Eur J Plant Pathol (2009) 123:367–375
DOI 10.1007/s10658-008-9373-3

PCR-based monitoring of recent isolates of tobacco blue mold from Europe reveals the presence of two genetically distinct phenotypes differing in fungicide sensitivity

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Received: 23 November 2007 / Accepted: 8 September 2008 / Published online: 16 October 2008

Abstract Bioassays testing the fungicide sensitivity against metalaxyl of *Peronospora tabacina* isolates collected in German tobacco fields in 2005 revealed the presence of two phenotypes, resistant and sensitive. DNA fingerprints using SSR and minisatellite primers allowed separation of the samples into two groups. The differences in amplification patterns coincided with the sensitive and resistant reaction of the isolates in metalaxyl bioassays. New primers were developed which allowed PCR-based detection of *P. tabacina* and differentiation of the metalaxyl-sensitive and the metalaxyl-resistant phenotype, respectively. Screening of recent blue mold isolates from Germany and other European countries for metalaxyl sensitivity with leaf disk bioassays coincided completely with the PCR-based identification of the two phenotypes. In Germany, exclusively resistant isolates were found between 2002 and 2004. These still dominate. Since 2005 the co-occurrence of sensitive isolates has been shown. No similar monitoring has been done in any other European country. However, we found the resistant phenotype reaction in a French isolate of 2004 and in two out of three Italian isolates in 2007. Two isolates from Poland and Bulgaria in 2007 were sensitive to metalaxyl. For all 58 isolates tested since 2002 the metalaxyl bioassay-based resistance type and the PCR-based tests for sensitive and resistant genotype coincided. Using genotype-specific primers for future population studies may help to trace the sources of the pathogen from which yearly propagation starts.

Keywords Metalaxyl · Oomycete · *Peronospora tabacina* · Resistance

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Introduction

In 2002, metalaxyl-resistant *Peronospora tabacina* Adam (syn. *P. hyoscyami* De By. f. sp. *tabacina* (Adam) Skalicky) caused massive problems in Germany. In response, tobacco blue mold infections and the fungicide resistance of the pathogen population were monitored. Field isolates of the pathogen were collected and cultivated for fungicide tests and analysis of population genetics (Krauthausen et al. 2003). The epidemics in 2002 and 2003 had been caused by seedling infection and a subsequent nation-wide distribution of diseased plants (Keil and Spring 2005). No incidence of metalaxyl-resistance has been reported from other European countries until now. This is in contrast to the situation in North America, where metalaxyl-resistant strains of *P. tabacina* were found in the early 1980s (Bruck et al. 1982) and infections

seem to spread seasonally from subtropical areas into temperate regions via long-distance dispersal of sporangiospores (Aylor et al. 1982). Assuming that natural infections in temperate areas of Europe are wind-borne by sporangiospores from pathogen populations hibernating in North Africa, it could be expected that the local problem with fungicide-resistant strains of *P. tabacina* in Germany in 2002 and 2003 would be negligent in subsequent years through phytosanitary measures in seedling cultivation. Surprisingly, the resistant phenotype continuously reappeared from 2004 to 2007, although strict controls prevented seedling infection in Germany. This left the question whether, despite the lack of evidence for oospore formation (Blancard 1998), the resistant pathogen had successfully hibernated in Germany. Another explanation could be that fungicide-resistant strains also occur in North Africa and seasonally invade Europe. Until 2005, 39 German isolates and one from France were tested and were found to be resistant to metalaxyl. Only a single sensitive strain was found late in the season of 2005 in a field near Heilbronn. Later on, PCR fingerprinting using simple-sequence-repeat (SSR) primers revealed clear differences between isolates of the resistant and the sensitive phenotype (Spring et al. 2007). In contrast, the amplification patterns within the two groups were uniform, indicating homogeneity within the two groups.

A PCR-based test was developed for differentiation between the two phenotypes found in Germany and bioassays on leaf disks were performed to test coincidence with the fungicide-resistance phenotype. Questions on the origin and geographic distribution of the two phenotypes led us to intensify our investigation and to extend sampling. We report here on the results of recent samplings of tobacco blue mold from five European countries.

Materials and methods

Origin of *Peronospora tabacina* isolates

From 2002 to 2007, occurrence of tobacco blue mold in major regions of tobacco cultivation in Germany was identified and recorded with the aid of the German extension service (Beratungsdienst Tabakbau and the Landesanstalt für Pflanzenbau, Forchheim). Infected leaf samples from 52 accessions from different regions of Germany were sent to our laboratory in Hohenheim for cultivation and characterisation. Table 1 shows the origin and characteristics of two representative strains from each of the years 2002 to 2006 and all 2007 samples used for this study. An additional sample from France (sample no. 570) was included in the testing for comparison.

A 2007 blue mold information system for the Euro-Mediterranean Zone was established by the CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) and was published on the internet (http://lel-web.de/app/ds/lap/tabak/2007/bminfo07.pdf). This enabled the extension of our study to five strains from Bulgaria, Italy and Poland (see isolates of 2007 in Table 1).

Pathogen isolation and maintenance

The infected leaf samples obtained were used for the harvest of sporangiospores which served for DNA extraction and infection experiments. Pathogen cultivation was performed transferring sporangiospores to detached leaves of the tobacco cvs Jupiter and ITB697 (LAP Forchheim, Germany). Inoculated leaves were incubated in Petri dishes on wet filter paper and kept in a climate chamber at 16°C with a 14 h photoperiod. Infection was monitored as pathogen sporulation on the leaf surface which usually occurred after 10 to 12 days. Fresh sporangia were used for metalaxyl tests or stored at −70°C.

Single sporangiophore isolates were established in the same way by picking a single sporangiophore with a fine pair of tweezers under a dissection microscope and transferring it to a tobacco leaf disk. Leaf disks were allowed to float upside down on deionised water in a 25-well replica plate (Multimed, Kirchheim, Germany) and were cultivated in the climate chamber until sporulation occurred.

Fungicide test

Resistance to metalaxyl was tested in leaf disk experiments as described previously (Rozynek and Spring 2001). Leaf disks were inoculated on the lower leaf side with fresh sporangiospores (5,000 per leaf disk) and floated upside-down on either a water control or increasing concentrations of metalaxyl-M (solution of pure compound in water) in the range of 0.01 to 100 μg ml^{-1}. The infection rate was monitored over a

Table 1 List of isolates of *P. tabacina* used for this study

Year	No. and origin of isolates tested	Representative samples shown in Figs. 2, 3, 4 and their collection site	Collection date	Metalaxyl phenotype	PCR genotype
2002	14	No. 483 Neuried, BW, Germany	24.07.2002	Resistant	Pt_Tol +
	BW	No. 489 Oftersheim, BW, Germany	05.09.2002	Resistant	Pt_Tol +
2003	13	No. 545 Bad Krozingen, BW, Germany	02.06.2003	Resistant	Pt_Tol +
	BW, RP, BA, BB, NS, France	No. 570 Bergerac, France	Nov. 2003	Resistant	Pt_Tol +
2004	7	No. 618 Forchheim, BW, Germany	08.07.2004	Resistant	Pt_Tol +
	BW, NRW, SH	No. 621 Rheinmünster, NRW, Germany	22.07.2004	Resistant	Pt_Tol +
2005	6	No. 724 Speyer, RP, Germany	19.07.2005	Resistant	Pt_Tol +
	BW, RP, BB	No. 801 Forchheim, BW, Germany	20.09.2005	Sensitive	Pt_Sens +
2006	5	No. 862 Horkheim, BW, Germany	06.09.2006	Sensitive	Pt_Sens +
	BW	No. 863 Forchheim, BW, Germany	13.08.2006	Resistant	Pt_Tol +
2007	13	No. 921 Forchheim, BW, Germany	28.06.2007	Resistant	Pt_Tol +
	BW, RP	No. 922 Ettlingen, BW, Germany	28.06.2007	Resistant	Pt_Tol +
		No. 923 Bad Krozingen, BW, Germany	01.08.2007	Resistant	Pt_Tol +
		No. 924 Dundenheim, RP, Germany	16.07.2007	Resistant	Pt_Tol +
		No. 926 Speyer, RP, Germany	12.07.2007	Resistant	Pt_Tol +
		No. 928 Ottersheim, BW, Germany	14.07.2007	Resistant	Pt_Tol +
		No. 938 Horkheim, BW, Germany	03.09.2007	Resistant	Pt_Tol +
		No. 968 Forchheim, BW, Germany	18.09.2007	Resistant + sensitive	Pt_Tol + Pt_Sens +
	Italy	No. 920 Verona, Italy	20.06.2007	Resistant[a]	Pt_Tol +
		No. 933 Verona, Italy	20.07.2007	Sensitive	Pt_Sens +
		No. 935 Lazio, Italy	27.07.2007	n.t.	Pt_Tol +
	Bulgaria	No. 925 Plovdiv, Bulgaria	04.07.2007	Sensitive	Pt_Sens +
	Poland	No. 934 Wytyczno, Poland	20.07.2007	n.t	Pt_Sens +

The upper part of the list contains two representative strains of each season from 2002 to 2006. The lower part lists all isolates collected in 2007.

BA Bayern, *BB* Brandenburg, *BW* Baden–Wuerttemberg, *NRW* Nordrhein–Westfalen, *NS* Niedersachsen, *RP* Rheinland–Pfalz, *SH* Schleswig–Holstein, *n.t.* not tested in bioassay

[a] Metalaxyl treatment in the field did not stop sporulation

period of 14 days and was recorded as ratio of sporulating disks. Sensitive strains did not sporulate at concentrations >0.1 ppm metalaxyl-M, whereas resistant strains still sporulated at concentrations >10 ppm.

DNA isolation

Sporangiospores of the samples were disrupted in a mixer mill (9 Hz, 3 min) and DNA extracted using the Genomic DNA Purification Kit (Fermentas, Germany). The purified DNA was dissolved in 100 µl sterile water and used directly for PCR or stored at −70°C. For comparison in PCR experiments, DNA from uninfected tobacco leaf tissue (kept under the same culture conditions as infected plants) and from sporangiospores of other oomycetes (e.g. *Plasmopara halstedii*) was used. As an additional control for contamination with external DNA, a bacterial culture was established from two samples of *P. tabacina*. Sporangiospores of the samples no. 489 and no. 724 were washed in LB-medium (Lennox, Roth, Germany) containing 20 µg ml^{-1} Geneticin (G418, Roth, Germany) to control contaminating eukaryotes. The suspension was filtered through a 5 µm mesh to separate sporangiospores from bacteria. The filtered suspension was incubated at 37°C for 24 h. Bacteria were concentrated to a final concentration of 1.6×10^9 cells per ml^{-1} for DNA extraction. DNA was subjected to PCR with the fingerprint primers as listed below. The bacterial DNA samples did not give amplification products comparable to those of the oomycetes.

Primers and PCR conditions

Sequences of all primers used in this study are shown in Table 2. For PCR fingerprints the following

Table 2 Primers used in this study for differentiation of *P. tabacina*

Primer name	Sequence 5′ to 3′
$(CAC)_5$	CACCACCACCACCAC
$(GACA)_4$	GACAGACAGACAGACA
$(GTG)_5$	GTGGTGGTGGTGGTG
T3B	AGGTCGCGGGTTCGAATCC
Pt_Co_for	CGACCCACATGGACACATGC
Pt_Co_rev	CGTAGCTTGGCCACAGTGCC
Pt_Tol_for	CGTTAAGCTCTGGGACGTCG
Pt_Tol_rev	GGTACACACTCTGCGCAC
Pt_Sens_for	GGTGGTTGGCAGCAACCTG
Pt_Sens_rev	GCAGAATATCGGTCGTGCAGAG

minisatellite primers were used: $(CAC)_5$, $(GACA)_4$, $(GTG)_5$ and T3B. PCR conditions were set according to Intelmann and Spring (2002): initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 60 s, annealing 30 s at 48°C and 54°C (for $(GTG)_5$ and T3B), respectively, elongation for 75 s at 74°C, 30 cycles repetition for the last three steps. Final extension was 5 min at 74°C. PCR conditions for the specific primers Pt_Co, Pt_Tol and Pt_Sens were 95°C for 1 min, (94°C for 30 s, 65°C for 40 s, 72°C for 1 min)—32 times, 72°C for 4 min. Amplification products were separated on 1% agarose gels or stored at −20°C for subsequent experiments.

Gels were stained with ethidium bromide (0.5 μg ml^{-1}) for 30 min and visualised with UV light on a transilluminator. Amplified fragments from different samples were documented and compared using EasyWin-Software (HeroLab, Germany) and fragment size was determined on the basis of a DNA ladder (GeneRuler 1 kb DNA Ladder or 1 kb DNA Ladder Plus, Fermentas, Germany).

Additional sample analysis was performed via capillary electrophoresis on a 2100 Bioanalyzer (Agilent, Böblingen, Germany) following the protocol of the manufacturer.

Phenotype-specific primers

Phenotype-specific primers Pt_Co, Pt_Tol and Pt_Sens (for sequences, see Table 2) were designed on the basis of sequence data from PCR products (marked with arrows in Figs. 2 and 3) found in all samples of *P. tabacina* and in either metalaxyl-resistant or sensitive strains, respectively. The amplification products obtained in PCR with the constructed primer pairs Pt_Co, Pt_Tol and Pt_Sens were 1,083, 598 and 434 bp in length. Primer specificity (Fig. 1) was tested with the host plant *Nicotiana tabacum* (kept under the same culture conditions as infected plants) and with other oomycete species. Using the primers Pt_Co, Pt_Tol and Pt_Sens in PCR with DNA from healthy tobacco leaf tissue and from sporangiospores of *Peronospora parasitica*, *Peronospora chenopodii* and *Plasmopara halstedii* did not give any amplification. Control experiments revealing the functionality of the DNA samples were performed with *RuBisCO*-specific primers for tobacco DNA and *cytochrome-c-oxidase*-specific primers for oomycete DNA (Fig. 1a). Amplification products with primers Pt_Co, Pt_Tol and Pt_Sens were exclusively obtained in samples containing DNA of *P. tabacina*.

Primer sensitivity was tested using sonication-treated sporangiophores with sporangiospores of *P. tabacina* as the template. Single *P. tabacina* sporangiophores were picked with a pair of tweezers from the tobacco leaf surface, placed into 12.5 μl of sterile water, sonicated for 2 min (Bransonic B-1200 E2, Branson Ultrasonics, Danbury, USA) and used directly for PCR. We were able to detect one single sporangiophore (carrying 20–30 sporangiospores) with each of the three primer pairs (Fig. 1b). The sonication as described by Wiglesworth et al. (1994) turned out to be an appropriate method for DNA extraction from minute sample amounts, despite the risk of DNA fragmentation as discussed by Caiazzo et al. (2006).

Cloning and DNA sequencing

Three selected PCR amplicons, one uniformly present and two suitable for differentiation of the two pathogen phenotypes (marked with arrows in Figs. 2 and 3), were excised from the agarose gel and gel-extracted using the PerfectPrep Gel Cleanup Kit (Eppendorf, Germany) according to the manufacturer's protocol. The isolated DNA fragments were then cloned into a sequencing plasmid using the GeneJet PCR Cloning Kit (Fermentas, Germany) and multiplied in *Escherichia coli*. Plasmids were prepared from selected positive bacterial clones with the GeneJet Plasmid Miniprep Kit (Fermentas, Germany) and subjected to nucleotide sequencing for subsequent database comparison.

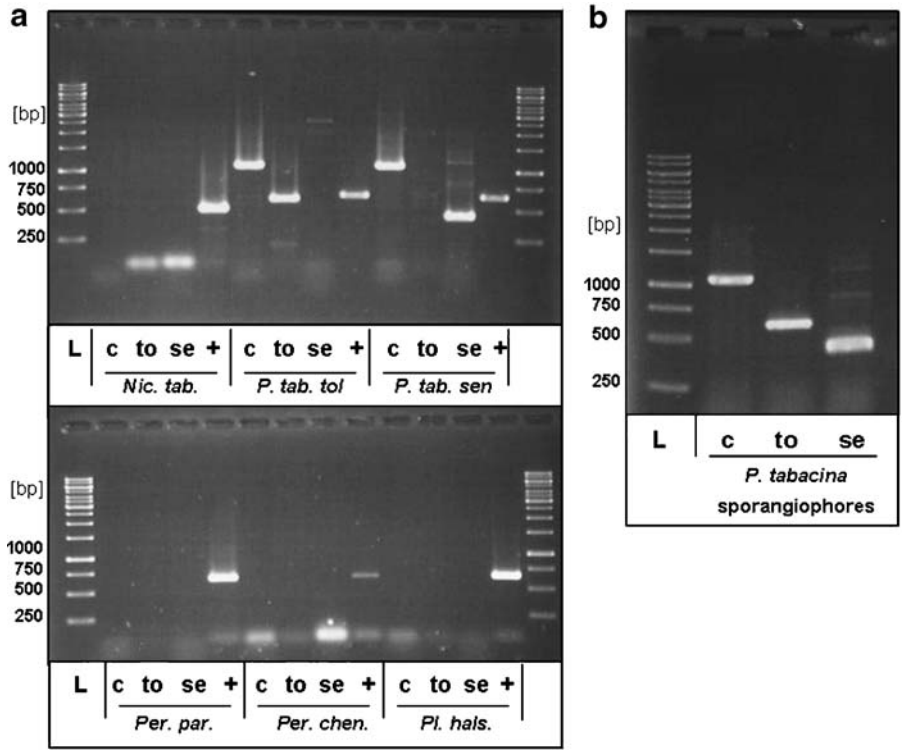

Fig. 1 Specificity (**a**) and sensitivity (**b**) of the primers. *c* = primers Pt_Co; *to* = primers Pt_Tol; *se* = primers Pt_Sens; + = positive control (*RuBisCO*-primers for *N. tabacum*, *cytochrome-c-oxidase*-primers for oomycetes). **a** Primer specificity was tested with DNA from *Nicotiana tabacum* (*Nic. tab.*), *P. tabacina* metalaxyl-tolerant (*P. tab tol*), *P. tabacina* metalaxyl-sensitive (*P. tab sen*), *Peronospora parasitica* (*Per. par.*), *Peronospora chenopodii* (*Per. chen.*) and *Plasmopara halstedii* (*Pl. hal.*). **b** Amplification products of the primer pairs Pt_Co (*lane c*), Pt_Tol (*lane to*) and Pt_Sens (*lane se*) when tested with single-sporangiophore DNA from the field isolate no. 968 (a mixed phenotype collected 18.9.2007, Forchheim, Germany)

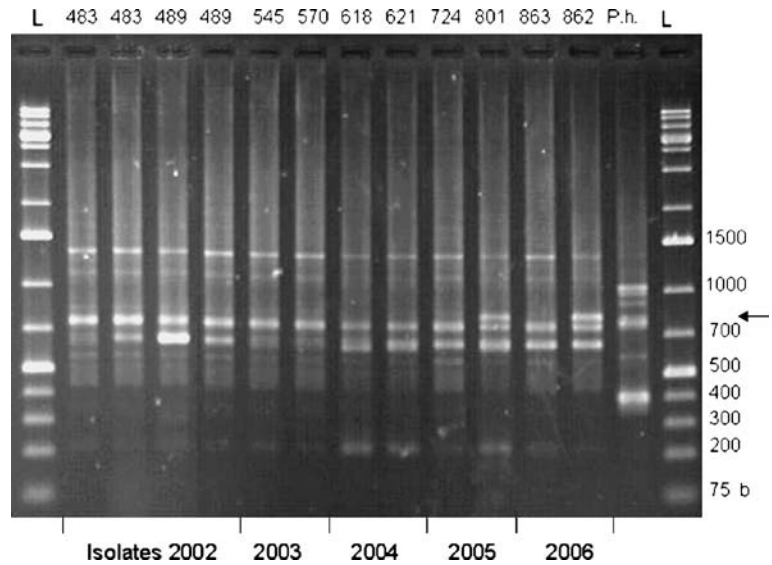

Fig. 2 PCR-fingerprints with primer T3B on agarose gel. Lanes: DNA-ladder (*L*), metalaxyl-resistant strains (no. 483a/b, 489a/b, 545, 570, 618, 621, 724, 863) from 2002 to 2006, metalaxyl-sensitive strains (no. 801, 862) of 2005 and 2006, *Plasmopara halstedii* (*P.h.*), DNA ladder. *Arrow* marks position of the additional amplicon in the two sensitive strains

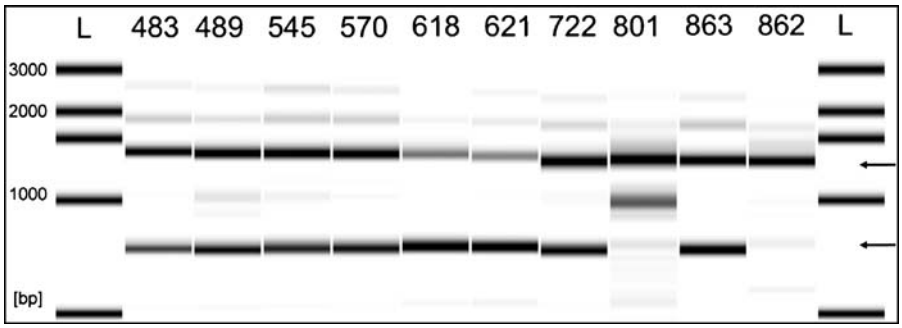

Fig. 3 Comparison of $(GACA)_4$-PCR amplification products in high resolution capillary electrophoresis. Lanes: metalaxyl-resistant strains (no. 483, 489, 545, 570, 618, 621, 722, 863) from 2002 to 2006, metalaxyl-sensitive strains (no. 801, 862) of 2005 and 2006. The *upper arrow* marks the uniformly present amplicon, the *lower arrow* marks the amplicon typical for the tolerant strains

Results

PCR fingerprinting of metalaxyl-resistant and sensitive strains

A total of 44 strains of tobacco blue mold collected from 2002 to 2006 from different regions of Germany and one strain from France were subjected to PCR fingerprinting with SSR primers for investigation of genetic diversity. DNA samples from *P. tabacina* resulted in characteristic fingerprints which clearly differed from patterns of other oomycetes (e.g. *P. halstedii*; Fig. 2). Patterns obtained with DNA from the host plant *N. tabacum* showed no similarity and samples from the bacterial culture established from *P. tabacina* sporangiospores gave no amplification products (data not shown). The latter control was performed, because Sukno et al. (2002a) had reported contamination problems with bacterial DNA in RAPD-based population studies on tobacco blue mold isolates in the USA.

Amplification patterns of sporangial DNA with primers $(CAC)_5$ or $(GTG)_5$ resulted in identical patterns for all *P. tabacina* samples (data not shown), whereas the minisatellite primer T3B produced an additional amplicon of 805 bp in length only in the two samples no. 801 and no. 862 derived from metalaxyl-sensitive isolates of the pathogen (Fig. 2). In addition, fingerprints with primer $(GACA)_4$ showed a prominent product at 830 bp only in resistant isolates (Fig. 3, lower arrow). A faint product of similar size occurred in the sensitive strains, but high resolution capillary electrophoresis revealed a slightly bigger size (844 bp). Hence it was not identical with the 830 bp fragment from the resistant isolates.

Two amplicons, 1,271 and 830 bp in length, from the metalaxyl-resistant strain no. 483 and the 805 bp amplicon from the sensitive strain no. 801 were subjected to DNA sequencing (GenBank accession numbers: EU721730, EU721731, EU721732). Comparison (BLAST) with DNA databases did not reveal significant similarity to other published DNA sequences.

Screening of recent blue mold isolates from European countries

Ten out of the 13 tobacco blue mold field isolates from 2007 could be cultivated and were subjected to metalaxyl leaf disk bioassays. The isolates from Bulgaria, Poland and one out of three accessions from Italy tested sensitive to a fungicide concentration of 1 μg ml^{-1} (Table 1). In contrast, all six isolates from Germany were resistant in the bioassay (tested up to a concentration of 100 μg ml^{-1}). One field isolate from Forchheim, Germany (no. 968) showed reduced sporulation at metalaxyl concentrations above 1 ppm, thus indicating a putative mixture of the sensitive and the resistant phenotype.

DNA samples of all 2007 isolates together with the above mentioned isolates from 2002 to 2006 were subjected to PCR using the primers Pt_Co, Pt_Tol and Pt_Sens. With primers Pt_Co, all samples gave the amplification product of 1,083 bp in length, specific for *P. tabacina*. All isolates resistant in the bioassay gave the expected amplicon of 598 bp in PCR with primers Pt_Tol. No amplification of this segment occurred in isolates which had been tested sensitive in

the metalaxyl bioassay (no. 862, no. 925, no. 933, no. 934). Vice-versa, PCR with primers Pt_Sens identified the sensitive isolates with a product of 434 bp (Fig. 4).

Interestingly, DNA from the sporangiospore sample of field isolate no. 968 (Forchheim, Germany) gave amplification products with both primers Pt_Tol and Pt_Sens, although it had sporulated at high concentrations of metalaxyl in the bioassay. When PCR experiments were repeated with DNA from mechanically collected single sporangiophores, the amplicons of different samples either indicated the fungicide-resistant phenotype or the fungicide-sensitive phenotype (Fig. 1b). This confirmed the presence of mixed populations of both phenotypes in the original field sample and explained the reduced sporulation rate in the bioassays at high metalaxyl concentrations. Employing single-sporangiospore infections under metalaxyl selection, we meanwhile succeeded to establish phenotypic and genotypic homogeneous sensitive and resistant strains (no. 968s and no. 968r) of the 2007 Forchheim field isolate.

Discussion

Detection of pathogens with specific molecular markers was shown in the early 1990s for animal (Dalrymple 1990; Miyakawa et al. 1992) and few plant pathogens (Goodwin et al. 1990; Kuske et al. 1991; Nazar et al. 1991). The technique was successfully established for further plant pathogens in the following years (Wiglesworth et al. 1994; Goodwin et al. 1995; Sreenivasaprasad et al. 1996; Tooley et al. 1997; Lindqvist et al. 1998; Bakan et al. 2002; Casimiro et al. 2004). PCR-based diagnosis of tobacco blue mold infection has already been described in 1994 (Wiglesworth et al. 1994) and has been renewed recently using ITS DNA (Ristaino et al. 2007). The aim of these studies was either to detect early infections (Caiazzo et al. 2006) or differentiation from other tobacco pathogens (Ristaino et al. 2007). The results presented here, show for the first time, that metalaxyl-sensitive and resistant fungicide-phenotypes of *P. tabacina* can be determined by means of specific PCR-primers. This accelerates the identification of metalaxyl-resistant strains to few hours, while bioassays require more than a week (Keil and Spring 2005). In addition, bioassays are often impeded when isolates cannot be secured before the farmers have tried to save their crop by applying alternative fungicides to kill the pathogen. Such a case occurred in our study with one of the two isolates from Verona, Italy. While the accession no. 933 was identified as sensitive in PCR as well as in the bioassay, isolate no. 920 could not be cultivated and tested in the bioassay, but its DNA showed the resistant PCR-type. The material we received from this isolate had been treated with metalaxyl in the field, but continued to spread until dimetomorph had been applied to prevent further propagation (M. Zorzi, personal communication). The results of the PCR test with DNA of no. 920 coincided with the metalaxyl-resistant phenotype of the isolate shown in the field. This underlines the capacity of the PCR test to assign even unviable *P. tabacina* material to the corresponding phenotype.

In all blue mold samples tested so far, the occurrence of the two differentiating fragments coincided with the metalaxyl-phenotype of the pathogen isolates. However, this does not necessitate that

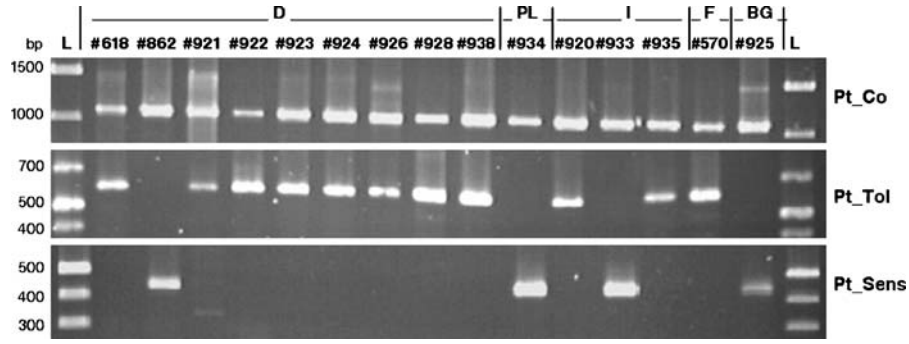

Fig. 4 PCR products of DNA samples from *P. tabacina* isolates collected in Germany (*D*), Poland (*PL*), Italy (*I*), France (*F*) and Bulgaria (*BG*) obtained with the primer pair Pt_Co (specific for *P. tabacina*), Pt_Tol (specific for the metalaxyl-resistant phenotype) and Pt_Sens (specific for the metalaxyl-sensitive phenotype)

functional information related to resistance is located on the DNA fragments used for differentiation. Our database comparison did not reveal any significant similarity of the differentiating fragments to published DNA sequences and does not imply any functional information. The function of the test depends on the genetic variability of the pathogen and so far only two genotypes were detected with PCR fingerprints in more than 50 samples from five European countries. Additional sequencing of the *cox2*-region of mitochondrial DNA from representative samples of our collection revealed nearly identical sequences, independent of the geographic origin and fungicide phenotype of the isolates (data not shown). This underlines the overall high similarity in DNA fingerprints of the blue mold accessions we had tested so far. Our results from European isolates of the pathogen coincide with the genetic uniformity found in over 30 North American populations of tobacco blue mold screened by Sukno et al. (2002b).

The metalaxyl-resistant phenotype of *P. tabacina* in Europe was first observed in 2002 in German isolates (Krauthausen et al. 2003) and was recorded continuously in this area since that time (Keil and Spring 2005; Spring et al. 2007). This inevitably raises questions on the origin, the distribution and hibernation of this phenotype. The occurrence of metalaxyl-resistant strains in other European countries has not yet been reported, but our results, although based on only a few accessions, undoubtedly show that resistant strains exist, at least in France and Italy.

The PCR-based screening of the two European genotypes could be used in coming years to trace the sites of hibernation and the seasonal spread of the two populations. The CORESTA blue mold information system has listed for 2007 first incidences in Iran and Tunisia in April, while early outbreaks in Europe were registered in June almost simultaneously for Bulgaria, France, Italy and Germany (http://lel-web.de/app/ds/lap/tabak/2007/bminfo07.pdf). This may indicate a long-distance dispersal of sporangia from south to north, but other possibilities for wintering (Spring et al. 2007) cannot be excluded, unless a broader screening includes sampling of the North African and Asian sources.

Acknowledgements We are indebted to A. Billenkamp and A. Lengersdorf, Landesanstalt für Pflanzenbau Forchheim, W. Schwär and E. Uckele, Beratungsdienst Tabakbau, for providing isolates of tobacco blue mold. We would like to thank Syngenta Agro GmbH Germany for partial funding of the monitoring. Technical support from Agilent Technologies GmbH Germany in capillary electrophoresis is gratefully acknowledged.

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