Detection of Pathotypes and Genetic Diversity of *Cercospora beticola*

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The pathotypes of *Cercospora beticola*, causal agent of sugar beet leaf spot disease, were identified by application of pathogenicity test using 100 isolates obtained from the provinces with intensive sugar beet cultivation. For the identification of pathotypes, five sugar beet cultivars were used each with different resistance factors. Cultivar reactions were determined by inoculation of cultivars with the isolates under controlled conditions and measuring disease severity on the 15th day according to the 1-9 KWS Scale. Based on the reactions of the five cultivars, a total of 15 pathotypes were detected. All employed sugar beet cultivars were resistant to Pathotype no:1 comprising most of the isolates. Genetic diversity of the causal agent was characterized by AFLP reaction. The products acquired at the end of AFLP reaction were detected by means of Beckman CEQ 8800 DNA Capillary Series Analysis and the results obtained were evaluated according to the similarity index UPGMA. For the genetic analysis of *C. beticola* isolates, 9874 polymorphic fragments of sizes between 100 and 500 bp were analysed which were generated by nine primers. The dendrogram derived from AFLP analysis depicted the existence of five different subgroups. The polymorphism rate among isolates was 91.13% and the dendrogram distribution of the pathotypes obtained by pathogenicity indicated that pathotypes were not discriminated and did not compose any groups.

**Keywords**: AFLP, *Cercospora beticola*, genetic diversity, sugar beet, pathotype

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Cercospora Leaf Spot (CLS) disease of sugar beet caused by the fungus *Cercospora beticola* (Sacc.) has been accepted as the most destructive and economically damaging foliar disease in hot and humid regions with sugar beet cultivation (Duffus and Ruppel, 1993; Weiland and Koch, 2004). The pathogen has been stated to cause more than 30% damage in sugar beet fields (Weiland and Koch, 2004). Extensive fungicide application has been performed in order to protect the sugar beet fields from CLS. In the fields planted with sugar beet cultivars resistant and moderately-tolerant to CLS, fungicides can protect the crops in high disease pressure (Moretti et al., 2006). Due to the facts that the pathogen frequently undergoes genetic change (Ruppel 1972) and the resistance to CLS is controlled qualitatively by a minimum of five loci (Setiawan et al., 2000), today breeders still have difficulty in developing a CLS-resistant sugar beet cultivar. For development of resistant cultivars to be used for the control of the disease, the consideration of genetic diversification of the pathogen and the detection of the pathotypes are extremely important. There are only a few studies worldwide on the detection of the pathotypes (Solel and Wahl, 1971, Ruppel, 1972, Whitney and Lewellen 1974, Lewellen and Whitney, 1976) and genetic diversity (Weiland et al., 2001; Moretti et al., 2004, Vereijssen et al., 2004, Groenevald, 2005) associated with the disease. For this purpose; pathogenicity test and Amplified Fragment Length Polymorphism (AFLP) technique have been applied to 100 *C. beticola* isolates obtained from sugar beet fields with high disease prevalence in the provinces of Thrace, Central Anatolia and Western Black Sea regions in order to detect the pathotypes and genetic diversity of the disease agent.

**Materials and Methods**

**Obtainment and storage of fungal isolates.** The isolates were obtained from the infected samples collected from 24 sugar beet cultivation areas under the domain of seven Turkish sugar factories in 2006. Out of a total 100 isolates, 8 was collected from Alpulu, 22 from Adapazari, 18 from Arımaşa, 18 from Çarşamba, 2 from Niksar, 15 from Kastamonu and 19 from Susurluk. Each isolate was obtained from a leaf collected from a different field proportional to the acreage of the region.

Isolation of *C. beticola* was performed in sugar beet leaf extract agar (SBLEA) (15g agar/litre, 250g fresh leaves/litre). Small pieces taken from the clean parts of the sporulating lesions from the infected leaves were transferred to the tubes containing 500 µl sterile water. Hundred
µl was taken from these tubes and plated on SBLEA agar. Subsequently, the mycelial pieces taken from the sides of the single spore derived colonies were transferred to the tubes with SBLEA and after growth; they were stored in refrigerator and cryo tubes containing 10% glycerin at −80 °C.

Differential cultivars and their growth. A total of five sugar beet cultivars widely grown in our country were used in our study, namely Arosa and Kassandra with low field resistance, Leila and Evelina with moderate resistance and Visa with high resistance to \textit{C. beticola}. The varieties Arosa, Evelina, Kassandra and Leila were provided from KWS Türk Tarım Ticaret A.Ş.; organize Sanayi Bölgesi 14. Cad. No: 22, 26110, Eskişehir, visa from Sesvanderhave, N. V.; Industriepark Soldantenplein Zone 2, Nr. 15, 3300, Tienen, Belgium). Test plants were grown from the seeds in greenhouse at a temperature of 25 ± 5°C for a duration of 10 weeks (Bargabus et al., 2004).

Preparation of inoculum. Isolates were grown in petri dishes containing SBLEA for a duration of seven days. Inoculum suspension was obtained by removal of the grown fungus from agar surface by means of sterile water. Inoculum suspension was obtained by removal of the discs containing SBLEA for a duration of seven days.

Preparation of inoculum. Isolates were grown in petri dishes containing SBLEA for a duration of seven days. Inoculum suspension was obtained by removal of the grown fungus from agar surface by means of sterile water containing 0.05% Tween 20. The density of this suspension was determined by using a Thoma Slide. The prepared inoculum was sprayed onto the sugar beet cultivars until they are completely wet by means of a hand sprayer. Sterile water was given to the plants in the control pots. The inoculated plants were incubated under 95-100% relative humidity for a duration of four days in humidified, polyethylene growth chambers.

Three leaves were randomly selected from each plant on the 15th day of inoculation, examined for the disease and the disease categories were evaluated according to the 1-9 KWS Scale (Shane and Teng, 1992). For the determination of reactions, sugar beets with a scale value below 5 were accepted as “resistant (R)” while those with a scale value of 5 and above were accepted as “susceptible (S)”.

DNA isolation. \textit{C. beticola} isolates were incubated in 100 ml Potato Dextrose Broth (PDB) medium for a duration of one week in an orbital shaker (170 rpm) at 26 ± 1°C in dark. Total genomic DNA was extracted according to method of Vilgays and Gonzalez (1990). The pureness and concentrations of DNA’s were detected in 1% agarose gels and with Nanodrop spectrophotometer. Total DNA concentration was set as 250 ngµl⁻¹. The method stated by Vos et al. (1995) was employed in AFLP reactions. Genomic DNA (250 ngµl⁻¹) was digested by EcoRI and 	extit{MseI} restriction enzymes and ligated with the addition of appropriate adaptors. Final amplification was realized by means of nine selective primer combinations (Table 1). The acquired products were detected by Beckman CEQ8800 Genetic Analysis System with use of sample loading solution and mineral oil of the producer company.

| Primer No | Primer Combinations | Total Bands | Polymorphic Bands | Polymorphism (%) |
|-----------|---------------------|-------------|--------------------|------------------|
| 1         | M-A E-G             | 1203        | 1112               | 92.43            |
| 2         | M-C E-A             | 1133        | 1081               | 95.41            |
| 3         | M-C E-C             | 985         | 843                | 85.58            |
| 4         | M-C E-T             | 1367        | 1277               | 93.41            |
| 5         | M-G E-T             | 1110        | 1003               | 90.36            |
| 6         | M-G E-G             | 1428        | 1342               | 93.97            |
| 7         | M-T E-A             | 985         | 910                | 92.38            |
| 8         | M-T E-C             | 1483        | 1313               | 85.53            |
| 9         | M-T E-T             | 1141        | 993                | 87.02            |
| Total     |                     | 10835       | 9874               | 91.13            |

Table 1. Total and polymorphic DNA bands and polymorphism rates (%) on \textit{Cercospora beticola} the causal agent of sugar beet (\textit{Beta vulgaris}) leaf spot isolates obtained by various primer combinations in AFLP analyses

Statistical methods used in the evaluation of results. The differences among pathogenicity levels induced by \textit{Cercospora} isolates collected from a variety of field sites were analysed by one-way analysis of variance (ANOVA) Kruskal Wallis Test (Kruskal and Wallis, 1952) on the 15th day of the greenhouse experiment. Following the detection of PCR products by use of the equipment Beckman CEQ 8800 DNA Capillary Series Analysis, the obtained results were scored in electronic medium as “existent (1)” and “non-existent (0)” for each primer pair. The obtained raw data were evaluated by means of MVSP (Multi-Variate Statistical Package, version 3.1) software according to the similarity index UPGMA (Unweighted pair group method with arithmetic averages) of Sorensen (1948). Phylogenetic analyses were also supported by Principle-Co-Ordinate Analysis (PCO).

Results and Discussion

The fifteen pathotypes obtained by the reactions of 100 \textit{C. beticola} isolates on five genetically different cultivars on the 15th day of the inoculation is presented in Table 2. The most prevalent pathotype was Pathotype no: 1. All studied sugar beets were resistant to this pathotype. The second most prevalent pathotype was Pathotype no: 15 which comprised 24 isolates. This pathotype lead to severe disease in all tested sugar beets. The pathotypes noes: 2, 3, 5, 6, 7,