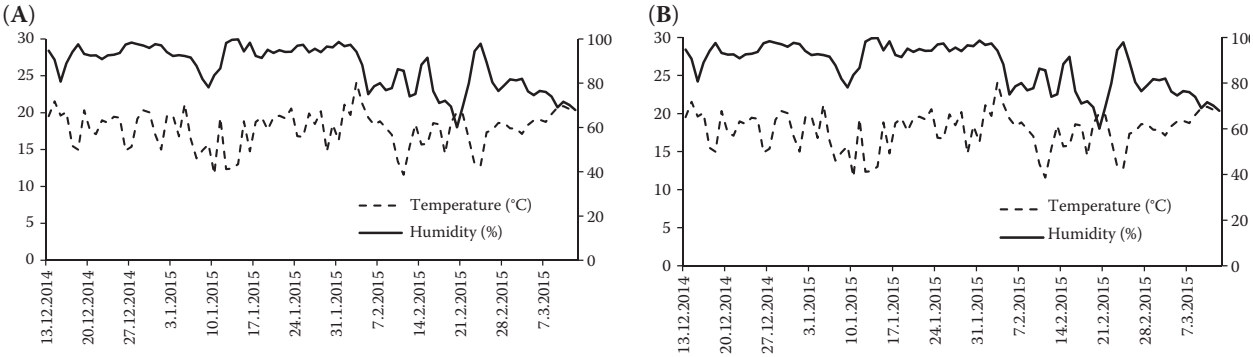

Figure 1. Temperature and relative humidity in potted (**A**) and greenhouse (**B**) assay

and genetic diversity of pure lines have been determined using ISSR markers.

MATERIAL AND METHODS

Isolate 40 obtained from a project (Polat *et al.* 2016) in Turkey (36°230532 E, 40°18238 N, 18 m s.l.) and B05.10 (reference) provided by University of Wageningen, The Netherlands, were used. The reaction levels of long, capia, bell and banana pepper types of a pure line (F_6) in the BATEM genepool were determined both in potted peppers and in a greenhouse. The experiment was set in a completely randomised parcel design and treatments were replicated three times, and repeated twice. Temperature and relative humidity data during assays were obtained by a data logger (Extech, RHT20). Disease severity was calculated according to the Townsend-Heuberger formula and evaluated using COSTAT Statistic Analysis Program. Significant differences at a P-level < 0.05 were determined using ANOVA and Duncan's analysis.

Potted assays. Two seedlings for each pure line were sown in each of 5 pots. When the seedlings were blooming at a height of 20–30 cm, they were sprayed with a spore suspension (carrot juice + 1% gelatin) of each isolate individually (10^5 spore/ml) until the runoff using a hand sprayer. In the control, carrot juice + 1% gelatin without spore suspension was used. All plants were kept in a greenhouse with approximately 15 h light and 9 h dark. The plants were examined and disease severity was recorded 10 days after inoculation using a 0–4 scale (Delen *et al.* 1985).

Greenhouse assays. Ten **s**eedlings for each pure line were sown in **a** plastic house and inoculated with spore suspensions using a hand sprayer after 2 weeks. In the control, carrot juice + 1% gelatin without spore suspension was used. Disease severity was recorded at harvest time using a 0–4 scale (Delen *et al.* 1985).

Molecular identification of pure lines. Genomic DNA was extracted from young leaves by the CTAB method (Doyle & Doyle 1990). Twenty-six ISSR primers were used. PCR amplifications and reactions were carried out according to Polat *et al.* (2014).

Amplified bands from each primer were scored as present (1) or absent (0). Statistical analysis was carried out using the PAST (Paleontological Statistics) software (Hammer *et al.* 2001). The genetic similarity matrix, neighbour joining (NJ) and principal coordinate analysis (PCoA) were constructed based on Dice's coefficient (Dice 1945). Polymorphism rate (Pr) and polymorphism information content (PIC) values were evaluated as described by Smith *et al.* (1997).

RESULTS

Potted assays. The temperatures and humidity values were in the range of 12–24°C and 62–100%, respectively (Figure 1A). The reaction assay pointed

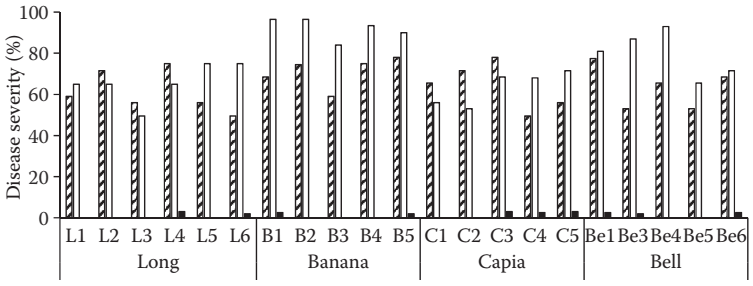

Figure 2. Disease severity of pure lines in germplasm in potted assay (%)

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Table 1. Disease severity (%) of pure lines in potted and greenhouse assays (mean ± SD)

Fruit type	Pure line	Potted			Greenhouse		
		isolate 40	isolate B05.10	control	isolate 40	isolate B05.10	control
Long	L1	65.0 ± 3.0^{hij}	59.0 ± 3.0^{ijk}	0.0 ± 0.0^{m}	9.5 ± 2.5^{pqrs}	33.5 ± 11.5^{hijk}	0.0 ± 0.0^{s}
	L2	65.0 ± 3.0^{hij}	71.5 ± 3.5^{efgh}	0.0 ± 0.0^{m}	11.0 ± 4.0^{opq}	17.0 ± 5.0^{mnop}	0.0 ± 0.0^{s}
	L3	49.5 ± 6.5^{l}	56.0 ± 6.0^{jkl}	0.0 ± 0.0^{m}	9.5 ± 2.5^{pqrs}	13.5 ± 3.5^{nop}	0.0 ± 0.0^{s}
	L4	65.0 ± 3.0^{hij}	75.0 ± 0.0^{cdefg}	3.0 ± 0.0^{m}	17.5 ± 2.5^{mnop}	38.5 ± 6.5^{gh}	1.6 ± 1.5^{rs}
	L5	75.0 ± 0.0^{cdefg}	56.0 ± 0.0^{jkl}	0.0 ± 0.0^{m}	16.0 ± 1.0^{nop}	28.5 ± 11.5^{ijkl}	0.0 ± 0.0^{s}
	L6	75.0 ± 0.0^{cdefg}	49.5 ± 6.5^{l}	2.0 ± 1.0^{m}	28.5 ± 1.5^{ijkl}	21.0 ± 1.0^{lmn}	0.6 ± 1.2^{rs}
Banana	B1	96.5 ± 3.5^{a}	68.5 ± 6.5^{efgh}	2.5 ± 0.5^{m}	27.5 ± 2.5^{jkl}	17.5 ± 2.5^{mnop}	2.3 ± 0.3^{qrs}
	B2	96.5 ± 3.5^{a}	74.5 ± 6.5^{defgh}	0.0 ± 0.0^{m}	10.0 ± 0.0^{pqr}	36.0 ± 6.0^{hij}	0.0 ± 0.0^{s}
	B3	84.0 ± 16.0^{bc}	59.0 ± 9.0^{ijk}	0.0 ± 0.0^{m}	10.0 ± 5.0^{pqr}	32.5 ± 7.5^{hijk}	0.0 ± 0.0^{s}
	B4	93.5 ± 6.5^{a}	75.0 ± 0.0^{cdefg}	0.0 ± 0.0^{m}	12.5 ± 2.5^{nop}	37.0 ± 0.0^{hi}	0.0 ± 0.0^{s}
	B5	90.0 ± 3.0^{ab}	78.0 ± 3.0^{cde}	2.0 ± 0.0^{m}	20.0 ± 5.0I^{mno}	18.5 ± 1.5^{mnop}	1.0 ± 1.0^{rs}
Capia	C1	56.0 ± 0.0^{jkl}	65.5 ± 9.5^{ghi}	0.0 ± 0.0^{m}	47.5 ± 17.5^{ef}	58.5 ± 3.5^{cd}	0.6 ± 1.2^{rs}
	C2	53.0 ± 3.0^{kl}	71.5 ± 9.5^{efgh}	0.0 ± 0.0^{m}	40.0 ± 5.0^{fgh}	46.0 ± 9.0^{efg}	1.1 ± 1.3^{rs}
	C3	68.5 ± 6.5^{efgh}	78.0 ± 3.0^{cde}	3.0 ± 1.0^{m}	21.0 ± 4.0^{lmn}	17.5 ± 2.5^{mnop}	1.6 ± 1.2^{rs}
	C4	68.0 ± 0.0^{fghi}	49.5 ± 6.5^{l}	2.5 ± 0.5^{m}	51.0 ± 4.0^{de}	62.5 ± 2.5^{bc}	1.8 ± 0.8^{rs}
	C5	71.5 ± 3.5^{efgh}	56.0 ± 0.0^{jkl}	3.0 ± 0.0^{m}	26.0 ± 1.0^{klm}	38.5 ± 6.5^{gh}	1.6 ± 1.2^{rs}
Bell	Be1	81.0 ± 6.0^{cd}	77.5 ± 9.5^{cdef}	2.5 ± 0.5^{m}	33.5 ± 3.5^{hijk}	62.0 ± 10.0^{bc}	1.5 ± 0.9^{rs}
	Be3	93.0 ± 0.0^{a}	65.5 ± 9.5^{ghi}	2.0 ± 0.0^{m}	58.5 ± 11.5^{cd}	76.0 ± 1.0^{a}	1.5 ± 0.5^{rs}
	Be4	65.5 ± 9.5^{ghi}	53.0 ± 3.0^{kl}	0.0 ± 0.0^{m}	69.5 ± 7.5^{ab}	51.0 ± 1.0^{de}	0.0 ± 0.0^{s}
	Be5	71.5 ± 3.5^{efgh}	68.5 ± 6.5^{efgh}	0.0 ± 0.0^{m}	52.5 ± 2.5^{de}	50.0 ± 0.0^{de}	0.0 ± 0.0^{s}
	Be6	74.5 ± 6.5^{defgh}	65.5 ± 9.5^{ghi}	2.5 ± 0.5^{m}	63.5 ± 1.5^{bd}	73.5 ± 6.5^{a}	2.1 ± 0.6^{qrs}

Isolate 40, isolate B05.10, and control were evaluated together to lettering

out that all the tested pure lines showed a disease severity ranging from 49.5 to 96.5. Among all the pure lines L6 and C4 showed the lowest disease severity with 49.5 according to the reference isolate. However, B1, B2, B4 and Be3 showed the highest disease severity according to isolate 40 (Table 1 and Figure 2)

In greenhouse assays. The temperature values varied between 10 and 23°C, and the humidity values varied between 60 and 95% (Figure 1B). At harvest time, the reaction assay indicated that disease severity of all tested and characterised pure lines ranged from 9.5 to 76.5. Among all the pure lines, L1 and L3 showed the lowest disease severity with 9.5. Generally, isolate 40 caused higher disease severity than the reference isolate in analysed samples. The highest disease severity was determined in Be3 and Be6 bell-type pepper (Table 1 and Figure 3).

Molecular identification of pure lines. A total of 26 ISSR primers were screened, and a total of 205 alleles revealing 27% polymorphism were obtained, with an average of 2.38 polymorphic alleles per locus from eighteen polymorphic primers. Monomorphic band was obtained from 818, 819, 826, 830, 885, 886, 887, and 824 primers. PIC values varied from 0.13 to 0.99 with an average of 0.53 (Table 2). Cophenetic correlation (*r*) was 0.95. Bell-type pure line Be3 was the most distant sample from all other accessions with similarity level of 0.33 (Figure 4). NJ analysis (Figure 5)

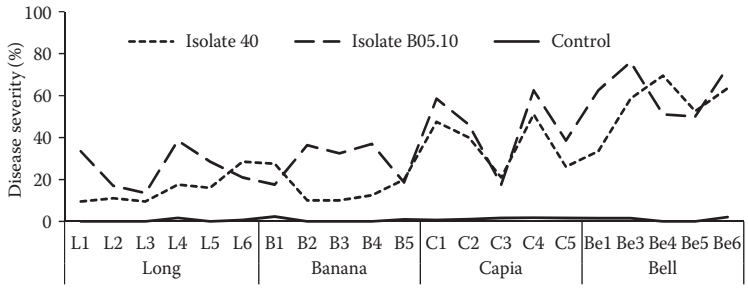

Figure 3. Disease severity of pure lines at harvest time in greenhouse assay (%)

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Table 2. ISSR markers used for the molecular characterisation of pure lines and polymorphism values

Primers	Fragment size ranges (bp)	Total fragment	Polymorphic fragment	Polymorphism rate (%)	PIC
868	350–1700	9	8	0.88	0.13
866	300–1500	9	7	0.77	0.77
859	400–1600	9	7	0.77	0.41
858	300–1600	10	4	0.40	0.81
873	800–3000	8	6	0.75	0.71
856	400–1700	9	1	0.11	0.99
818	200–1000	6	0	0.00	0.00
823	400–2000	7	1	0.14	0.77
857	300–1700	7	2	0.28	0.48
849	300–1300	8	1	0.12	0.99
847	500–1600	8	2	0.25	0.88
846	350–1300	10	4	0.40	0.76
835	650–1800	8	1	0.12	0.78
819	350–1100	6	0	0.00	0.00
826	250–1000	6	0	0.00	0.00
825	300–1500	10	1	0.10	0.99
842	200–1200	11	4	0.36	0.88
830	300–1400	7	0	0.00	0.00
834	150–1500	9	5	0.55	0.73
885	300–1300	7	0	0.00	0.00
886	250–1400	6	0	0.00	0.00
887	250–1500	7	0	0.00	0.00
880	200–2000	9	5	0.55	0.83
112	300–1800	6	2	0.33	0.99
818	200–1200	7	1	0.14	0.96
824	350–1200	6	0	0.00	0.00
Total		205	62	–	–
Mean		7.88	2.38	0.27	0.53

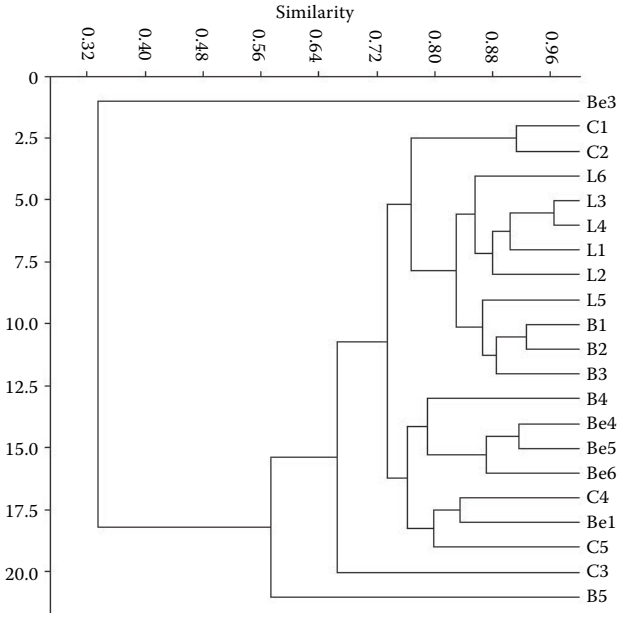

Figure 4. Dendrogram of 21 pure lines using the UPGMA method obtained from ISSR markers

and PCoA (Figure 6) showed that the long-type pure lines were separated from the other accessions.

DISCUSSION

B. cinerea causing the grey mould disease is one of the important factors limiting pepper cultivation as an airborne plant pathogen with necrotrophic lifestyle attacking (Williamson *et al.* 2007). Effective control of this disease is still based on the frequent application of fungicides, but serious disease problems have been associated with the extensive appearance of resistant strains (Myresiotis *et al.* 2007; Shao *et al.* 2015). Moreover, the chemicals used against the pathogen cause negative effects on the environment and human health and additionally increase expenses. Therefore, the use of resistant/tolerant varieties seems to be one of the best solutions to disease management. However, breeding of resistant pepper against *B. cinerea* is dif-

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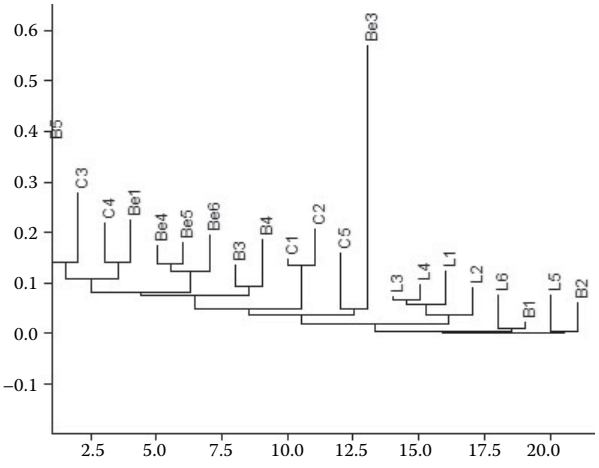

Figure 5. Neighbour joining of 21 pure lines using the UPGMA method obtained from ISSR markers

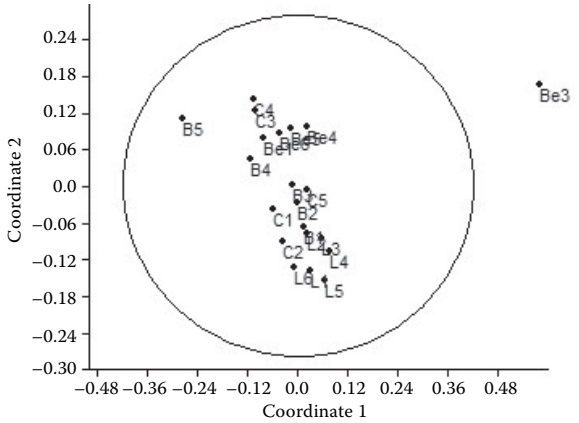

Figure 6. Principal coordinate analysis (PCoA) of 21 pure lines using ISSR markers

ficult due to the lack of resistant or tolerant parents in the world (Williamson *et al.* 2007; Anonymous 2013). However, plant varieties/types of reactions to *B. cinerea* can show significant differences. Sezer and Dolar (2012) investigated pathogenicity of the agent and reactions of some common hazelnut cultivars to *B. cinerea*. It was concluded from the reaction that the Çakıldak cultivar with 33.33– 61.11% disease severity rate was more tolerant than the other five examined cultivars. Likewise, this study showed variations of reactions among different fruit type pure lines.

Molecular marker-assisted breeding is an important component of modern breeding technology. ISSR markers involve the amplification of DNA segments between two identical microsatellite repeat regions. It is a PCR based method that is potentially useful in revealing polymorphisms that could be used as markers to determine genetic diversity (Pradeep Reddy *et al.* 2002). The ISSR molecular markers were already used to evaluate the genetic diversity in most vegetables, such as cucumber, melon (Parvathaneni *et al.* 2011), and tomato (Mansour *et al.* 2010).

Genetic variability among 30 landraces and one commercial Greek pepper cultivar was assessed using ISSR markers (Tsaballa *et al*. 2015). Likewise, ISSR markers were used to determine the genetic diversity of five cultivated pepper species. Studies showed that pepper genetic diversity and that cultivated species identification and gene exchange between cultivated and wild species should be strengthened in pepper breeding to improve heterosis (Lijun & Xuexiao 2012). Among *Capsicum* accessions high variability by ISSR markers was detected (Dias *et al.* 2013). In this work, ISSR data showed remarkable genetic diversity within the pure lines because of the cophenetic correlation between ultrametric similarities of the tree and the similarity matrix was found to be high (r = 0.95) when the cluster analysis strongly represents the similarity matrix. Aka-Kacar *et al.* (2005) reported that the interpretation of the correlation coefficient matrix is as follows: $r \geq 0.9$ is very good, $0.8 \leq r < 0.9$ is good, $0.7 \leq r < 0.8$ is poor and $r < 0.7$ is very poor.

PIC provides an estimate of the discriminatory power of a locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of those alleles. PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles at equal frequencies) (Uzun *et al.* 2011). If a PIC value is greater than 0.7, it is considered to be highly informative. However, a PIC value of 0.44 is considered to be moderately informative (Hildebrand *et al.* 1992). Therefore, primer 868 was a poor discriminative marker in our study.

Up to date, there has been no report on the disease severity caused by *B. cinerea* on pepper pure lines in germplasm. For this reason, this report is important to pepper breeders. This is the first report on the screening of host resistance in pepper pure lines as breeding material in germplasm. These results provide important information for future breeding programs.

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