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Development of a rapid and highly sensitive direct-PCR assay to detect a single conidium of *Botrytis cinerea* Pers.:Fr *in vitro* and quiescent forms *in planta*

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Abstract

"Direct-PCR" amplifications of *Botrytis cinerea*-specific genomic sequences, without any DNA purification step or time consuming sample preparation, were developed. A single copy sequence of 0.7 Kb in the *Botrytis cinerea* genome was amplified in reactions containing no more than 1 x 10^5 to 1 single conidium. As a demonstrative application, this assay was applied to detect *B. cinerea* in different parts of immature grape berries (at 'pea size'), when previously inoculated with conidia at flowering. Using this method we showed the presence of quiescent *Botrytis* in the receptacle area only. Cloning and sequencing of the fragment confirmed the single sequence gene of *B. cinerea*. These results demonstrate that the method is easy to apply and of sufficiently high sensitivity to detect the presence of *B. cinerea* in immature grape berries. Its use for studies on the development of grey mould and improved control of the disease in vineyards is discussed.

K e y w o r d s : *Botrytis cinerea*, "Direct-PCR", *Vitis vinifera.*

Introduction

The polymerase chain reaction (PCR) has become a widely used technique with applications in medical, agricultural, biotechnological, phylogenetical and basic research (Mullis and Faloona 1987; Saiki *et al.* 1988; Ehrlich *et al.* 1991). From diagnostics to site-specific mutagenesis, PCR generally involves prior purification of nucleic acids. PCR on whole bacterial cells and associated bacteriophages without any purification step was shown upon lysis at the high temperature of amplification, thus providing an easy tool to characterize constructs simply by adding the organism to the reaction mix (Güssow and Clackson 1989). Direct single copy sequence amplification was achieved on tobacco leaves and root pieces (Berthomieu and Meyer 1991), on fungal spores of *Aspergillus* (Aufauvre-Brown *et al.* 1993) and was developed for use in diagnostics of viral and bacterial infections (Olive 1989). The technique however fails in cases of inefficient cell lysis, degradation, modification or capture of DNA, or when the polymerase enzyme is blocked or degraded. Rossen *et al.* (1992) and Wilson (1997) summarized the various compounds found in sample and DNA extraction solutions which inhibit or facilitate the enzymatic amplification reaction. The amplification of rare target sequences is further hindered by the loss of genetic template during multiple extraction steps.

Molecular (PCR) and serological (ELISA) tools are now used widely in agricultural field diagnostics, for example to detect a pathogen during quiescence prior to an epidemic outbreak, thus leading to better disease forecasting (Parry and Nicholson 1995; Pryor and Gilbertson 2001; Dewey and Yohalem 2004). Quiescent *Botrytis* in symptomless grapes is generally evaluated by the time-consuming plating-out of surface-sterilised plant tissues on selective media and mycological identification of isolated fungi. Recently, a specific molecular marker for *B. cinerea*, based on a 757-bp nucleotide sequence, was characterized (Rigotti *et al.* 2002). This marker allows rapid detection of the fungus during quiescence inside plant tissues.

The present work describes a method for PCR amplification of this specific genomic sequence and the detection of *B. cinerea* when it remains quiescent in immature grape berries without any DNA purification step or time-consuming sample preparation.

Material and Methods

O r g a n i s m s a n d g r o w t h c o n d i t i o n : *Botrytis cinerea* strain P69 was grown in Petri dishes on potato dextrose agar (Difco) at 21 °C under alternating 12 h light and dark periods. Conidia were collected by vacuum aspiration according to Pezet and Pont (1990), sealed in plastic tubes and stored dry at -80 °C, according to Gindro and Pezet (2001) until PCR analysis and/or infection studies. Inflorescences of Vitis vinifera L. cv. Pinot gris grown in the field were sprayed at full bloom with an aqueous suspension of 1 x 10^4 conidia ml^{-1} as described by Viret *et al.* (2004). At véraison, individual berries were harvested, washed with sterile water, sealed in plastic tubes and stored at -80 °C. Uninfected berries were used as controls.

S a m p l e p r e p a r a t i o n a n d P C R : PCR on fungal cells. PCRs were performed on different dilutions of aqueous conidial suspensions. The number of conidia was measured for each dilution level with an heamocytometer. For the amplification experiments on a single conidium, an aqueous

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suspension of 10^2 conidia was plated on agar medium and conidia were collected separately under a magnifying glass with a sterile glass capillary tube and put in polyethylene microtubes containing 18 µl of water. The collected fungal cells were briefly swirled in 18 µl sterile water. The tubes were transferred to a thermocycler pre-heated to 97 °C for 15 min (Biometra PC-Personal Cycler) to break the cells. The thermocycler was then held at 4 °C to add 7 µl of the PCR mix, namely 2 units of Taq (Qiagen Taq DNA Polymerase), 0.4 µM of each primer according to RIGOTTI *et al.* (2002), 0.2 mM of each dNTP, 3 mM $MgCl_2$ and 1x PCR buffer. Amplification was performed with one cycle at 95 °C for 3 min, followed by 34 cycles at 94 °C for 20 s, 54 °C for 20 s, 72 °C for 30 s. Gel electrophoresis of PCR products was performed on 1 % agarose gel. Control amplifications were performed on 1 ng of fungal purified genomic DNA. The experiments were done in triplicate.

Direct PCR on crushed grape berries containing *B. cinerea.* The receptacle area and the papillate stigma were cut from an immature berry developed from inoculated flowers and stored on ice . The other regions of the berries were divided with a sterile scalpel into 6 parts (Fig. 2) and these were also stored on ice. Each part was weighed and crushed in 1.5 ml tubes containing 100 µl sterile water mg^{-1} fresh weight on ice, using a rough plastic pestle. Homogenized extracts (18.3 µl) were transferred into microtubes and placed in a pre-heated thermocycler (Biometra PC-Personal Cycler) at 97 °C for 15 min to break open the cells and to inactivate most cell enzymes, then cooled to 4 °C before adding the 7.2 µl PCR mix and starting the amplification, as described above. The experiments were repeated 4 times.

Control DNA extraction and purification: Fungal DNA was used as control for classical PCR methods. *B. cinerea* was grown in liquid cultures (100 ml potato dextrose broth in 250 ml flasks) at 21 °C for 3 d under constant agitation. Mycelium was pelleted by centrifugation, washed with water, frozen and lyophilized. Fungal DNA was extracted according to CENIS (1992), including an RNase treatment and a chloroform-isoamyl alcohol extraction. DNA from *V. vinifera* cv. Pinot gris was extracted from leaves using a sucrose-CTAB method (KIRKPATRICK et al. 1987; DOYLE and DOYLE 1990; AHRENS and SEEMÜLLER 1992).

Cloning and sequencing: A final extension at 72 °C for 3 min was added to allow for cloning the PCR product into pCR4Blunt-TOPO® (Invitrogen) according to their procedure. Plasmids were purified on spin columns from Qiagen and sent to Microsynth for sequencing. The sequences were aligned and analyzed using Blast search (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

A single copy sequence of 0.7 kb in the *B. cinerea* genome (RIGOTTI *et al.* 2002) was amplified in reaction mixtures containing 10 to 1 x 10^5 conidia. Amplification did not occur in a 25 ml reaction volume containing 2.5 x 10^5 and more conidia. A negative control without conidia did not show amplification (Fig. 1 a). Single conidia were manually selected and amplification of each sampled conidium was successful (Fig. 1 b). These results showed that Direct-PCR was sensitive and sufficiently specific to amplify a single copy genomic sequence in a single heat-lyzed conidium, probably due to the multinucleate state of *B. cinerea* asexual spores (EPTON and RICHMOND 1980; PEZET *et al.* 2004). For example, several hundred copies of ITS sequences are found in one genome of *B.* cinerea and ITS-based detection can be 100 times more sensitive than the detection limit for a single copy amplicon (NIELSEN *et al.* 2002; DEWEY and YOHALEM 2004). Direct-PCR was then performed on grape berries to detect the quiescent form of the fungus. Different parts of the berries were analyzed (Fig. 2), and an amplification of 0.7-kb band was only visible in the receptacle area (Fig. 3). Cloning and sequencing of the fragment confirmed the single sequence gene of *B. cinerea* (RIGOTTI *et al.* 2002; EMBL accession number AJ422103). The same sequence was obtained from DNA purified from *B. cinerea* alone. This con-

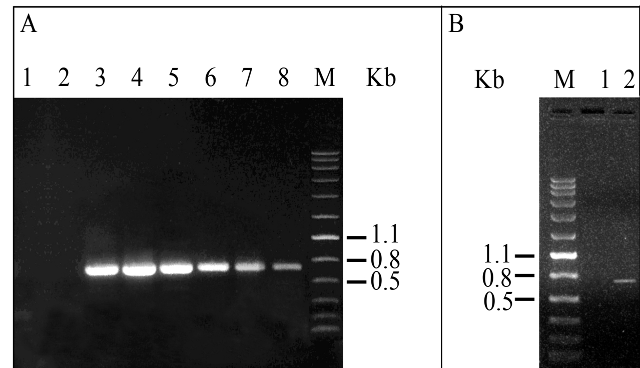

Fig. 1: "Direct-PCR" amplification on succesive dilutions of *B. cinerea* aqueous conidia suspensions and on one single conidium with primers C729+/C729-. **A**: All concentrations in 25 µl PCR final reaction volume. Lane 1: control without conidia; Lane 2: 2.5 x 10^5 conidia; Lane 3: 1x10^5 conidia; Lane 4: 2 x 10^3 conidia; Lane 5: 7 x 10^2 conidia; Lane 6: 3 x 10^2 conidia; Lane 7: 1 x 10^2 conidia; Lane 8: 10 conidia; M: Leon DNA ladder. **B**: Lane 1: control without conidia; Lane 2: amplification of the 0.7-kb fragment in one single conidium; M: Leon DNA ladder.

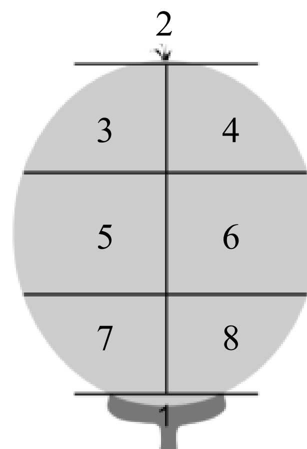

Fig. 2: "Direct-PCR" amplification and detection of *B. cinerea* in an immature grape berry with primers C729+/C729-. Lane 1: amplification of the 0.7-kb fragment in the receptacle area; Lane 2: control amplification on non-infected berry; M: Leon DNA ladder.

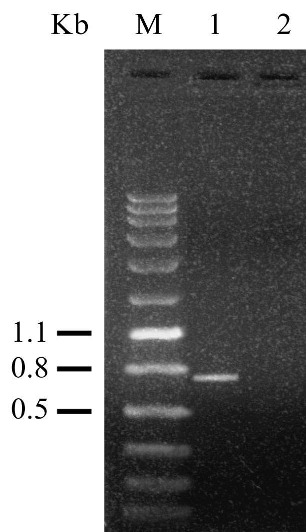

Fig. 3: Schematic representation of an immature grape and the parts used for direct amplification and detection of quiescent *B. cinerea*. **1**: receptacle area; **2**: papillate stigma; **3** to **8**: grain divided in 6 parts.

firms previous microscopic observations which have shown that conidia and conidial germ tubes were mostly present in a region at the interface between the ovary to the receptacle (VIRET *et al.* 2004). No product was detected after amplification of DNA purified from *V. vinifera* purified alone.

It is known that amplification reaction can be inhibited by different parameters such as excessively high cell numbers, cell wall fragments, DNA-binding proteins, polysaccharides, phenolics, detergents or insufficient lysis of the cells (MOREIRA 1998; DEWEY and YOHALEM 2004). In our experiments, the concentration of cells in the mix and the percentage of lysed cells were measured carefully and these components did not interfere in PCR amplification. No amplification had occurred without pre-treatment at 97 °C for 15 min prior to starting PCR cycles. This heat-shock step can break cell walls and membranes and inactive enzymes by irreversible degradation of proteins. Lysozymes and ribonucleases are inactivated at 90-100 °C in hepatocytes when irreversible degradation of cellular proteins occurs at much lower temperatures (LEPOCK *et al.* 1993). Phenolics are the most important PCR-inhibitory plant cell compounds (NIELSEN *et al.* 2002); this heat treatment probably favours their degradation because it was suggested for condensed tannins that are degraded into anthocyanins by heat (PORTER *et al.* 1985). Amplification was not achieved if the conidial concentration exceeded 1 x 10^5. These results are in agreement with WILSON (1997) describing factors which reduced nucleic acid amplification, such as more than 10^3 bacteria in the mix. However, direct DNA amplification from up to 10^6 conidia in a 50 ml reaction mixture was demonstrated in *Aspergillus* spp by AUFAUVRE-BROWN *et al.* (1993).

Amplification applied to a single fungal conidium may be useful to study the genetic variability of fungi, to identify markers for fungicide resistance (SCHOONBEEK *et al.* 2002), or to study gene-disruption events in transformants (AUFAUVRE-BROWN *et al.* 1993), but also to detect the presence of quiescent mycelium or propagules of *B. cinerea* in immature grape berries, as described previously (MCCLELLAN and HEWITT 1973; PEZET and PONT 1986; VIRET *et al.* 2004). These authors considered that *B. cinerea* can infect flowers and stay in immature berries until véraison, when grey mould can rapidly destroy entire clusters, depending on the meteorological conditions. "Direct-PCR" can be easily applied to determine the infection potential of quiescent *B. cinerea* in berries and to obtain information for epidemiological studies and disease control.

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