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SHORT NOTES

Genetic diversity of some Tunisian *Botrytis cinerea* isolates using molecular markers

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Summary. The genetic diversity of *Botrytis cinerea* in Tunisia was studied using molecular markers, and the level of resistance to the fungicide fenhexamid was shown. Isolates from different plants (grape, tomato, cucumber, onion, strawberry, gerbera and rose) and different parts of the country were analysed in order to determine whether the two groups, *transposa* and *vacuma*, that were detected in French vineyards, are also present in Tunisia. A combined PCR and Dot Blot method was developed to identify the transposable elements *Boty* and *Flipper* that distinguish between these two *B. cinerea* groups. Both the *transposa* and *vacuma* groups, and isolates containing the transposable element *Boty*, were found in Tunisia. Moreover, analysis of the *Bc-hch* locus by PCR and restriction enzyme digestion identified only the *B. cinerea* group corresponding to one allelic type. Finally, by using the level of resistance shown by *B. cinerea* to the fungicide fenhexamid as a marker, it was confirmed that this was the only group of *B. cinerea* in the Tunisian population.

Key words: *Botrytis cinerea, transposa*, *vacuma, Bc-hch,* fenhexamid.

Introduction

Botrytis cinerea Pers., Fr. (anamorph of *Botryotinia fuckeliana* [de Bary] Whetz) is an ubiquitous plant-pathogenic fungus that causes grey mould on many economically important crops, including vegetables, ornamentals, fruits and particularly grapes. The fungus can destroy plant tissues by acting either as a parasite or as a saprophyte. Recent studies have shown that *B. cinerea* has great genetic diversity and morphological variability (Der Vlugt-Bergmans *et al*., 1993; Diolez *et al*., 1995; Van Giraud *et al*., 1998).

Investigations conducted in a Champagne vineyard in France suggest that *B. cinerea* is composed of two sympatric sibling species, *B. transposa* and *B. vacuma*, characterised by the absence or presence of two transposable elements or transposons, *Boty* and *Flipper*: *B. transposa* has both these elements (Giraud *et al*., 1998), whereas *B. vacuma* has none. The fungus seems to have a certain degree of host specialization. It has been suggested that *transposa* isolates are better adapted to in-

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fect grapes berries, while *vacuma* isolates preferably infect the grape leaves (Giraud *et al*., 1998). Screening for polymorphism using PCR-RFLP of the *Bc-hch* gene (the *B. cinerea* homolog of the *Neurospora crassa het-c* vegetative incompatibility locus) confirmed the presence of two groups, in this French vineyard, of which one was named *Botrytis pseudocinerea*, and the second *B. cinerea* (Fournier *et al*., 2003). Control of *B. cinerea* is sometimes very difficult and this may reflect the existence of distinct populations. Many families of synthetic fungicides to control this fungus exist but, most of them are restricted in use because strains resistant to them have appeared. The hydroxyanilide fenhexamid is a sterol biosynthesis inhibitor (SBI) and botryticide whose primary target site is 3-keto reductase, an enzyme involved in sterol biosynthesis. Fenhexamid does not affect spore germination but inhibits germ-tube elongation and mycelial growth at low concentrations (Leroux *et al*., 2002a). The *B. cinerea* genotypes HydR1 have developed resistance to this fungicide. These genotypes show increased sensitivity to other SBI molecules having different target enzymes, such as 14α-demethylase inhibitors (DMI) and Δ14-reductase inhibitors. More specifically, the HydR1 strains contain two expressed mutations, phenylalanine at position 15, and serine at position 105, which in other phenotypes, are isoleucine and asparagine respectively (Albertini *et al.,* 2002; Leroux *et al*., 2002a; Albertini *et al.,* 2004).

The aims of the present study were: 1. to evaluate the genetic diversity of *B. cinerea* in Tunisia by carrying out a search for the two forms *transposa* and *vacuma*; 2. to identify the two groups *B. pseudocinerea* and *B. cinerea*; and 3. to evaluate the level of resistance of some Tunisian isolates to fenhexamid.

Materials and methods

Fungal isolates and their maintenance

The fungal material consisted of 80 isolates of *B. cinerea* collected from different parts of Tunisia and different host plants between 1998 and 2003, two laboratory isolates (SAS56, SAS405) from Italy (Dipartimento di Protezione delle Piante, University of Bari, Italy), used as positive controls of the presence of the transposable element *Boty* (SAS56) and *transposa* (SAS405), and three French isolates (collection numbers 780, 900, 945) kindly provided by INRA, Versailles, France, and which belonged to group I (*B. pseudocinerea*). Fungal colonies were purified and maintained on potato dextrose agar (PDA). After monosporal purification, isolates were stored in 10% glycerol at -20°C.

Table 1 gives details of only 11 isolates, collected in 1998, 1999 and 2000, which were used in the entire study. The remaining 69 isolates were tested by PCR only in order to determine the polymorphism of the *Bc-hch* gene.

Table 1. Characteristics of a subset of *Botrytis cinerea* isolates used in this study to test for transposons: origin, host plant and year of collection.

Isolate	Origin	Host plant	Year of collection
Bc 1.98	Tunisia	Grape (Muscat d' Italie)	1998
Bc 2.98	Tunisia	Grape (Super Seedless)	1998
Bc 6.98	Tunisia	Tomato	1998
Bc 13.99	Tunisia	Grape (Chardonnay)	1999
Bc 15.99	Tunisia	Grape (Muscat d' Italie)	1999
Bc 23.00	Tunisia	Grape (Muscat d' Italie)	2000
Bc 22.00	Tunisia	Tomato	2000
Bc 24.00	Tunisia	Grape (Muscat d' Italie)	2000
Bc 25.00	Tunisia	Grape (Muscat d' Italie)	2000
SAS405[1]	University of Bari, Italy	Laboratory isolate	-
SAS56[1]	University of Bari, Italy	Laboratory isolate	-

[1]SAS405 and SAS56 are reference strains obtained from a single ascospore from crosses between compatible isolates in laboratory.

DNA extraction

Fungal isolates were grown on Czapeck-pectine liquid medium (2.5 g $NaNO_3$; 0.5 g KCl; 10 mg $MgSO_4 \cdot 7H_2O$; 10 mg $FeSO_4 \cdot 7H_2O$; 1 g K_2HPO_4; 10 mg MoNa; 10 g pectin; 1 l distilled water) for 3 days at 20°C. Mycelial mats were harvested, washed with sterile water, and frozen in liquid nitrogen. Genomic DNA was extracted as described by Moeller *et al*. (1992). Frozen mycelium was ground in a mortar with liquid nitrogen, then disrupted in a tube in an extraction buffer (100 mM Tris-HCl at pH 8, 10 mM EDTA and 2% SDS). The tube was incubated at 60°C for 1h and the mixture was amended with 3 M NaCl and 10% CTAB and then incubated at 65°C for 10 min. Chloroform was added to the mixture and kept for 30 min on ice before centrifugation at 13,000 rpm (Rotor No. 12002, Sigma, Osterode, Germany) for 10 min. The supernatant was added to 5 M acetate ammonium, kept for 30 min on ice and centrifuged at 13,000 rpm (Rotor No. 12002, Sigma) for 5 min. Isopropanol was added to the resulting solution, and the mixture was incubated for 1 h at -20°C. DNA was collected by centrifugation for 10 min at 13,000 rpm (Rotor No. 12002, Sigma). The pellet was washed with 70% ethanol, dried and resuspended in TE (10 mM Tris-HCl at pH 8 and 1 mM EDTA). Lastly, the RNA was removed by adding 2 μl of Rnase (10 mg ml^{-1}) and incubating at 37°C for 1 h. DNA was determined by electrophoresis on 1% agarose gel in 1×TBE buffer (54 g Tris-HCl, 27.5 g boric acid and 3.72 g EDTA), stained with ethidium bromide and observed under UV light (312 nm).

Detection of the transposable element *Flipper* by PCR

Flipper is a transposable element of 1872 pb (Levis *et al*., 1997) (Accession No. U74294). A pair of primers (Flipper-F: 5'-ATGCGTAAGGCTTGCTCAG-3'; Flipper-R: 5'-GCTTTCTGATAGCAGGCA-3') were developed to PCR-amplify 1 kb of the sequence. The amplification was conducted in a final volume of 50 μl reaction containing 20 μM of each primer, 10 mM of each dNTP, 1.5 mM $MgCl_2$, 100 ng of fungal DNA, 2.5 U Taq DNA polymerase (Appligène, France) and 5 μl of its corresponding buffer. Reactions were performed in a Genius thermal cycler programmed as follows: 1 cycle of 2 min at 94°C, followed by 40 cycles of 50 s at 94°C, 50 s at 60°C, 1 min and 30s at 72°C. A final extension of 10 min was carried out at 72°C. Amplified products were separated by electrophoresis on 1% agarose gel in 1×TBE, stained with ethidium bromide, and photographed under UV light using Polaroid film.

Detection of the transposable element *Boty* by dot blot

Boty is a 6 Kb retro-transposon (Diolez *et al*., 1994) (Accession No. X81790 and X81791). DNA (1 μg) was denatured in 1 M NaOH for 15 min, spotted onto a Hybond-N (Amersham, Buckingham, England) nylon membrane and fixed under UV light. Hybridisation was carried out with a sequence of the transposable element *Boty* (kindly provided by C. Levis, INRA Versailles, France) labelled with ^{32}P using a random primer labelling kit (Gibco, CA, USA).

***Bc-hch* amplification and digestion**

Two primers: 262 (5'-AAGCCCTTCGATGTCTTGGA-3') and 520L (5'-ACGGATTCCGAACTAAGTAA-3'), described by Fournier (Fournier *et al*., 2003), were used to amplify the *Bc-hch* gene. These primers amplified a 1171 pb fragment between position 701 and 1171 of the *Bc-hch* gene. The amplification was conducted in a final volume of 100 μl containing 3 μM of each primer, 10 mM of each dNTP, 2 mM $MgCl_2$, 100 ng of fungal DNA, 2.5 U Taq DNA polymerase (Promega) and 10 μl of its corresponding buffer. Reactions were performed in a Genius thermal cycler programmed as follows: 1 cycle of 2 min at 94°C, followed by 40 cycles of 50 s at 94°C, 50 s at 55°C, 1 min and 30 s at 72°C. A final extension of 10 min was carried out at 72°C. Amplified products were separated by electrophoresis on 1% agarose gel in TBE, stained with ethidium bromide, and photographed under UV light using Polaroid film. Digestion was carried out on 40 μl of the PCR product using 2 units of *Hha* I restriction enzyme (Appligène, France). Incubation was performed overnight in a Genius thermal cycler programmed at 37°C. Digested products were separated by electrophoresis on 2% agarose gel in TBE buffer, stained, and photographed as above.

Anti-fungal assays

To study the effect of fenhexamid on mycelial growth of *B. cinerea*, the PDA medium containing different fungicide concentrations was inoculated with 6 mm mycelium plugs (taken from the margin of 2-day-old colonies) and the plates incubated

at 20°C in the dark. The fungicide was dissolved in ethanol and added aseptically to PDA after autoclaving. The mycelial growth rate was evaluated by measuring the diameter of colonies during 3 days and the EC_{50} values (concentrations causing a 50% reduction in mycelium growth) were calculated from the dose-response curves. From these data the level of resistance was deduced.

Results and discussion

Presence of *transposa* and *vacuma* isolates in Tunisia

PCR amplification using Flipper-F and Flipper-R primers revealed the transposable element *Flipper* in some *B. cinerea* isolates (Bc 1.98, Bc 15.99, Bc 22.00, Bc 23.00 and SAS405) as shown in Figure 1. In addition, dot blot analysis, with the sequence of *Boty* as a probe, revealed DNA hybridisation for the isolates Bc 2.98, Bc 13.99, Bc 15.99, SAS56 and SAS405 as shown in Figure 2. Three of the Tunisian isolates (Bc6, Bc 24.00 and Bc 25.00) lacked the two transposable elements *Boty* and *Flipper*, showing that both *B. vacuma* and *B. transposa* occur in Tunisia (Table 2). It was also confirmed that strain SAS405, which belongs to the *B. transposa* type (C. Levis, personal communication) contains both *Boty* and *Flipper* elements. The transposable element *Boty* was present in strains collected in 1998 and 1999, but not in any strains collected in 2000. The transposable element *Flipper* was present in strains

Table 2. Groups of *Botrytis cinerea* detected in Tunisia.

Isolate	Transposable element	Group
Bc 1.98 Bc 22.00 Bc 23.00	*Flipper*	'*Flipper* isolates'
Bc 2.98 Bc 13.99 SAS56	*Boty*	'*Boty* isolate'
Bc 15.99 SAS405	*Flipper* / *Boty*	*transposa*
Bc 6.98 Bc 24.00 Bc 25.00	None of the two transposable elements	*vacuma*

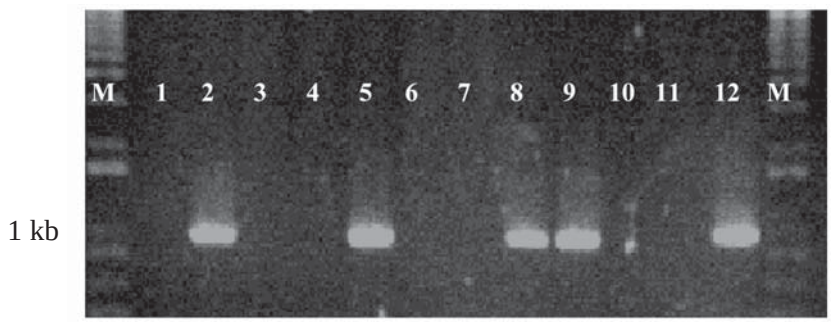

Fig. 1. Electrophoresis on 1% agarose gel showing the result of the *Flipper* PCR amplification. Lane 1, SAS56; lane 2, SAS405; lane 3, Bc 2.98; lane 4, Bc 13.99; lane 5, Bc 1.98; lane 6, Bc 6.98; lane 7, Bc 24.00; lane 8, Bc 22.00; lane 9, Bc 23.00; lane 10, Bc 25.00; lane 11, Bc 15.99; lane 12, Bc 22.00; M, marker, 1 kb DNA ladder.

Fig. 2. Dot-blot analysis, from right to left and top to bottom of : reference strains SAS56, SAS405 and Tunisian isolates Bc 1.98, Bc2.98, Bc 6.98, Bc 24.00, Bc 13.99, Bc 15.99. Hybridisation was carried out using a ^{32}P labelled *Boty* probe.

collected in 1998 (Bc 1.98), 1999 (Bc 15.99) and 2000 (Bc 22.00 and Bc 23.00). Furthermore, some isolates (Bc 2.98, Bc 13.99 and SAS56) were found to harbor only the transposable element *Boty*, which was observed also in the Champagne vineyard and in Chile (Munoz *et al*., 2002). These isolates were named '*Boty* isolates'. Some other isolates (Bc 1.98, Bc 22.00 and Bc 23.00), not identified elsewhere, contained only the transposable element *Flipper*. This last phenomenon could probably be either the result of a migration of the transposable element *Flipper*, which may be invading isolates of the *vacuma* group, or the other transposable element may not have been detected because the molecular technique employed was not suitable. It may well be that PCR amplification, used to detect *Flipper*, was not as accurate as the dot blot hybridisation adopted for *Boty* detection.

Table 3. EC_{50} and phenotypes of *Botrytis cinerea* detected in Tunisia.

Isolate	EC_{50} (ppm)	Phenotype [1)]
Bc 1.98	0.037	HydS
Bc 22.00	0.156	HydS
Bc 23.00	0.216	HydS
Bc 2.98	0.238	HydS
Bc 13.99	0.164	HydS
SAS56	0.172	HydS
Bc 15.99	0.033	HydS
SAS405	0.08	HydS
Bc 6.98	0.076	HydS
Bc 24.00	1.5	HydR2
Bc 25.00	0.284	HydS

[1)] HydS, hydroxyanilide sensitive; resistance level lower than 2. HydR2, hydroxyanilide resistant; resistance level higher than 2.

Sensitivity to fenhexamid

In vitro studies conducted on the different isolates revealed only hydroxianilide sensitive (HydS) strains (Table 3). HydS strains have a level of resistance lower than 2. This confirmed that the Tunisian isolates analysed by PCR-RFLP belonged to the second group (*B. cinerea*) and not the first (*B. pseudocinerea*). The Tunisian isolates thus have not developed resistance to this sterol biosynthesis inhibitor (SBI) recently introduced to Tunisia. Furthermore, *in vitro* anti-fungal studies indicated that fenhexamid, like other SBI, did not affect spore germination but at low concentrations inhibited germ-tube elongation and mycelial growth (Leroux *et al*., 2002). In the French vineyard, all strains belonging to the first group were of the type *B. vacuma*, and strains of the second group were either *B. vacuma* or *B. transposa* (Leroux *et al*., 2002b. This was confirmed for the Tunisian isolates also, which presented only the second group *B. cinerea*, with the two types *B. vacuma* and *B. transposa*).

***Bc-hch* PCR-RFLP analysis**

PCR analysis with primers 262/520L, used to amplify the fragment corresponding to the region of the *Bc-hch* gene, gave the same result for all isolates tested, with a 1171 bp PCR product (Fig. 3). Digestion with the *Hha* I enzyme revealed some polymorphism between the Tunisian isolates and the French isolates used as a positive control and

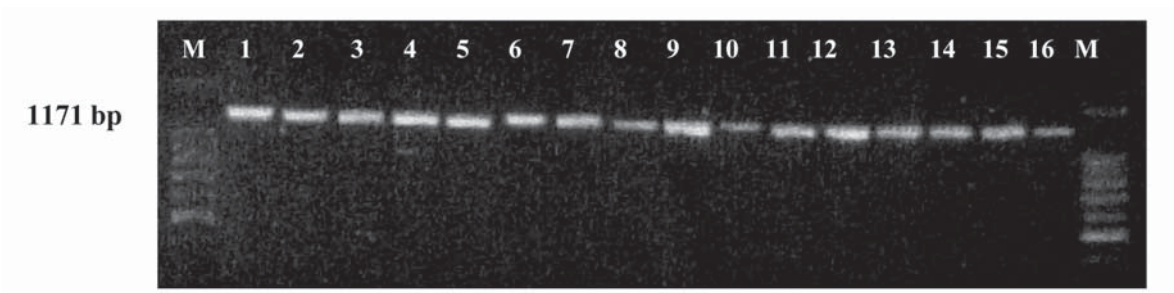

Fig. 3. Electrophoresis on 1% agarose gel showing the result of the PCR amplification of the *Bc-hch* gene by the 262/520L primer; M, 100 bp marker.

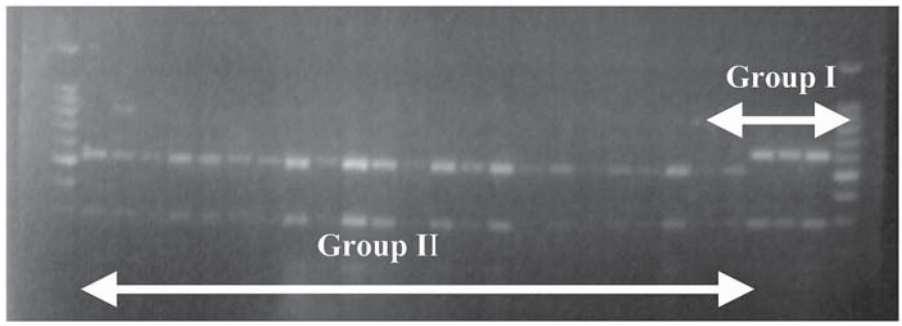

Fig. 4. Electrophoresis on 2% agarose gel showing the result of *Bc-hch* digestion with the *Hha* I restriction enzyme. Group I, 3 *Botrytis pseudocinerea* French isolates. Group II, a sample of 23 Tunisian isolates.

belonging to the first group (*B. pseudocinerea)*. As a consequence, two restriction patterns were detected, differing in the size of the upper band *Bc-hch2*, 517 bp; *Bc-hch1*, 601 bp. The restriction enzyme *Hha* I had 5 restriction sites in the *Bc-hch2* allele, whereas the *Bc-hch1* allele had only 4. The restriction site in position 367 had mutated in the *Bc-hch* allele (Fournier *et al*., 2003): this was revealed by a 600 bp band detected in the DNA of strains from group I (Fig. 4).

Conclusions

In this work we found that the two sympatric types *B. vacuma* and *B. transposa* occured in the Tunisian isolates. The group *pseudocinerea,* detected in 5% of French isolates (Fournier, personal communication) was not found. That group is characterized by the absence of the transposable elements *Boty* and *Flipper* and by a reduced sensitivity to fenhexamid. In fact, the sensitivity of the Tunisian isolates to fenhexamid confirmed the absence of the *pseudocinerea* group and proved the efficacy of this novel fungicide against grey mould in Tunisia. To study the genetic diversity and population structure of *B. cinerea* in Tunisia other powerful molecular markers, such as AFLP or SSR, are needed to explore more fully the polymorphism existing between strains. We are also analysing the sequence of *Bcpme*2, a gene controlling the pectin methyl esterase, an enzyme which is involved in cell-wall degradation of the plant host and is supposed to be a primary determinant of pathogenicity (Valette-Collet *et al*., 2003), examining whether PCR amplification and sequencing DNA reveal Single Nucleotide Polymorphisms (SNPs) between isolates from different plants. All these molecular markers will shed light on whether the *B. cinerea* collection of strains is specialised according to the region or the plant host.

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