MOLECULAR METHOD FOR DETECTION OF
CERCOSPORA BETICOLA SACC.

Andreea-Mihaela BALAU [1], Francesco FARETRA [2]

[1] University of Agricultural Sciences and Veterinary Medicine of Iaşi
[2] University of Studies "Aldo Moro", Bari, Italia

Abstract

The most important foliar disease of sugar beet (*Beta vulgaris* L.) is Cercospora leaf spot, caused by *Cercospora beticola* Sacc. Losses caused by this pathogen appear insignificant at first but in reality heavy pressure from the disease which is caused by *Cercospora beticola* Sacc. results in significant loss in root weight and reduction of recoverable sugar in sugarbeet.

This work present an protocol for the detection of *Cercospora beticola* from sugar beet plants. This method is based on PCR (*Polymerase Chain Reaction*) and is useful for identification of *Cercospora beticola* and can determine how early in the growing season sugarbeet tissues are colonized by the fungus. A rapid detection of disease and accurate identification of the causal agent is necessary for the development of an effective control system.

Leaf disks from sugar beets plants were used for this PCR method. After DNA purification, aliquts of the homogenate were added to PCR reaction and amplified using the Cercospora actin gen specific. Fragment size of the amplified products was correlated with the size of that amplified from DNA extracted from *Cercospora beticola* cultures to identify the fungus.

Key words: *Cercospora beticola*, detection, PCR protocol.

Cercospora leaf spot (*fig. 1*) in sugar beet (*Beta vulgaris* L.) caused by *Cercospora beticola* Sacc. occurs worldwide and may cause a 25-50% reduction of gross sugar yield. In severe epidemics the foliage will be totally destroyed and the beet starts to produce new leaves.

Figure 1 **Cercospora infected sugarbeet leaf**

Root weight and sugar content are strongly negatively influenced by the extend of the growth (Irena, Gaurilčikiene, Irena, Deveikyte, Egle, Petraitiene, 2006).

The timely application of fungicides in conjunction with forecastin models that predict the likelihood of Cercospora infection has become an important complement to genetic resistance in leaf spot control (Windelset et al., 1998). Therefore a rapid detection of disease and accurate identification of the causal agent is necessary for the development of an effective control system (Lartey *et* all, 2003). With the aid of a light microscope, the presence of conidiophores and conidia in lesions can be used to identify Cercospora leaf spot. In the absence of conidiophres and conidia in the lesion Cercopora leaf spot could be confused with spots produced by *Phoma betae* Franc and *Ramularia beticola* Fautrey and Lambote (Whitney and Duffus, 1986) and leaf blotches caused by abiotic factors or bacteria (Wolf and Vereet, 2002).

We present a PCR protocol for the detection and identification of *Cercospora beticola* that utilizes a simple and rapid extraction system from *Cercospora beticola* cultures and tissues.

MATERIAL AND METHOD

The polymerase chain reaction (PCR) method involves three essential steps, all conducted in succession under somewhat different controlled temperatures.

The first step known as "denaturation step" involves melting of the target DNA (template). In this reaction, standard DNA template is first denaturated by incubation at high temperature. The two dissociated DNA strans remain free in solution until temperature is lowered. The second step ("annealing step") involves annealing of two oligonucleotide primers to the denaturated strands by complimentary base pairing. The third step, "primer extension" involves extension of the primer by a thermostable DNA polymerase enzyme. In the PCR method, this typical set of steps is called a cycle and the amplified product begins to amplify within three cycle. The amount amplified product doubles after each cycle leading to accumalation of the specific regions of DNA, wich is later visualised by electroforesis of the PCR products.

The ability to amplify DNA from crude mycelial preparations is an important factor in the identification of fungi from plant material, and this is the primary advantage in using PCR, wich requires only small amounts of the test material and the tehnique is applicable even to partly degraded materials of poor quality. The polymerase chain reaction can be used to detect groups of strains, pathotypes, species or higher taxa, provided that specific oligonuclotide primers for these taxa are available.

DNA extraction

To reveal the molecular method for detection of Cercospora beticola fesh plant leaf material was used (from two sugarbeet species, Barsa and Brasov) and fungal mycelia were either scraped directly from the PDA culture Cultures were grown on PDA at 23°C in the dark. Leaf disks from diseased tissues and disease free green house plants were tested. DNA extraction from this cultures was carried out basically according to Murray and Thompson (1980) slightly modified as Rita M. De Miccolis et all, 2010.

After 1 week at 23°C, in the dark (200-300 mg fresh weight), grown on PDA overlapped by a sterile cellophane disks in Petri dishes (100mm) was transfered in Eppendorf tubes of 2 ml, containing one steel spheres (5 mm diam.), immersed in liqiud nitrogen and then agitated for 45 sat maximal frequency, oscillation coresponding to 1500 oscilations using the mixer mill Retsch MM 301. (Retsch Gmbh, Hann, Germany).

The powedered mycelium was suspended in 600µl of hot CTAB [100 mM Tris-HCl, pH 8,0; 1,4 M NaCl; 20mM EDTA, pH 8,0; 2% CTAB (p/v); 0,2% β mercaptonol (v/v)], mantained at 75 °C for 30 min and the mixture, frozen in liquid nitrogen and unfrozen at 75°C three times, was maintained at 75°C for 1 hour.

Extraction was carried out in 600µl of cloroform, nucleic acids, collected by centrfugation for 15 min. At 14.000 rpm, were precipitated with 600 µl of isopropanol at -80°C for 30 min., and collected by centrifugation (15 min at 14.000rpm). The pellet, washed with ethanol (70%), was suspended in 200 µl of TE (10mM Tris-HCl; 1m Na2 EDTA, pH 8,0).

The extracts, added with 0,1 µg ml-1 of RNAase A (Sigma, Saint Louis, Missouri, USA), were incubated at 37 °C for 2h. After the digestion, the DNA, precipitated at - 80°C , for 30 min in presence of 0,6 vol of ammonium acetate 5M (120µl) and 2 volof absolute ethanol, were collected through centrifugation at 14.000 rpm for 15 min. and suspended in 50-100 µl of ultra pure water.

DNA were then quantified through measuring the absorbance at 260 and 280 nm bu a spectrophometer (Du 640, Beckman Intruments, Inc. Palo Alto, California) and diluted to a final concentration of 50 ng µl-1.

PCR

The set of used PCR primers (*tab. 1*) were designed with Primer Primer (Premier biosoft International, palo Alto , California) from the Cercospora actin gene sequence in GenBank (Lartey and Weiland, Accesion # AF443281). The primers was designed to amplify an approximately 959 bp fragment of *Cercospora beticola* actin gene sequence [Robert T. Lartey et all, 2003] and were supplied by Sigma Aldrich.

Table 1

Caracteristics of primers designed from Cercospora actin gene sequence

OLIGO NAME	Primer sequences (Forward/Reverse)	Product size (bp)
CBACTN595L	5'AGCACAGTATCAT/ GATTGGTATGG3'	959
CBACTN595R	5'CACTGATCCAGAC/ GGAGTACTTG3'	

PCR reaction was carried out with Taq polimerase, nucleotides, MgCl2 and buffer supplied by Promega Corporation, Madison USA. (*tab. 2*)

Table 2

The PCR reaction mixtures

Amplifications reactions	µl
1x Green Go Taq® Flexi Buffer	5
2mM MgCl2	2
Nucleotide dNTPs	0,75
Primer Forward CBACTIN595L	0,5
Primer Revers CBACTIN595R	0,5
Enzima GoTaq DNA polymerase (5U/µl)	0,15
DNA	2
Ultra pure steril water	14,1
Total volume	25

Amplifications were carried out in a My Cycler™ thermal cycler (Bio Rad Laboratoires, Hercules California, USA) and the conditions were as follows: Initial denaturation of 5 min. at 95°C; 30 cycles of 1 min at 95°C (denaturation); 1 min. at gradient temperature : 60°C (annealing); 1 min. at 72°C (extension); Final extension of 7 min. at 72°C.

After amplification, 10 µl of each PCR product were loaded on 1,5 % agrarose gel (Molecular Biology Certified Agarose) and run in a TBE Buffer (0,5%) at 110 volts for 110 minutes in an horizontal gel submerge electrophoretic cell (Sub Cell Electrophoresis Cell, Bio Rad Laboratoires).

Agarose gel were stained in 1µg ml-1 of ethidium bromide solution for 30 min. and washed for 10 min. in distilled water. Gel images were captured by System Gel Doc 100 (Bio Rad Laboratoires) using Quantity One® software version 4 (Bio Rad).

The expected lenghts of the amplified DNA fragments were estimated by comparsion with a 100 bp DNA Ladder (New England Bio-Labs, USA) and quantification of PCR products werw done by comparsion with Precision Molecular Mass Standard (Bio Rad Laboratoires).

RESULTS AND DISCUSSIONS

The polymerase chain reaction (PCR) is a powerful molecular tool with widespread application in plant disease diagnostis.

Results of the present amplifications of DNA are presented in the *figure 1*.

The fragments of the *Cercospora beticola* genome were amplified with actin specific primers. The CBACTIN959L and CBACTIN959R set provided consistent amplification of the *Cercospora beticola* actin gene segment (Fig.2).
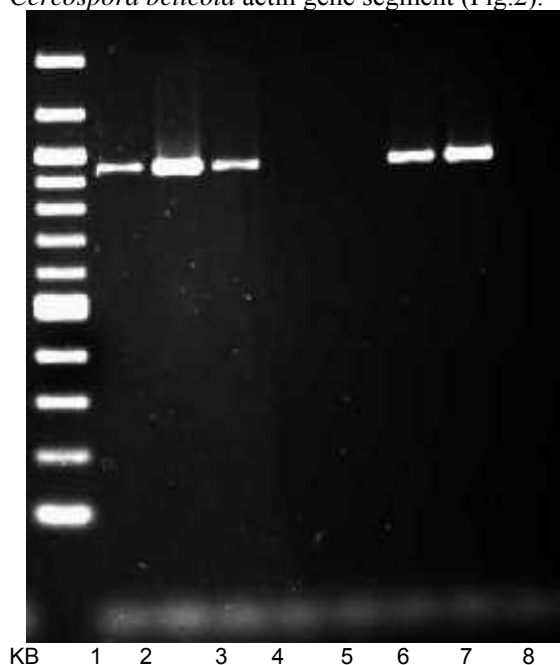

Figure 2 **PCR amplified fragments of Cercospora beticola genome***: KB = 1KB Ladder 1,2,3 – Genomic DNA extract from fungal culture; 4,5 - Uninfected sugar beet leaf ; 6,7 – Infected sugar beet leafs without Cercospora isolation, 8 – Control blank

The expected fragment was about 1 kbp. Regardless of the source of the template; DNA extract from fungal culture (lanes 1, 2 and 3),

direct from infected sugarbeet tissues (lanes 6 and 7) ; a fragment of the expected size was amplified. No amplification was observed from the uninfected control sugarbeet leaves (lanes 4 and 5) or the blank (lane 8).

We present in this research a protocol for rapid detection and identification of Cercospora beticola, the causal agent of Cercospora leaf spot of sugarbeet without laborious manipulation of the sample. Indeed, without culture of fungal propagules and genomic DNA extraction, we were able to amplify unique fragments of the *Cercospora beticola* genome from infected tissue.

The fact that the present work was done using field samples suggests that the test is useful in the initial stages of disease diagnosis. Besides sugarbeet. *C. beticola* produces leaf spots on most *Beta vulgaris* such as red garden beets (McKay and Pool., 1918).

CONCLUSSIONS

1. Rapid detection of disease and accurate identification of the causal agent is necessary for the development of an effective control system.

2. This work is useful in the initial stages of disease diagnosis and enable for a rapid detection of *Cercospora beticola.*

3. PCR protocol utilizes a simple and rapid extraction system from sugarbeet leaf tissue.

BIBLIOGRAPHY

Irena, Gaurilčikiene, Irena, Deveikyte, Egle, Petraitiene, 2006 – *Epidemic progress of Cercospora beticola Sacc. in Beta vulgaris L. under different conditions and cultivar resistance*, Biologija, Nr.4 , Akademia Lithuania, p.54-59.

Windelset, C.E., Lamey, H.A., Hilde, D., Widner, J., Knudsen, T., 1998 - *A Cercospora Leaf Spot Model for Sugar Beet*, Plant disease, Vol. 82, nr.7, p. 716-725.

Wolf , P.F.J., J.A. Vereet, 2002 – *An integrated pest management system in Germany for the control of fungal leaf diseases in sugar beet*, The IPM sugar beet model, Plant Disease, vol. 86, p. 336-344.

Whitney, E.D., Duffus, J.E., , 1986 – *Compendium of Beet diseases and insects*, St. Paul, MN, APS Press, p. 8-9.

Lartey, R.J., Weiland, J.J., Can, Caesar, Ton, That, Sarah, Bucklin-Comiskey, 2003 – *A PCR Protocol for Rapid Detection of Cercospora beticola in Sugarbeet Tissues,* Journal of Sugar Beet Research, Vol.40, Nos 1-2, p.10.

McKay, Pool., 1918 - *Field studies of Cercospora beticola*, Phytopathol, vol. 8, p.119-136.

Rita, M., De Miccolis and all, 2010 - *Selection, characterization and genetic analysis of laboratory mutants of Botryotinia fuckeliana (Botrytis cinerea) resistant to the fungicide boscalid,* Volume 128, Number 2, 185-199, DOI: 10.1007/s10658-010-9643-8.