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Dedicated

To

My Parents

Jürgen Derpmann

Characterization of Fitness Parameters and Population Dynamics of *Botrytis cinerea* for the Development of Fungicide Resistance Management Strategies in Grapevine

Gray mold caused by the fungus *Botrytis cinerea* is an economically important disease in grapevine. The pathogen has a high tendency to become resistant to frequently applied systemic fungicides. Only a few years after introduction of the fungicide class of benzimidazoles (MBC), resistant strains appeared frequently in European vineyards. Since the discontinuation of the use of benzimidazoles to control *B. cinerea* in 1975, the frequency of MBC-resistant strains decreased significantly. In the present study, the influence of fungicide resistance management strategies on the population dynamics of *B. cinerea* isolates resistant to fungicides was investigated in a three year field trial at three sites near Bordeaux. The tested strategies were mixture, alternation and annual alternation of thiophanate-methyl (TM) and mepanipyrim (MP). Strategies were compared to the solo application of TM and conventional fungicide treatments, where no TM was applied. Frequencies of fungicide-resistant isolates were determined in monitoring procedures conducted prior and subsequent to fungicide applications.

In all three years, spray programs including TM resulted in significantly higher frequencies of TM-resistant isolates (BenR1 phenotype) compared to those detected in conventionally treated plots. In the first year, all strategies tested led to similar BenR1 isolate frequencies compared to the solo application of TM (23%). In the second year, solo application of MP as part of the annual alternation resulted in significantly lower BenR1 isolate frequencies (16%) compared to spray programs including TM (39%). However, at the end of the study no significant differences in BenR1 isolate frequencies were detected between the strategies tested and the solo application of TM (47%). Different single nucleotide polymorphisms (SNP) in the β-tubulin gene confer resistance to MBC fungicides. Allele-specific polymerase chain reactions (as-PCR) as well as EvaGreen® real-time as-qPCR showed a high correlation between the BenR1 isolate and E198A allele frequency. Over the winter period 2009/10, a decrease of BenR1 isolate frequency was detected (-12%), which points to difference in fitness of MBC-sensitive (BenS) and BenR1 isolates. Therefore, various fitness parameters were tested comparing ten BenS with ten BenR1 isolates. At favourable conditions, no significant differences were detected between the two sensitivity groups. At unfavourable conditions, mycelium growth, lesion size and spore production of BenS isolates were significantly higher than those of BenR1 isolates. In a competitive assay on leaf discs as well as on grapevine plants a decrease in BenR1 conidia frequency of 7 % per generation was observed.

Fitness costs associated with resistance could have reduced the frequency of BenR1 isolates within the primary inoculum, when the fungus was confronted with unfavourable development conditions. If no MBC fungicides are applied during the season, then the short-distance dispersal of BenS conidia from the infected flowers and other sources leads to a decrease of the resistant fraction in the consecutive berry-associated population, as well. Over time, the difference in fitness leads to a linear decrease resulting in the low frequencies of BenR1 isolates as observed in German and French vineyards nowadays. A registration of the mixture of thiophanate-methyl with mepanipyrim would contribute to the diversity of modes of action controlling *B. cinerea*. Due to the emergence and development of resistance to ‘single-site’ fungicides of all chemical classes, a resistance management strategy combining all tools available in an integrated pest management will be needed. Thus, a registration of the mixture of thiophanate-methyl with mepanipyrim will lead to a prolongation of the lifespan of newly introduced active ingredients to control *B. cinerea* in grapevine in the future.

Jürgen Derpmann

Untersuchungen zur Fitness und Populationsdynamik von *Botrytis cinerea* zur Entwicklung einer Fungizid-Resistenzmanagement-Strategie im Weinbau

Der Erreger des Grauschimmels *Botrytis cinerea* verursacht hohen wirtschaftlichen Schaden durch Qualitätseinbußen und Ertragsverluste im Weinbau. Das Pathogen verfügt über eine hohe genetische Diversität, wodurch bei intensivem Fungizid-Einsatz resistente Stämme auftraten. Dies führte im Falle der 1971 eingeführten Benzimidazole (MBC) nach wenigen Jahren zu dem Entzug der Genehmigung für den Weinbau in Deutschland. Über 30 Jahre später wurde eine Abnahme des Anteils MBC-resistenter Isolate auf unter 10% festgestellt. In der aktuellen Studie wurde der Einfluss von Antiresistenz-Strategien auf die Entwicklung des Anteils Fungizid-resistenter *B. cinerea* Isolate im Rahmen eines dreijährigen Feldversuches an drei Standorten in der Nähe von Bordeaux geprüft. Als Strategien wurden der jährliche Wirkstoffwechsel, die Mischung und die Alternierung von Thiophanate-Methyl (TM) und Mepanipyrim (MP) geprüft. Diese Strategien wurden mit der Soloanwendung von TM und konventionellen Spritzfolgen, in denen kein TM angewendet wurde, verglichen.

In allen drei Jahren führten Spritzfolgen mit TM im Vergleich zu den konventionell gespritzten Flächen zu signifikant höheren Anteilen TM-resistenter Isolate (BenR1). Im ersten Jahr führten alle geprüften Strategien im Vergleich zu der Soloapplikation von TM zu ähnlichen Anteilen von BenR1 Isolaten (23%). Im zweiten Jahr führte die Soloapplikation von MP im Rahmen des jährlichen Wirkstoffwechsels zu signifikant niedrigeren Anteilen von BenR1 Isolaten (16%) im Vergleich zu den anderen Strategien (39%). Am Ende der Studie zeigten sich nach Anwendung der geprüften Strategien und der Soloapplikation von TM ähnlich hohe Anteile von BenR1 Isolaten (47%). Resistenzen gegenüber MBC-Fungiziden werden durch verschiedene Punktmutationen auf dem β-Tubulin-Gen verursacht. Diese Mutationen wurden mittels allel-spezifischer Polymerase-Kettenreaktionen (as-PCR) und EvaGreen® real-time as-PCR nachgewiesen. Dabei zeigte sich eine enge Korrelation zwischen dem Auftreten von BenR1 Isolaten und dem Nachweis der E198A-Mutation. Im Anschluss an die Winterperiode 2009/10 wurde eine Abnahme des Anteils von BenR1 Isolaten festgestellt (-12%). Daher wurden Fitnessparameter von zehn BenS und zehn BenR1 Isolaten miteinander verglichen. Unter günstigen Wachstumsbedingungen zeigten sich keine Unterschiede zwischen den Sensitivitätsgruppen. Unter ungünstigen Wachstumsbedingungen wurden signifikant höhere Myzelwachstumsraten, Läsionsdurchmesser und Sporenproduktion von BenS im Vergleich zu BenR1 Isolaten gemessen. In kompetitiven Untersuchungen auf Blattscheiben sowie Weinreben wurde eine Abnahme des Anteils von BenR1 Konidien von 7% je Generation gemessen.

Dieser Fitnessunterschied könnte den Anteil von BenR1 Isolaten innerhalb des Primärinokulums, wenn der Pilz mit ungünstigen Entwicklungsbedingungen konfrontiert wird, reduziert haben. Wenn keine Benzimidazole appliziert werden, dann würde die Verbreitung der MBC-sensitiven Isolate von den infizierten Blüten aus zu einer Abnahme des Anteils von BenR1 Isolaten in der anschließend die Beeren infizierenden Population führen. Über einen längeren Zeitraum betrachtet würde dies zu einer linearen Abnahme des Anteils der BenR1 Isolate führen bis hin zu den niedrigen Anteilen, die derzeit in deutschen und französischen Weinbergen beobachtet werden. Eine Zulassung von Thiophanate-Methyl in Mischung mit Mepanipyrim kann nur durch genau definierte Empfehlungen für das Resistenzmanagement erfolgen. Dadurch würde die Diversität der Wirkstoffe erweitert und eine Verlängerung des Nutzungszeitraums von neu entwickelten Wirkstoffen zur Bekämpfung von *B. cinerea* im Weinbau in der Zukunft ermöglicht werden.

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ABBREVIATIONS

%	Percent
°Oechsle	Degree Oechsle
°C	Degree Celsius
µg	microgram
µL	microliter
AniR	Phenotype, which shows a reduced sensitivity to anilinopyrimidines
AniR1	Phenotype, which shows a resistance to anilinopyrimidines
ATP	Adenosine-5'-TriPhosphate
BBCH	Scale used to identify the phenological development stages (BBCH officially stands for "Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie")
BenR	Phenotype, which shows a resistance to benzimidazoles
BenR1	Phenotype, which shows a resistance to benzimidazoles, but not to N-phenyl-carbamates
BenR2	Phenotype, which shows a resistance to benzimidazoles and N-phenyl-carbamates
BSM	*Botrytis* Selective Medium
CAA	Carboxylic Acid Amides
cm	Centimeter
CZA	Czapek-Dox-Agar
DMI	DeMethylation Inhibitors
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyriboNucleoside TriphosPhate
E198A	Mutation at codon 198, which leads to substitution of glutamatic acid by alanine
E198K	Mutation at codon 198, which leads to which leads to substitution of glutamic acid by lysine
E198V	Mutation at codon 198, which leads to substitution of glutamatic acid by valine
EC	European Commission
EDTA	EthyleneDiamineTetraacetic Acid
EPPO	European and mediterranean Plant Protection Organization
et al.	*et alii*
EU	European Union
F200Y	Mutation at the codon 200 tyrosine replaces phenylalanine
FGA	Fructose Gelatin Agar
FRAC	Fungicide Resistance Action Committee
g	Gram
GPS	Global Positioning System
ha	Hectare
HydR1	Phenotype, which shows a resistance to fenhexamid
IDW	Inverse Distance Weighting
INRA	Institut national de la recherche agronomique
IUPAC	International Union of Pure and Applied Chemistry
kPa	Kilopascal
kg	Kilogram
km	Kilometer
L	Liter
LOD	Level of detection
LOQ	Level of quantification

M	Mol
m²	square meter
mA	mili Ampere
MBC	Methyl Benzimidazole Carbamates
MDR	Multi Drug Resistance
MFS	Major Facilitator Superfamily transporters
m	meter
min	minute
mL	mili liter
mm	mili meter
mM	mili Mol
ng	Nanogram
NPC	N-Phenyl-Carbamate
PA	Phenylamides
PCNB	Pentachloronitrobenzene
PCR	Polymerase Chain Reaction
PDA	Potato-Dextrose-Agar
PDB	Potato-Dextrose-Broth (PDB)
ppm	Parts per million
QiI	Quinone inside Inhibitor
QoI	Quinone outside Inhibitor
qPCR	quantitative real-time Polymerase Chain Reaction
RF	Resistance factor
RNA	Ribonucleic acid
RSD	Relative Standard Deviation
rpm	Rounds per minute
SADIE	Spatial Analysis by Distance IndicEs
SBI	Sterol Biosynthesis Inhibitor
SC	Suspension concentrate
SD	Standard Deviation
SDHI	Succinate DeHydrogenase Inhibitor
SDW	Sterile Distilled Water
SE	Standard Error of the mean
SNP	Single Nucleotide Polymorphisms
spp.	*species pluralis*
TAE	Tris-Acetate-EDTA
U.S.	United States of America (U.S.A.)
V	Volt
v/v	Volume to volume
w/v	Weight to Volume
WA	Water Agar
WGS-1984	World Geodetic System 1984

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1 INTRODUCTION

Botrytis cinerea Pers.: Fr. is the anamorph form of the ascomycete *Botryotinia fuckeliana* (de Bary) Whetzel. It is a perthotrophic, facultative fungus attacking more than 200 crop hosts worldwide, particular on economically significant plants like tomato, strawberry, onion and grapevine (WILLIAMSON *et al.* 2007). *B. cinerea* causes soft rotting of aerial plant parts and rotting of transported and stored fruits leading to prolific conidiophores bearing macroconidia typical of the gray mold disease (WHETZEL, 1945).

The fungus survives the winter saprophytically as mycelium or sclerotia on plant debris. The epidemic starts in the spring by formation of conidiophores, which produce macroconidia as short-lived propagules during the season (HOLZ, COERTZE and WILLIAMSON, 2004). Macroconidia are spread by wind, rain and insects such as the vinegar fly *Drosophila melanogaster* and the crossed grapevine moth *Lobesia botrana* (LOUIS *et al.* 1996; FITT et al. 1985, FERMAUD and MENN, 1989). Also, humans or other vertebrates can transport *B. cinerea* inoculum, so that the fungus is present around the world from the cool temperate zones of Alaska to subtropical areas (ELAD *et al.* 2004). If the fungus is subjected to adverse conditions, then microconidia will be produced by mature hyphae, sclerotia and germ tubes of macroconidia (JARVIS, 1962). Ascospores produced in apothecia of the teleomorph *Botryotinia fuckeliana* are rarely observed in the field (LORBEER, 1980). Therefore, the name of the anamorphic stage *Botrytis cinerea* is used commonly.

An overcast sky and temperatures of 18 to 23°C are optimal for conidial production, dispersal and germination of conidia. In addition, appreciable mycelial growth occurs at temperatures of 0 to 10°C. For germination a high relative humidity of about 90 % or free water is needed (BLAKEMAN, 1980). Additionally, the presence of endogenous nutrients like saccharides is required for germination and pathogenicity (PHILLIPS, MARGOSAN and MACKEY, 1987).

After germination on the plant surface, the fungus has various ways to penetrate the host tissue. *B. cinerea* can penetrate directly through wounds caused by biotic (e.g. feeding) or abiotic factors (e.g. hail). Also, it can penetrate through natural openings like stomata or lenticels (FOURIE and HOLZ, 1995). Additionally, *B. cinerea* is able to penetrate directly through intact host tissue by formation of pseudo-appressoria (JENKINSON *et al.* 2004). Subsequent to successful penetration, *B. cinerea* kills the host cells by secretion of phytotoxic metabolites, such as botrydial, host-selective toxins and by induction of oxidative burst during cuticle penetration (KAN, 2006). This causes lesions of the host tissue, on which prolific grey conidiophores are formed, which produce the secondary inoculum and lead to further spread within the field (HOLZ, COERTZE and WILLIAMSON, 2004).

In grapevine, *Vitis vinifera* L. the susceptibility of plant organs changes in the course of the vegetation period. *Botrytis cinerea* can infect leaves, buds, flowers, shoots and especially ripening grapes. In spring, primary inoculum is produced by sclerotia in the soil, on fruit mummies, on infected pieces of cane or herbicide damaged weeds (Figure 1-1). At that time, flowers of grapevine are highly susceptible to *B. cinerea* infection (JERSCH *et al.* 1989). The fungus can penetrate through the stigma and enters the ovule by systemic hyphal growth. Additionally, it can enter through wounds caused by the drop of senescent petals at the end of flowering. After latent infection of the flower, the fungus survives the summer in the stylar tissue or saprophytic within aborted flower tissue (over-summering, KELLER *et al.* 2003). At berry ripening, a decrease in thickness of the cuticle, an increase in sugar content and a reduction in organic acids involved in plant defense of the berry are observed. Therefore, susceptibility of berries increases and latent infections lead to visible symptoms (ELMER and MICHAILIDES, 2004). These early infections, starting at sugar contents below 50° Oechsle, lead to the formation of the sour rot. Massive quantitative losses are caused by destruction of the rachis structure, so that the entire cluster falls to the ground at ripening (SCHRUFT and VOGT, 2000). Qualitative losses are caused by reduction of sugar content due to discontinuation of the ripening process. In red wines, loss of color due to degradation of anthocyanin reduces the quality (BAUER, 2002).

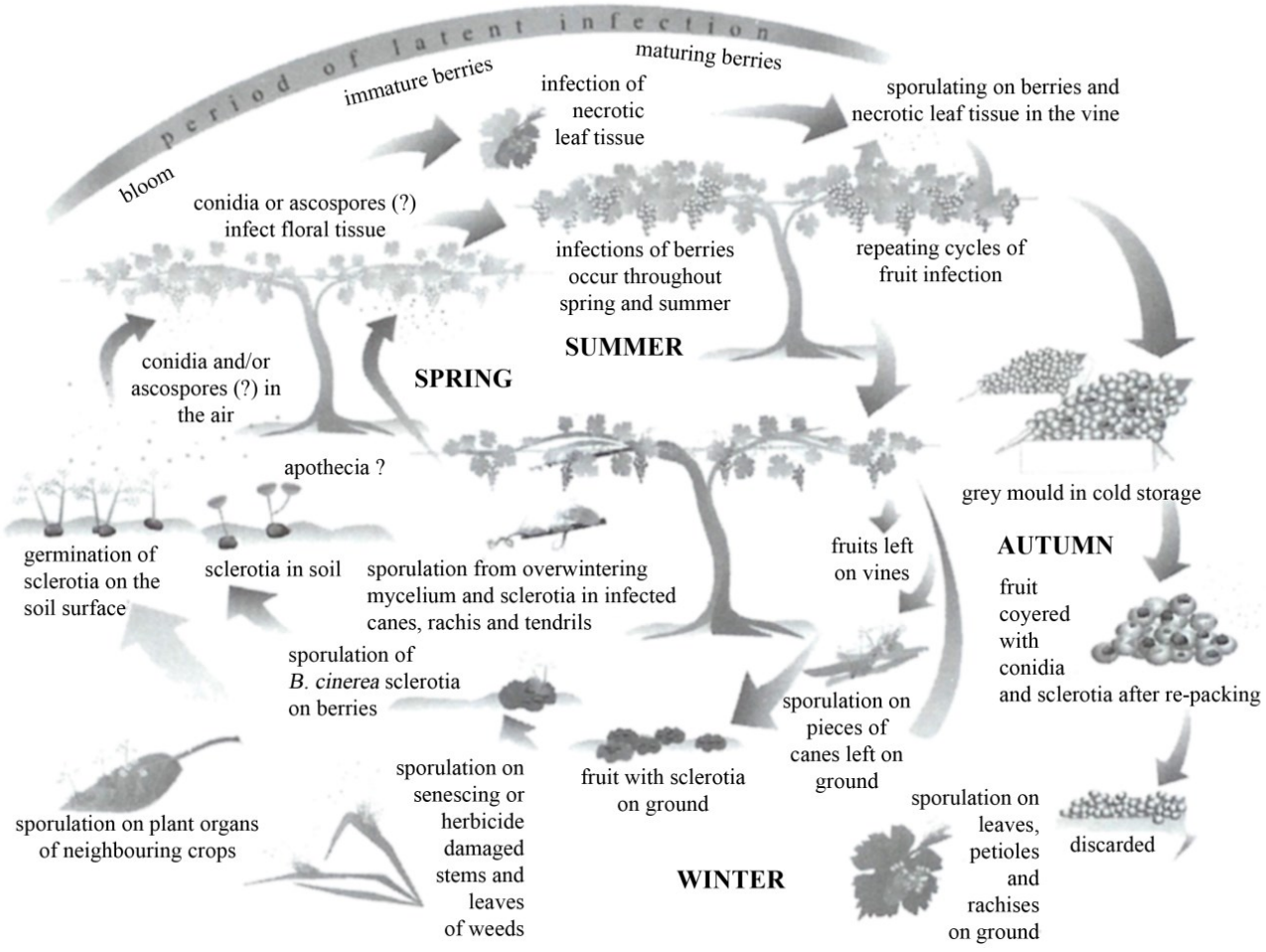

Figure 1-1 Proposed life cycle of *Botrytis cinerea* and disease cycle of grey mold in vineyards according to ELMER and MICHAILIDES (2004).

A late attack of *B. cinerea*, at sugar contents of about 70° Oechsle, results in increased sugar content due to higher transpiration through the perforated cell wall. In white grapevine cultivars these infections can lead to the production of noble rot wines, e.g. 'Trockenbeerenauslese' in Germany and 'Sauternes' in France (ROSSLENBROICH and STUEBLER, 2000).

In viticulture, with a cultivation area of about 8 million hectares worldwide, *Botrytis* infections lead to annual losses of about 2 billion U.S. dollars (VIVIER and PRETORIUS, 2002). Growers use different strategies to reduce the infestation of their plants with *B. cinerea*.

The choice of variety is one of the most important factors in the control of *B. cinerea*. Resistance of mature berries is mostly due to morphological characteristics such as an increased cuticle thickness or a reduced number of pores and lenticels on the berry surface (GABLER *et al.* 2003). However, such a breeding strategy while maintaining the qualitative and quantitative characteristics takes a lot of resources. 28 years were required to breed a new variety (cv. Regent), which is resistant to *Botrytis cinerea*, downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*). This cultivar is mainly used in organic viticulture (NAIR and HILL, 1992). Customized fertilization (especially nitrogen), a consistent weed management and cultural practices such as pruning type and cutting of leafs reduce *Botrytis* infestation. Additionally, reducing the number of flowers per panicle, application of potassium water glass at flowering or grape partitioning at bunch closure can be applied to reduce cluster compactness (VAIL and MAROIS, 1992). All these measures increase exposure to light and air circulation leading to an accelerated drying of the plant. Thus, the fungus has unfavourable conditions for germination and development (STEEL, 2001, PERCIVAL et al. 1993). Another method of reducing *Botrytis* infestation is the mechanical removal of floral debris from fruit clusters. Thus, the basis of the saprophytic over-summering phase of the fungus is withdrawn (WOLF et al. 1997).

In recent decades several promising biological control agents were tested to prevent or delay *B. cinerea* infection. These include antagonistic fungi of the genera *Trichoderma*, *Gliocladium* and *Ulocladium*, bacteria of the genera *Bacillus* and *Pseudomonas*, as well as various yeasts as summarized by ELAD and STEWART, 2004). However, control of *B. cinerea* under field conditions has been inconsistent when compared with that observed under glasshouse or laboratory conditions (ELMER and REGLINSKI, 2006).

The most effective way to counter a *Botrytis cinerea* attack is the use of fungicides. This has resulted in a global market share of fungicides used against *Botrytis* spp. of 15 – 25 million U.S. dollars per year (ELAD *et al.* 2004). In the past, up to eight applications were performed per year. Based on research conducted in the last decades, knowledge about the biology of the pathogen was used to decrease the number of applications to two to four sprays (BROOME *et al.* 1995). Applications at the end of flowering (BBCH 68) prevent the colonization of flowers, thus reducing the latent infections within bunches of berries (KAST, 2007). The application just before bunch closure (BBCH 77) is the

final possibility to apply the active ingredient within the cluster on the rachis. This application is especially important for compact red grapevine cultivars (KAST, 2007). The last possible application is at beginning of ripening (veraison, BBCH 83). It is dependent on the retention period of the active ingredient(s), usually three to four weeks prior to harvest. This application should protect the berries with high fungicide application rates from secondary attack by wind spread conidia. However, this time of application results in high residual fungicide concentrations in the products consumed by humans (KELLER *et al.* 2003). Late treatments can also have negative effects. Instead of colonization by *B. cinerea* such treatments can enhance the establishment of other rot pathogens, for instance *Penicillium* spp. Such pathogens can affect the quality of the wine more negatively compared to *B cinerea* due to the production of mycotoxins (SCHWENK *et al.* 1989).

Chemical control of *B. cinerea* can be achieved by several chemical classes of fungicides. They can be classified by their biochemical modes of action. The oldest ones are non-systemic 'multi-site' fungicides, which have more than one target in the fungus. They can be divided into three main chemical classes. There are dithiocarbamates, such as thiram, maneb and mancozeb, chloromethyl-mercaptan derivatives, such as captan, folpet, and phthalonitriles, such as chlorothalonil. However, their practical use is restricted, because they can delay fermentation in wine production. Their preventive activity is mainly due to the suppression of spore germination, which is related to the inhibition of several thiol-containing enzymes (LEROUX *et al.* 2002).

Modern anti-fungal compounds are mainly 'single site' fungicides, which interfere with a specific target in the fungus, thus inhibiting its growth. An overview of the chemical classes used to control *B. cinerea* is given in Table 1-1.

Using chemical control it has to be noted, that *Botrytis cinerea* has a high tendency to become resistant to frequently applied systemic fungicides. It is a high risk pathogen due to a high number of generations per year, a high number of progeny, a wide host range and a high genetic variability within a population (BRENT and HOLLOMON, 2007).

Due to the qualitative character of benzimidazole resistance, isolates highly resistant to benomyl were observed (BenR1: resistance level > 1000, LEROUX and CLERJEAU, 1985). This phenotype resistant to benzimidazoles was widespread in German vineyards after three years of benomyl application. A loss of control was observed under field conditions (SCHUEPP and LAUBER, 1977; SMITH, 1988). Therefore, the registration of benzimidazoles for control of *B. cinerea* was not prolonged in Germany and other countries in 1974 (SCHRUFT, 2001; GEORGOPOULOS and SKYLAKAKIS, 1986). BenR1 strains are sensitive to N-phenyl-carbamates, like diethofencarb (ELAD *et al.* 1988). This negative cross-resistance pattern led to the introduction of the mixture diethofencarb and carbendazim in the late 1980s (FUJIMURA *et al.* 1990). A view years after application, isolates resistant to diethofencarb as well as carbendazim (resistance level: 30 – 100, BenR2) were detected (LEROUX *et al.* 1999).

Table 1-1 Classification of "single site" fungicides according to its' fungicide class, target site and first year of registration to control *Botrytis cinerea*.

Fungicide class (Abbreviation)	Fungicide(s)	Target site(s)	Year	Reference
Benzimidazoles (MBC)	benomyl, carbendazim, thiophanate-methyl	microtubule assembly (β-tubulin subunit)	1969	LEROUX *et al.* (1985)
Carboximides	Carboxin	fungal respiration (succinate dehydrogenase)	1969	SCHEWE *et al.* (1995)
Dicarboximides	iprodione, vinclozolin	lipid metabolism and osmotic regulation	1978	GRIFFITHS *et al.* (2003)
Phenylpyridinamines	fluazinam, dinocap	fungal respiration (oxidative phosphorylation)	1990	GUO *et al.* (1991)
N-phenyl-carbamates (NPC)	diethofencarb	microtubule assembly (β-tubulin subunit)	1987	FUJIMURA *et al.* (1990)
Anilinopyrimidines	cyprodinil, pyrimethanil, mepanipyrim	methionine biosynthesis (cystathionine-β-lyase)	1992	MASNER *et al.* (1994)
Phenylpyrroles	fludioxonil, fenpiclonil	lipid metabolism and osmotic regulation	1995	FORSTER *et al.* (1996)
Hydroxyanilides	fenhexamid	sterol biosynthesis (3-keto reductase)	1998	DEBIEU *et al.* (2001)
Strobilurines (QoI)	azoxystrobin, pyraclostrobin	fungal respiration (cytochrome bc1)	1996	MYRESIOTIS *et al.* (2008)
Second generation of carboximides (SDHI)	boscalid, bixafen, fluopyram,	fungal respiration (succinate dehydrogenase)	2003	AVENOT *et al.* (2010)

The molecular bases of benzimidazole resistance are single nucleotide polymorphisms (SNPs) in the structural gene *Mbc1* encoding the β-tubulin. The BenR1 phenotype correlates with a SNP at codon 198, which leads to substitution of glutamate by alanine (E198A). It is the most common SNP leading to benzimidazole-resistance in field isolates of *B. cinerea* (YARDEN and KATAN, 1993; LUCK *et al.* 1994; MA and MICHAILIDES, 2005; BANNO *et al.* 2008). According to AKAGI *et al.* (1995), the E198A mutation alters the binding site of the β-tubulin to carbendazim by change of an ethyl sized pocket (Figure 1-2). The substitution of glutamic acid by valine at codon 198 (E198V) was detected rarely in field isolates show a resistance phenotype similar to E198A mutants (BANNO *et al.* 2008). The phenotype BenR2, which is resistant to benzimidazoles and N-phenyl-carbamates, was analyzed by YARDEN and KATAN (1993). The authors identified two SNPs. At the codon 200 tyrosine replaces phenylalanine (F200Y) and at codon 198 glutamic acid is substituted by lysine (E198K). Strains with the F200Y mutation are moderately resistant to benzimidazoles, while the E198K mutants, like the E198A mutants, are highly resistant to benzimidazoles.

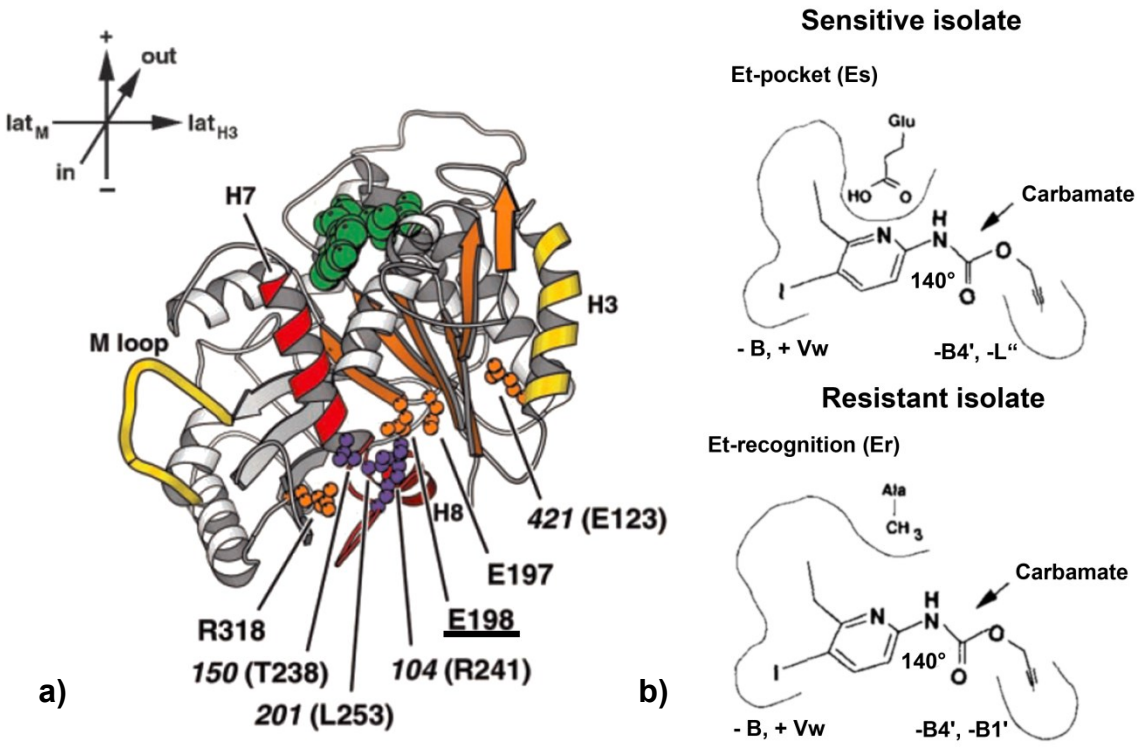

Figure 1-2 (a) Locations of benomyl-resistant β-tubulin alleles of *Saccharomyces cerevisiae.* Cutaway view of the core of β-tubulin with the interior-facing loop removed (RICHARDS *et al.* 2000). (b) Receptor mapping of benomyl-resistant and sensitive β-tubulin of *Botrytis cinerea* (AKAGI *et al.* 1995).

Due to the fact, that the primary mode of action of anilinopyrimidines has not been clarified, resistant strains could only be identified by their phenotype. Resistant isolates were detected in different monitoring procedures a few years after introduction of the active ingredient (LEROUX *et al.* 1999; FORSTER and STAUB, 1996; LATORRE *et al.* 2002). Highly resistant isolates (AniR1) showed resistance levels of more than 100. Additionally, high anilinopyrimidine resistance was not associated with decreased sensitivity to other fungicides (LEROUX *et al.* 1999). Molecular basis of this resistance is unknown, because no mutations in the *Cbl* or *metC* genes coding the cystathionine β-lyase correlated with resistance phenotypes (FRITZ *et al.* 2003). Strains showing lower resistance levels (5 – 15) were distinguished according to their spectrum of cross-resistance towards other fungicides (LEROUX *et al.* 1999). Recent research showed, that these multi drug resistant (MDR) phenotypes were caused by active efflux of fungicides due to ATP-dependent membrane transporters, such as ABC and MFS transporters (KRETSCHMER *et al.* 2009; HAYASHI, 2003, MERNKE *et al.* 2011). The molecular basis of MDR is a constitutive overexpression of these transporters. In the MDR1 phenotype (syn. AniR2) the *bcatrB* gene coding for an ABC transporter is overexpressed by mutations in the transcription factor Bcmrr1. In the MDR2 phenotype (syn. AniR3) a specific rearrangement in the promoter of the *bcmfsM2* gene with the insertion of a 1326 bp sequence causes an overexpression. The latter emerged MDR3 phenotype is a meiotic recombination of the MDR1 und MDR2 phenotypes, thus carrying the mutated *bcatrB* and *bcmfsM2* genes (KRETSCHMER *et al.* 2009).

Mutations associated with fungicide resistance may display pleiotropic effects, which become apparent in the absence of fungicide selection pressure (JEGER, WIJNGAARDEN and HOEKSTRA, 2008). The evolution of resistance to fungicides in fungal populations is largely dependent on the fitness of the resistant fraction of the population (BARDAS *et al.* 2008). If a mutation leading to resistance does not influence the fitness, then a stable resistance frequency in absence of the fungicide selection pressure will be observed (KARAOGLANIDIS *et al.* 2011).

Botrytis cinerea is a high risk pathogen capable of sexual and asexual reproduction, but ascospore production is rarely observed (GIRAUD *et al.* 1997). Therefore, the haploid, mitotic stage of the fungus is used to investigate the evolution of resistance. The fitness cost of resistance can be assessed by culturing sensitive and resistant *B. cinerea* strains and testing them for a variety of fitness parameters including conidial production and aggressiveness on plants (PRINGLE and TAYLOR, 2002).

Several fitness studies on *B. cinerea* have been published. These studies have revealed fitness cost of strains resistant to dicarboximide (HSIANG and CHASTAGNER, 1991; SUMMERS *et al.* 1984; RAPOSO *et al.* 2000), phenylpyrrole (ZIOGAS *et al.* 2005; GULLINO, LEROUX and SMITH, 2000) and hydroxyanilide fungicides (SUTY, PONTZEN and STENZEL, 1999; BILLARD *et al.* 2012). Such fitness costs have led to a decrease of resistant strains in absence of fungicide application (Figure 1-3). However, resistances to benzimidazoles or to anilinopyrimidines have no significant effect on the fitness parameters tested (HSIANG, 1991; ELAD *et al.* 1992; FORSTER and STAUB, 1996; BARDAS *et al.* 2008). Similarly, there seems to be little or no fitness cost associated with multidrug resistance (KRETSCHMER *et al.* 2009). Benzimidazole resistance has been stable for several years (HOFFMANN and LOECHER, 1979, SCHUEPP and LAUBER, 1981). However, a decrease of the benzimidazole-resistant fraction of the population in Germany was observed since the use of benzimidazoles was discontinued in viticulture thirty years ago (DERPMANN *et al.* 2010, LEROCH *et al.* 2010). These observations might be explained by fitness costs, which can only be detected under conditions that are suboptimal for the fungus (BROWN *et al.* 2006).

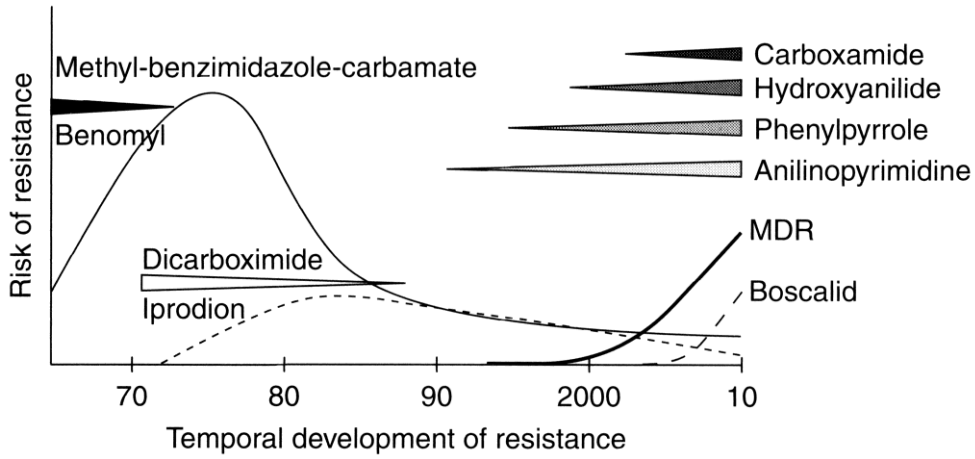

Figure 1-3 Resistance development of *Botrytis cinerea* to different fungicide classes in Germany (KRETSCHMER, 2012)

The existence of fitness costs of benzimidazole-resistant strains could provide the possibility for a resistance management strategy. Such strategies are requested by the European and Mediterranean Plant Protection Organization (EPPO) and the Regulation (EC) No. 1107/2009 of the European Parliament concerning the placing of plant protection products with an inherent resistance risk on the market. In practice, resistance management strategies must combine the long-term conservation of fungicide effectiveness with a pattern of use, which satisfies the needs of the farmer and to provide a reasonable pay-back to the manufacturer (BRENT and HOLLOMON, 2007b). In order to delay the evolution of resistance, suggested or pre-packed mixtures with other fungicides can be applied. The companion compound can be a multi-site fungicide known to have a low risk of inducing resistance or a single-site inhibitor, which is not cross-resistant. Also, fungicides at risk can be used as one component in a rotation or alternation of different fungicide treatments, thus restricting the number of treatments applied per season of the at-risk fungicide. In order to avoid high disease incidences caused by various pathogen populations able to adapt to selection pressure, protective applications are favored compared to eradicative or curative applications. Also, the use of disease resistant crop varieties, biological control agents, and appropriate hygienic practices, such as crop rotation and removal of diseased parts of perennial crop plants, reduces disease incidence and permits the more sparing use of fungicides. These measures should be applied uniformly over large areas in order obtain their full biological benefit (BRENT and HOLLOMON, 2007a).

At time of introduction in 1971, benzimidazoles were used without restrictions. After failure of control of *B. cinerea* in grapevine, use of benzimidazoles to control *B. cinerea* was discontinued in 1975 (SCHRUFT, 2001). A similar observation was made by DELP (1980) in Australia, where benzimidazoles were used to control *B. cinerea* on strawberries. If benzimidazoles were mixed with the multisite fungicide captan to control *Colletotrichum acutatum*, then no loss of control of *B. cinerea* by benzimidazoles was observed.

Dicarboximides introduced in 1976 controlled benzimidazole-resistant strains. However, frequent applications of dicarboximides, such as iprodione or vinclozolin, resulted in an increase of resistant strains (POMMER and LORENZ, 1995). Due to a reduced fitness of resistant strains (SUMMERS, 1984; HSIANG, 1991; RAPOSO, 2000), a decrease of the portion of resistant strains in absence of selection pressure in the period from October to the next fungicide application was observed (PAK *et al.* 1990; LOECHER *et al.* 1987). Therefore, a maximum of two treatments as well as combined treatments with multisite inhibitors, such as chlorothalonil or thiram, were advised (LEROUX *et al.* 1985).

Because of the loss of efficacy of benzimidazole and dicarboximide applications due to high percentages of resistant strains in populations of *B. cinerea* in the valuable Champagne vine growing area, fungicides with new modes of action were needed (LEROUX *et al.* 1985). In the mid-1990s anilinopyrimidines, such as cyprodinil and mepanipyrim, as well as fenhexamid were introduced. As a consequence of the experiences with the formation of resistance to benzimidazoles and dicar-

boximides, baseline monitoring procedures and resistance management strategies had to be developed prior to introduction of new products (RUSSELL, 2003). E.g. the number of fenhexamid treatments was limited to a maximum of one third of the treatments per season should contain fenhexamid with no more than two consecutive fenhexamid treatments (SUTY, PONTZEN and STENZEL, 1999; HAENSSLER and PONTZEN, 1999). Also, a preventive use was recommended, due to the presence of the naturally occurring resistance to fenhexamid (HydR1), which is not expressed in germ-tube elongation assays (LEROUX *et al.* 1999). The anilinopyrimidine fungicide cyprodinil was introduced to the market as a pre-packed mixture with fludioxonil, a phenylpyrrole fungicide. Additionally, the number of applications was limited to half of the treatments per season (FORSTER and STAUB, 1996). A long term monitoring conducted from 1995 to 2001 using a resistance management strategy of one treatment per fungicide class and season resulted in increased percentages of anilinopyrimidine- as well as fenhexamid-resistant phenotypes. However, the mixture of cyprodinil and fludioxonil as well as fenhexamid alone was still effective to control *B. cinerea* (BAROFFIO *et al.* 2003).

In 2003, the SDHI fungicide boscalid was introduced either as a single product or as a pre-packed mixture with pyraclostrobin, a QoI fungicide. Baseline studies detected no naturally occurring SDHI-resistant phenotypes (STAMMLER and SPEAKMAN, 2006; ZHANG *et al.* 2007; MYRESIOTIS *et al.* 2008). The number of treatments per season including SDHIs, preferably in mixture, was limited to two non-consecutive treatments in alternation with effective fungicides from different chemical classes (MCKAY *et al.* 2011). However, SDHI-resistant isolates occurred after a few years of use (AVENOT *et al.* 2010; FERNANDEZ *et al.* 2012; VELOUKA *et al.* 2013).

The resistance management strategies in the last decades resulted in a selection of not only target site resistances, but also of multi drug resistant phenotypes. They exhibit more than ten-fold resistance levels towards SDHIs, QoIs, DMIs, anilinopyrimidines, fludioxonil, and fenhexamid (KRETSCHMER *et al.* 2009; LEROCH *et al.* 2013; LEROUX and WALKER, 2013).

In order to develop a suitable resistance management strategy for benzimidazoles, the build-up of resistance must be monitored. Shifts in sensitivity in fungal populations can be measured by bioassays or molecular assays (SMITH *et al.* 1991; MA and MICHAILIDES, 2005). Additionally, efficacy data must be evaluated in order to correlate resistance frequency with field performance of the fungicide. At first, the sensitivity profile, which is the baseline sensitivity for an existing fungicide at a specific location, must be determined. Subsequently, monitoring procedures must be conducted in order to measure the dynamics of resistance build-up under selection pressure of different fungicide resistance management strategies (RUSSELL, 2003). By means of the methods described above, a suitable resistance management strategy can be identified and implemented in order to slow down the build-up of resistance, thus prolonging the lifespan of an active ingredient introduced to the market.

The aims of the present study were as follows:

- Determination of the influence of resistance management strategies for benzimidazoles on populations of *Botrytis cinerea* in three year field trials conducted at three sites near Bordeaux.
- Characterization of the genetic background of benzimidazole-resistant *B. cinerea* isolates collected in this study.
- Development of real-time PCR protocols to determine the frequency of resistance alleles in populations of *B. cinerea*.
- Conducting fitness experiments with benzimidazole-sensitive and –resistant isolates of *B. cinerea* in order to identify fitness costs associated with resistance to benzimidazoles.
- Analysis of the spatial and temporal distribution of benzimidazole-resistant isolates of *B. cinerea* to complement the results of field trials and laboratory experiments.
- Evaluation of the available data to develop recommendations for a use pattern of benzimidazoles to control *B. cinerea* in grapevine.

2 MATERIALS AND METHODS

2.1 ORGANISMS

2.1.1 PATHOGEN

For evaluation of the influence of resistance management strategies on population dynamics of *Botrytis cinerea*, a total of 5058 isolates were collected from three experimental sites near Bordeaux from June 2009 to August 2011. The code assigned to each isolate consisted of one letter and three numbers. Letters A, B and C indicated the experimental site near Grezillac, Saint Brice and Loupes, respectively. The first number indicated the treatment (1 – 5, see Table 2-4), the second number indicated the repetition (1 – 4) and the last number (1 – 22) indicated the sample number within the plot.

For characterization of fitness parameters isolates of *B. cinerea* were selected arbitrarily from a monitoring conducted in German vineyards in September 2007 (DERPMANN *et al.* 2010). A list of fungal isolates used in this study is given in Table 2-2.

As a reference for fungicide sensitivity assays *B. cinerea* isolates were chosen from a monitoring conducted in September 2009 according to results of a preliminary experiment (data not shown). A list of fungal isolates used is given in Table 2-1.

Table 2-1 Isolates of *Botrytis cinerea* collected from experimental sites near Bordeaux in September 2009 used for fungicide sensitivity assays.

Isolate code	Location of isolation	Sensitivity to benzimidazoles	Sensitivity to anilinopyrimidines
B-T2-R1-4	Saint Brice	resistant*	reduced sensitivity†
B-T4-R2-10‡	Saint Brice	resistant	resistant§
B-T4-R2-20‡	Saint Brice	sensitive**	sensitive*
C-T2-R3-5	Loupes	sensitive	sensitive
C-T2-R3-7	Loupes	resistant	reduced sensitivity
C-T2-R3-22‡	Loupes	resistant	reduced sensitivity

* mycelial growth of more than 50% at 1 ppm of thiophanate-methyl compared to control
† mycelial growth of more than 50% at 1 ppm of mepanipyrim compared to control
‡ isolate used as reference in fungicide sensitivity assay
§ mycelial growth of more than 50% at 15 ppm of mepanipyrim compared to control
** mycelial growth of less than 50% at 1 ppm of thiophanate-methyl compared to control

Two *B. cinerea* isolates with known sequence at codon 198 of the β-tubulin gene were used as reference isolates for nucleic acid based detection methods. These isolates were kindly provided by the culture collection of INIA (Instituto Nacional de investigación y Tecnologia Agraria Alimentaria, Spain). Isolate BC-266.6 showed the E198A-mutation and isolate BC-11.3 showed the wild-type.

Table 2-2 Isolates of *Botrytis cinerea* collected in September 2007 in German vineyards.

Isolate code	Location of isolation	Sensitivity to benzimidazoles	Sensitivity to anilinopyrimidines
May 6[†*]	Mayschoß	resistant[‡]	sensitive[§]
Marie 4	Marienthal	resistant	reduced sensitivity[**]
Rech 4[*]	Rech	resistant	reduced sensitivity
V3-3-4	Randersacker	resistant	sensitive
V5-3-1[*]	Heppenheim	resistant	sensitive
V5-3-5[*]	Heppenheim	resistant	sensitive
V5-5-2[*]	Heppenheim	resistant	sensitive
V6-1-4[*]	Höhnstedt	resistant	sensitive
V6-3-3[*]	Höhnstedt	resistant	sensitive
V6-3-4[*]	Höhnstedt	resistant	sensitive
V6-3-5[*]	Höhnstedt	resistant	sensitive
V6-3-6[*]	Höhnstedt	resistant	sensitive
May 3[*]	Mayschoß	sensitive[††]	sensitive
Rech 1[*]	Rech	sensitive	sensitive
V1-2-1[*]	Hammelburg	sensitive	sensitive
V1-5-2[*]	Hammelburg	sensitive	sensitive
V3-2-2[*]	Randersacker	sensitive	sensitive
V3-3-2[*]	Randersacker	sensitive	sensitive
V5-1-4[*]	Heppenheim	sensitive	sensitive
V5-2-4[*]	Heppenheim	sensitive	sensitive
V5-2-6[*]	Heppenheim	sensitive	sensitive
V6-4-3[*]	Höhnstedt	sensitive	sensitive

[*] isolates used in fitness experiments
[†] mycelial growth of less than 50% at 1 ppm of mepanipyrim compared to control
[‡] mycelial growth of more than 50% at 300 ppm of thiophanate-methyl compared to control
[§] mycelial growth of less than 50% at 1 ppm of mepanipyrim compared to control
[**] mycelial growth of more than 50% at 1 ppm of mepanipyrim compared to control
[††] mycelial growth of less than 50% at 1 ppm of thiophanate-methyl compared to control

2.1.2 PLANT

In-vivo experiments were conducted using grapevine (*Vitis vinifera* L.) cultivar 'Müller Thurgau'. It was rated medium susceptible to *B. cinerea* (German susceptibility score[*] 5). In the region of Bordeaux (France) field experiments were conducted using the cultivars 'Merlot Nior', 'Muscadelle' and 'Sauvignon'. The French *B. cinerea* susceptible scores[†] were 4, 5 and 5, respectively.

2.2 CHEMICALS AND MATERIAL

Axygen Inc. (Union City, CA, USA)

0.5 mL tubes, 0.2 mL thin-wall 8 strip PCR tube with lid

AppliChem GmbH (Gatersleben, Germany)

Chloramphenicol, ethanol, ethidiumbromide, isopropanol, TAE-buffer, magnesium sulfate ($MgSO_4$ $7xH_2O$), sodium nitrate ($NaNO_3$), tannic acid, glycerol

BASF SE (Ludwigshafen am Rhein, Germany)

Kumulus® WG (800 g kg^{-1} copper)

Certis Europe BV (Utrecht, Netherlands)

Frupica® SC (440 g L^{-1} mepanipyrim), Japica® SC (500 g L^{-1} mepanipyrim)

Biotium Inc. (Hayward, CA, USA)

Fast Plus EvaGreen® Master Mix qPCR with high Rox, EvaGreen® dye (20x)

Biomers.net GmbH (Ulm, Germany)

Oligonucleotides

Brand GmbH & Co KG (Wertheim, Germany)

Parafilm® M, Fuchs-Rosenthal hemocytometer

DuPont Inc. (Wilmington, DE, USA)

Rubigan® SC (120 g L^{-1} fenarimol)

[*] German susceptibility score from 1 to 9, where 1 is very low and 9 is very high susceptibility (ANONYM, 2008).
[†] French susceptibility scores from 1 to 5, where 1 is very low and 5 is very high susceptibility (FERMAUD, 2012)

Eflor GmbH (Muenchen, Germany)

Flory 2 Spezial (19-9-22)

Eppendorf AG (Hamburg, Germany)

1.5 mL, 2 mL, 15 mL and 50 mL tubes

Greiner Bio-One GmbH (Solingen, Germany)

96, 48 and 24 well flat bottom culture plates, petri dishes (60 mm and 90 mm in diameter), 15 mL and 50 mL tubes, 150 mm swab tubes

Hartmann AG (Heidenheim, Germany)

Cotton gauze

Merck KGaA (Darmstadt, Germany)

Czapek-Dox agar, potato dextrose agar, potato dextrose broth, Tween® 80

Nippon Soda Co. LTD. (Tokyo, Japan)

Topsin® 500 SC (500 g L^{-1} thiophanate-methyl)

Life technologies Inc. (Foster City, CA, USA)

MicroAmp™ Fast Optical 96-well reaction plate, MicroAmp™ Optical Adhesive Film

Promega GmbH (Mannheim, Germany)

Wizard® Magnetic DNA Purification System for Food, low range DNA ladder

Qiagen GmbH (Hilden, Germany)

DNeasy® Plant Mini Kit, QIAquick® Gel Extraction Kit

Roth GmbH (Karlsruhe, Germany)

Agar-Agar, 50 mL sample tubes with lid, humid chambers

Sartorius stedim biotech GmbH (Goettingen, Germany)

3 mm steel balls, 88 mm filter paper

Satisloh AG (Baar, Switzerland)

Silicon carbide (600 mesh)

Schott AG (Mainz, Germany)

Beakers, measuring cylinders, bottles, flasks

Sigma-Aldrich Co. LLC. (St. Louis, MO, Germany)

Glucose, fructose, potassium dihydrogen orthophosphate (KH_2PO_4), potassium chloride (KCl), agarose, gelatin, pentachloronitrobenzene (PCNB), diethofencarb, sodium hypochlorite

Sintagro AG (Langenthal, Switzerland)

Maneb 80 WP (800 g kg^{-1} Maneb)

Thermo Fisher Scientific Inc. (Waltham, MA, USA, former Fermentas GmbH)

DreamTaq™ DNA polymerase, DreamTaq™ Green Buffer (2 mM $MgCl_2$), 10 mM dNTPs, DNA ladder (low range, 100 bp)

Wilhelm Haug GmbH & Co. KG (Ammerbuch, Germany)

Plantosan® Topf 1.5 organic potting substrate

2.3 EQUIPMENT

BP 210 D	balance	Sartorius GmbH (Goettingen, Germany)
Behropur® B5	aqua dest. apparatus	behr LT GmbH (Duesseldorf, Germany)
LaM-3-20-MCS-J	autoclave	Sanoclav GmbH (Bad Uberkingen, Germany)
GFL 1083	water bath	GFL mbH (Burgwedel, Germany)
Antares 72	laminar flow bank	STERIL S.p.A.(Corsico, Italy)
Dispensette® Safety	dispenser	GmbH & Co KG (Wertheim, Germany)
RCT basic	Magnetic stirrer	IKA GmbH & Co. KG (Staufen, Germany)
Reverence®/Research®	pipette	Eppendorf AG (Hamburg, Germany)
Vortex-Genie® 2 G	vortexer	Scientific Industries Inc (Bohemia, NY, USA)
RuMed® Type 3501	incubator	Rubarth Apparate GmbH (Laatzen, Germany)
Multitron® Standard	incubation shaker	Infors AG (Bottmingen, Switzerland)
6027-65	freezer	AEG AG (Frankfurt, Germany)
1442-4	fridge	AEG AG (Frankfurt, Germany)
G10	digital camera	Canon Inc. (Tokyo, Japan)
GPS 12 XL	GPS handheld	Garmin GmbH (Olathe, KS, USA)

DMRB + HV-C20A	microscope + camera	Leica GmbH (Wetzlar, Germany)
Axiolab® re	upright microscope	Carl Zeiss GmbH (Oberkochen, Germany)
MZ16F + KL1500	stereomicroscope + camera	Leica GmbH (Wetzlar, Germany)
L2 + S4E	stereomicroscope	Leica GmbH (Wetzlar, Germany)
N 022 AN.18	membrane vacuum pump	KNF Neuberger GmbH (Freiburg, Germany)
Ultra Turrax® T25	homogenizer	IKA GmbH & Co. KG (Staufen, Germany)
Beta1-8k	lyophilisator	Martin Christ GmbH (Osterode, Germany)
Mikro-Dismembrator®	ball mill	Braun AG (Melsungen, Germany)
Direct Q 3 UV	ultrapure water apparatus	Millipore Co. (Billerica, MA, USA)
Thermomixer 5436	heating block	Eppendorf AG (Hamburg, Germany)
Centrifuge S41FR	tabletop centrifuge	Eppendorf AG (Hamburg, Germany)
BioPhotometer® plus	photometer	Eppendorf AG (Hamburg, Germany)
TGradient®	thermocycler	Biometra GmbH (Goettingen, Germany)
Sub-Cell GT	erlectrophoresis system	Bio Rad GmbH (München, Germany)
TI 1	transilluminator	Biometra GmbH (Goettingen, Germany)
MPS1000™	mini plate spinner	Lapnet International Inc. (Edison, NJ, USA)
Sprout®	mini centrifuge	Heathrow Co. LLC (Vernon Hills, IL, USA)
ABI® StepOne™ Plus	real-time thermocycler	Life technologies Inc. (Foster City, CA, USA)

2.4 CULTURE MEDIA

The following media were used for fungal isolation, *in vitro* experiments and mycelia production for DNA extraction. The stated recipes are per liter of distilled water. Culture media were autoclaved at 121°C for 30 minutes at 103 kPa allowed to cool to about 55°C and dispensed by means of a dispenser into 90 mm or 60 mm diameter disposable petri dishes.

Potato-Dextrose-Agar (PDA)

Potato-Dextrose-Agar	39.5 g

Potato-Dextrose-Broth (PDB)

Potato-Dextrose-Agar	24 g

Low Strength Potato-Dextrose-Agar (PDA_{low})

Potato-Dextrose-Agar	12.5 g
Agar Agar	13.3 g

Botrytis Selective Medium (BSM)

Glucose	2 g
$NaNO_3$	0.1 g
$KH_2PO_2´$	0.1 g
$MgSO_4\ 7xH_2O$	0.2 g
KCl	0.1 g
Tannic acid	5 g
chloramphenicol	0.2 g
pentachloronitrobenzene	0.01 g
Maneb 80	0.02 g
Rubigan® (12% fenarimol)	0.1 mL
Agar Agar	20 g

Prior to addition of agar the pH was adjusted to 4.5 with 1 mol L^{-1} NaOH. The medium was boiled, stirred and poured whilst still hot in 90 mm petri dishes (EDWARDS and SEDDON, 2001).

Fructose Gelatin Agar (FGA) according to TAKAGAKI *et al.* (2004)

$MgSO_4\ 7xH_2O$	0.5 g
KH_2PO_4	1.0 g
$NaNO_3$	2.0 g
Fructose	10.0 g
Gelatin	2.0 g
Agar Agar	15.0 g

Water Agar (WA)

Agar Agar	20.0 g

Low Strength Czapek-Dox-Agar ($CZA_{10\%}$)

Czapek-Dox-Agar	4.8 g
Agar Agar	10.0 g

2.5 CULTIVATION

2.5.1 PATHOGENS

2.5.1.1 Isolation

For the evaluation of resistance management strategies, plant organs possibly infected by *B. cinerea* were collected from June 2009 to August 2011.

Samples of plant organs collected in June 2009 were surface sterilized. Samples were placed in 1.2 % (v/v) sodium hypochlorite for 30 seconds and washed twice in sterile distilled water (SDW). Samples were dried and stored at -20°C. After de-freezing, five pieces of each sample were transferred to BSM-plates. After seven days of incubation mycelium of *B. cinerea* was re-cultivated on PDA_{low}.

Samples of flowers collected in June 2010 and May 2011 were frozen for at least 24 hours. After de-freezing, flowers were incubated for five days at 21°C with 14 hours of near-ultraviolet light at high relative humidity. Subsequently, sporulating mycelium of *B. cinerea* was transferred to a PDA_{low} plate amended with 0.2 g L^{-1} chloramphenicol and a dilution dash was performed. After an incubation time of seven days at 4°C single colonies were re-cultivated on PDA_{low}.

Samples of berries collected in September 2009, 2010 and May 2011 were checked for sporulation. If no conidiophores were visible, samples were transferred to a moist chamber and incubated up to seven days at 21°C with 14 hours of near-ultraviolet light at high relative humidity. Sporulating mycelium of *B. cinerea* was transferred to PDA_{low} plates amended with 0.2 g L^{-1} chloramphenicol and a dilution dash was performed. Samples in swab tubes were processed directly after arrival in the laboratory by performing a dilution dash. After an incubation time of seven days at 4°C single colonies were re-cultivated on PDA_{low}.

Subsequently, colonies on PDA_{low} were incubated for three days at 21°C in the dark and checked for contamination. After five to seven days of incubation at 21°C with 14 hours of near-ultraviolet light, a stereo microscope was used to identify *B. cinerea*. Finally, isolates were stored at 4°C until the fungicide sensitivity assay was performed.

2.5.1.2 Cultivation

Fungal isolates were sub-cultured onto PDA medium and incubated at 21°C in the dark for three days. To enhance sporulation isolates were incubated for additional ten days at 21°C with 14 hours of near-ultraviolet light. Subsequently, isolates were stored at 6°C in the dark and re-cultivated monthly as described above to avoid contamination. For long-term storage conidia were washed off as described in chapter2.7.2 and conidial suspensions were adjusted to a final concentration of 1 x 10^6 conidia mL^{-1} and 35 % (v/v) glycerol. Finally, samples were stored up to one year in a freezer at –20°C.

2.5.2 PLANTS

Grapevine plants (*Vitis vinifera* L. cv. 'Müller Thurgau') were produced by vegetative propagation from multiannual mother plants. Leaf axils were cut from green shoots and placed in matrixes, in which axillary buds took root. Subsequently, rooted cions were placed in 9 cm pots filled with organic potting substrate. Plants were grown under greenhouse conditions and treated bimonthly with 9 g L^{-1}(w/v) Kumulus® WG to prevent powdery mildew infections (*Uncinula necator* (Schw.) Burr.).

2.6 INOCULATION OF GRAPEVINE

2.6.1 PLANTS

For determination of the competitive ability of *B. cinerea* fungicide sensitivity groups, ten isolates sensitive (S) and ten isolates resistant to benzimidazoles (R, chapter 2.1.1) were pooled to two conidial suspensions according to their phenotype (S, R). Subsequently, mixed isolate inoculums were produced by intermingling of appropriate volumes of S and R so as to produce suspensions containing 100% R, 1% R : 99% S, 10% R : 90% S, 50% R : 50% S, 90% R : 10% S and 100% S. All conidial suspensions were adjusted to 1 x 10^5 conidia mL^{-1}.

Two months old grapevine plants (cv. 'Müller Thurgau') were cultivated as described in chapter 2.5.2. Leaves were injured by applying silicon carbide (150 g L^{-1}) with a brush in a circular movement. Plants were inoculated with the mixed isolate suspensions or sterile distilled water (SDW) with a sterilized hand atomizer until plants were dripping wet. Pots were covered with plastic bags and in-

cubated at high relative humidity for three days at 21°C or ten days at 6°C at 14 hours of daylight illumination. To prevent contamination of leaves by defoliation of plants, leaves were cut off, placed in humid chambers and incubated for additional three days at 21°C or 14 days at 6°C with 14 hours of near-ultraviolet light. After incubation, leaves were washed in 10 mL of SDW amended with tween (0.01 % v/v). Subsequently, conidial suspensions were filtered through double-layered cotton gauze and adjusted to a final concentration of 1 x 10^6 conidia mL^{-1} and 35 % (v/v) glycerol.

A second disease cycle was started by using the washed off conidial suspensions as inoculum for a new set of plants treated as described above. Five replicates were used per mixed-isolate inoculum. The repetition of the experiment was conducted on autoclaved leaf discs without fungicide application. Autoclaved leaf discs were prepared and inoculated as described in chapter 2.7.2.

2.6.2 DETACHED LEAVES

In order to characterize fitness parameters of selected *B. cinerea* isolates (chapter 2.1.1), aggressiveness of these isolates was tested on detached leaves of grapevine.

Detached leaves cut from two months old grapevine plants (cv. Müller Thurgau) were placed in humid chambers. Each of the three to five lobes was punctured with a pipette tip. Subsequently, 10 µL of conidial suspension (1x10^5 conidia mL^{-1}) or SDW amended with 2 g L^{-1} gelatin was pipetted on each wound. Leaves were incubated in the dark at 21°C for 3 or at 6°C for 10 days. Six replicates were used per isolate and the experiment was repeated twice.

2.6.3 BERRIES

For the validation of the quantitative polymerase chain reaction (qPCR) protocol as well for determination of differences in the sensitivity of the qPCR protocol and classical fungicide sensitivity assay, berries of grapevine (cv. ‘Birchstaler Muskat’) without synthetic chemical treatments were inoculated.

Berries were surface sterilized by submersion in 70 % (v/v) ethanol for 5 min and washed two times in SDW. Subsequent to drying, berries were inoculated by injection of 100 µL of the mixed isolate suspension (chapter 2.6.1) 100% R, 100% S, 10% R : 90% S (1x10^5 conidia mL^{-1}) or SDW into the middle of the fruit. Berries were placed in humid chambers and incubated at high relative humidity for five days at 21°C at 14 hours of daylight illumination. Thereafter, conidia were washed of the berries according to chapter 2.6.1. Washed berries were frozen at -20°C and DNA was extracted according to chapter 2.10.1. Two or four biological replicates were used per mixed-isolate inoculum.

2.7 ASSESSMENT OF FUNGAL GROWTH PARAMETERS

2.7.1 MYCELIAL GROWTH

2.7.1.1 Size of colony on synthetic medium

In order to characterize fitness parameters of selected *B. cinerea* isolates (chapter 2.1.1), a mycelial growth assay was performed. Inoculum was grown on water agar for five days. Subsequently, five-millimeter plugs were transferred to the center of PDA or $CZA_{10\%}$ plates and incubated for three days at 21°C or for ten days at 6°C in the dark. Colony diameter of each isolate was measured. Five replicates were used per isolate and the experiment was repeated twice.

For determination of discriminative concentrations of mepanipyrim used in the fungicide sensitivity assay, selected isolates of *B. cinerea* (chapter 2.1.1) were tested on FGA medium amended with 0, 0.01, 0.03, 0.1, 0.3, 1, 2, 3, 5, 10, 30 and 100 ppm of mepanipyrim (Frupica® SC). The inoculum was grown on water agar for five days. Subsequently, five-millimeter mycelial plugs were transferred from the edge of the colony to the center of FGA plates. After three days of incubation at 21°C in the dark, colony diameter was measured. Five replicates were used per isolate and fungicide concentration.

2.7.1.2 Microplate assay

In order to determine the phenotype of isolates of *B. cinerea* gained from plant organs of grapevine (chapter 2.5.1.1), their sensitivity to thiophanate-methyl, mepanipyrim and diethofencarb was tested in a fungicide sensitivity assay in 96-well microplates.

Sporulating mycelium free of nutrition medium was transferred from purified isolates to the center of wells filled with 100 µL of FGA culture medium either without amendment or with 1.5 ppm of thiophanate-methyl (Topsin® 500 SC), 1 and 15 ppm of mepanipyrim (Frupica® SC). Subsequently, isolates resistant to 1.5 ppm of thiophanate-methyl were tested in a second fungicide sensitivity assay on 0 and 10 ppm of diethofencarb (technical grade, dissolved in aceton) as described above. Two replicates were used per isolate and fungicide concentration.

After three days of incubation at 21°C with 14 hours of near-ultraviolet light, colonies in wells were checked for sporulation. If sporulation on fungicide amended medium was comparable to sporulation on unamended medium, the tested isolate was considered as resistant. Otherwise it was considered as sensitive.

Additionally, three reference isolates with known fungicide-resistant phenotypes (chapter 2.1.1) were included in the fungicide sensitivity assay.

For determination of frost tolerance of phenotypes resistant to different fungicide classes, selected *B. cinerea* isolates (Appendix Table 7-16) were transferred to 24-well culture plates filled with 1 mL of PDA_{low} per well. After an incubation time of two days at 21°C in the dark, isolates were frozen for seven days at -20°C. Subsequently, frozen mycelial plugs were transferred to 24-well culture plates. Presence or absence of mycelial growth was determined after three days of incubation at 21°C in the dark. Two replicates were used per isolate and the experiment was repeated twice.

2.7.2 SPORE PRODUCTION

For characterization of fitness parameters of selected *B. cinerea* isolates (chapter 2.1.1), spore production at 21°C was promoted by additional incubation of mycelium on PDA plates for eleven more days with 14 hours of near-ultraviolet light.

Spore production at 6°C was measured on autoclaved leaf discs cut from two to three month-old grapevine plants (cv. 'Müller Thurgau'). Leaf discs were inoculated by soaking them in conidial suspensions (1×10^5 spores mL^{-1}) or sterile distilled water (SDW) for five minutes. Subsequently, leaf discs were placed on pre-wetted filter paper, which was covered by sterilized parafilm, in a petri dish and incubated for five days at 21°C or for 14 days at 6°C with a 14 hour photoperiod.

After incubation, PDA-plates and leaf discs were washed with 2 mL of SDW amended with tween (0.01 % v/v) and conidial suspension was filtered through double-layered cotton gauze. Two replicate droplets were counted for each PDA-plate and leaf disc. Conidial concentration was measured with a haemocytometer. Results were expressed as number of conidia per square millimeter. Ten replicates were used per isolate. For the repetition of the experiment sporulation was tested on autoclaved leaf discs at 6°C as well as 21°C.

2.7.3 SPORE GERMINATION

For characterization of fitness parameters of selected *B. cinerea* isolates (chapter 2.1.1), conidial suspensions produced in the preceding experiment (chapter 2.7.2) were used.

200 µL of conidial suspension adjusted to 1×10^5 conidia mL^{-1} was pipetted onto a water agar plate and incubated for 18 hours at 21°C or for 60 hours at 6°C in the dark. The percentage of germinated spores was determined by counting at least 100 conidia per plate. A germ tube being twice as long as the conidium was considered as germinated (LEROUX *et al.* 1985). Three replicates were used per isolate.

2.7.4 GERM TUBE DEVELOPMENT

Competitive ability of *B. cinerea* fungicide sensitivity groups was tested on whole plants as well as on autoclaved leaf discs of grapevine. Subsequently to washing off (chapter 2.6.1), population dynamics of fungicide-resistant phenotypes in resulting conidial suspensions were evaluated by analyzing germ tube development of conidia on fungicide amended medium.

In order to create a selective medium, 10 ppm of PCNB (technical grade, dissolved in aceton) and 20 ppm of maneb (Maneb 80) were added to 2% WA medium. Each of the three fields on a diagnostic slide were coated with 60 µL of WA amended with 0, 10 ppm of thiophanate-methyl (Topsin® 500 SC) or 10 ppm of diethofencarb (technical grade, dissolved in aceton). Subsequently, 20 µL of conidial suspension adjusted to 1 x 10^4 conidia mL^{-1} was pipetted onto the surface of the culture medium.

After incubating slides 36 hours at 21°C and high relative humidity in the dark, germinated conidia, which showed normal or distorted germ tubes, were observed using a microscope at 200x magnification. At least 150 conidia were counted for each treatment and the percentage of conidia resistant to thiophanate-methyl was calculated by Equation 1.

$$BenR\% = \left[\left(\frac{N_{thiogerm}}{N_{thiogerm} + N_{thiodis}}\right) + \left(1 - \frac{N_{dietgerm}}{N_{dietgerm} + N_{dietdis}}\right)\right]/2 * 100$$

Equation 1 BenR%: percentage of *Botrytis cinerea* conidia resistant to 10 ppm of thiophanate-methyl; N_{thio}: number of counted conidia on FGA amended with 10 ppm of thiophanate methyl; N_{diet}: number of counted conidia on WA amended with 10 ppm of diethofencarb; $_{germ}$: germinated conidia showing normal germ tubes; $_{dis}$: germinated conidia showing distorted germ tubes.

2.7.5 LESION SIZE

For characterization of fitness parameters of selected *B. cinerea* isolates (chapter 2.1.1), aggressiveness of these isolates on detached leaves of grapevine was tested.

After incubation, leaves were photographed at constant light conditions and measured by image recognition software ImageJ® 1.45h (National Institute of Health, Bethesda, MD, USA). The lesion size of *B. cinerea* infection was expressed in square millimeters. Three to five lesions were measured per leaf and six biological replicates were used per isolate. The experiment was repeated twice.

2.8 APPLICATION OF FUNGICIDES

2.8.1 GREENHOUSE EXPERIMENTS

For determination of the competitive ability of fungicide-resistant isolates of *B. cinerea*, two months old grapevine plants (cv. 'Müller Thurgau') were cultivated as described in chapter 2.5.2. Plants were washed prior to fungicide application in order to remove residual sulfur. Thiophanate-methyl (Topsin® 500 SC) was applied with a hand atomizer at a rate equivalent to field rate (2.6 g L^{-1} a.i.). After spray of fungicide or sterile distilled water, plants were allowed to dry for 12 hours.

2.8.2 FIELD EXPERIMENTS

For the evaluation of resistance management strategies, thiophanate-methyl and mepanipyrim were applied in four different treatments from 2009 to 2011 (Table 2-4). Fungicides for control of *B. cinerea* were sprayed with a Stihl SR 320 mistblower (150 L ha^{-1}). Varying conventional fungicide treatments for control of *B. cinerea* were applied in the farmers' plots. All plots received the same commercial spray program for pests and pathogens other than *B. cinerea* (Appendix Table 7-1 to 7–3).

2.9 FIELD EXPERIMENTS

2.9.1 LOCATIONS AND EXPERIMENTAL SETUP

Field experiments were carried out from June 2009 to August 2011 under practical conditions at three commercial farms in the region of Bordeaux (France). The description of experimental sites and grapevine cultivars used are given in Table 2-3.

The experimental setup was as follows: Each plot had a size of 180 m². It consisted of five rows with twelve plants per row. Four plots per treatment were arranged in a completely randomized block design. Additionally, plots from surrounding fields of farmers were comprised in the trial as excluded controls.

Table 2-3 Experimental conditions of three experimental sites near Bordeaux (France).

	Location (Abbreviation)		
	Grezillac (A)	Saint Brice (B)	Loupes (C)
GPS-Data	N 44°80‘97“ E 0°22‘88“	N 44°69'44" E 0°18'33"	N 44°81'75" E 0°38'72"
Slope [%]	1	0	0
Soil type	Calcareous clay	Calcareous clay	Calcareous clay
Grapevine variety	Merlot	Muscadelle	Sauvignon
Rootstock	SO4	3309	3309
Pruning type	Guyot	Guyot	Guyot
Planting date	1981	1981	1995
Management system	Conventional	Conventional	Conventional
Distance within a row [m]	1	1	1
Row spacing [m]	3	3	3
Size of plot [m²]	180	180	180
Weather station	Saint Emilion	Saint Emilion	Latresne
Distance to station [km]	10	22	13

A draft of the experimental plan of the three sites is given in Appendix Figure 7-8 to 7–10. Different resistance management strategies were applied using thiophanate-methyl and mepanipyrim in four different treatments (Table 2-4). Weather data are given in Appendix Table 7-10 to 7–11 and Figure 7-4 to 7–7.

Table 2-4 Treatment schedules against *Botrytis cinerea* in the three experimental sites in the region of Bordeaux from 2009-2011.

Abbreviation	Application schedule and treatment	
	Stage A/B*	Stage C†
T1	Thiophanate-methyl (1050 g ha^{-1})‡	-
T2	Thiophanate-methyl (1050 g ha^{-1}) + Mepanipyrim (600 g ha^{-1})§	-
T3	Thiophanate-methyl (1050 g ha^{-1})	Mepanipyrim (600 g ha^{-1})
T4	Year 1 and 3: Thiophanate-methyl (1050 g ha^{-1}) Year 2 : Mepanipyrim (600 g ha^{-1})	-
T5	Conventional fungicide treatment	

* A: end of flowering (BBCH 65-68); B: before bunch closure (BBCH 77)
† C: beginning of berry ripening (BBCH 81-85)
‡ formulated product: Topsin® 500 SC (2.3 L ha^{-1})
§ formulated product: Japica® SC(1.2 L ha^{-1})

2.9.2 MONITORING OF *BOTRYTIS CINEREA*

2.9.2.1 Sampling

In June 2009 from each experimental site 120 to 150 samples were collected from plant organs of grapevine. Most samples were collected from old rachides with or without mummified berries and cane debris with visible sclerotia. Additionally, samples of flowers and leaves showing lesions were collected from plants. Geographical positions of collected samples were recorded by means of a GPS-handheld. For transportation samples were placed in 15 or 50 mL tubes with a dry paper tissue.

In June 2010 and May 2011 (BBCH 65 – 68) 96 samples of flowers were collected from three inner rows of each plot. The location of collection was noted and samples were placed in 48 well cell culture plates covered with a dry paper tissue and a lid.

In September 2009, 2010 and August 2011 at BBCH 89 up to 22 samples of berries infected by *B. cinerea* were collected in 50 mL sampling tubes with a dry paper tissue or samples were collected by lightly touching sporulating lesions with a cotton swab. Samples were taken from three inner rows of each plot and the location of collection was noted.

At transportation and after arrival in the laboratory samples were stored under cool conditions.

2.9.2.2 Disease assessment

From 2009 to 2011 disease incidence and severity of *B. cinerea* on 100 bunches of berries per plot were assessed at BBCH 89 prior to harvest. Disease incidence was expressed as percentage of bunches of berries infected by *B. cinerea* and disease severity was expressed as percentage of bunch area affected by *B. cinerea*.

2.10 MOLECULAR METHODS

2.10.1 DNA EXTRACTION

Mycelium of *B. cinerea* for DNA extraction was produced in 500 mL flask filled with 100 mL PDB by inoculation with three mycelial plugs per flask. After a five day incubation period at 22°C and 200 rpm, the content of the flask was homogenized and filtered using a vacuum pump. Mycelium was

collected in 2 mL tubes and frozen at -20°C. Samples were lyophilized and mycelium was homogenized using one to three 3 mm steel balls per tube in a mill (2000 rpm, 3 min). 20 mg of ground mycelium was transferred to a new 2 mL tube. For DNA extraction from *B. cinerea* mycelium the DNeasy® Plant Mini Kit protocol was used according to manufacturer's instructions (DNeasy® Plant Handbook – 08/2000). For DNA extraction from berries of grapevine infected with *B. cinerea* according to chapter 2.6.3 the Wizard® Magnetic DNA Purification System for Food protocol was used according to manufacturer's instructions (Instructions for use of products FF3750 and FF3751, revised 4/2009). Finally, quality and quantity of the extracted DNA was checked using a photometer and extracted DNA was stored at -20°C.

2.10.2 POLYMERASE CHAIN REACTION (PCR)

2.10.2.1 Design of primers

Primers were designed using Primer3 and BLAST-Software (NCBI, Bethesda, MD, USA), partial sequence data of β-tubulin of 24 *B. cinerea* isolates in EMBL Nucleotide Sequence Database (EBI, Cambridgeshire, UK, as of 04.2011) and the genome sequence of *B. cinerea* isolate B05.10 (Broad Institute, Cambridge, MA, USA). Primers designed in this study are given in Table 2-5.

2.10.2.2 Allele-specific PCR

In order to identify *B. cinerea* or to detect single nucleoid polymorphisms (SNPs) leading to benzimidazole resistance, several primer pairs were used (Table 2-5).

For each PCR reaction (25µl), 0.2 mM of each dNTP, 0.25 µM of each primer, 1 unit of DreamTaq™ DNA polymerase and 50 to 100 ng of template DNA were mixed with DreamTaq™ Green Buffer (2 mM $MgCl_2$) in thin-wall 8 strip PCR tubes. In each run DNA extracted from isolates BC-266.6, BC-11.3 and ultrapure water was used as references.

PCR assays were performed in a thermocycler using the following protocol: 94°C, 1 min, 1 cycle; 94°C, 30 s, 60°C, 30 s, 72°C, 1 min, 35 cycles; 72°C, 5 min, 1 cycle.

PCR products were loaded on 3 % (w/v) agarose gel and run in TAE buffer stained with ethidium bromide (0.5 µg mL^{-1}). A low range ladder was used as reference. Gel electrophoresis was run at 80 V and 400 mA for 90 min. Subsequently, DNA-fragments were visualized in a UV-transilluminator. The presence of specific fragments indicated presence of *B. cinerea* or SNP of interest.

Table 2-5 Sequence of primers designed for detection of *Botrytis cinerea*, partial sequencing of -tubulin gene and detection of single nucleotide polymorphisms (SNPs) leading to benzimidazole resistance; F: forward primer; R: reverse primer; Q: real-time PCR primer; AM: artificial mismatch in 3'-terminal region (underlined in sequence)

Name	Target gene/SNP	Nucleotide sequence (5'–3')	Reference
Detection of *B. cinerea*			
Bc-F	β-tubulin	GCTACCTTCTCCGTCGTC	LUCK *et al.* (1995)
Bc-R	β-tubulin	TTGAGTCAACTCTGGAACGG	LUCK *et al.* (1995)
Q-Bc1-F	IGS	GTTACTTGACATGCTCTGCCATT	SUAREZ *et al.* (2005)
Q-Bc1-R	IGS	CACGGCTACAGAAAGTTAGTCTACAA	SUAREZ *et al.* (2005)
Q-Bc3-F	β-tubulin	GCTGTAATTTCAATGTGCAGAATCC	SUAREZ *et al.* (2005)
Q-Bc3-R	β-tubulin	GGAGCAACAATTAATCGCATTTC	SUAREZ *et al.* (2005)
Partial sequencing of β-tubulin			
TUB-HPF1	β-tubulin	TGTCGAGCCATATAACGCAA	BANNO *et al.* (2008)
TUB-HPR1	β-tubulin	CCAACTTTCGGAGATCTGAG	BANNO *et al.* (2008)
Detection of SNP			
Bc-E198A	E198A	GGTTGAGAACTCTGACGC	LUCK *et al.* (1995)
Q-E198A	E198A	CAATTGGTTGAGAACTCTGACGC	This study
Q-E198A-AM	E198A	CAATTGGTTGAGAACTCTGACCC	This study
Bc-WT	Wild-type	GGTTGAGAACTCTGACGA	This study
Q-WT	Wild-type	CAATTGGTTGAGAACTCTGACGA	This study
Q-WT-AM	Wild-type	CAATTGGTTGAGAACTCTGACCA	This study
Q-F200Y	F200Y	GAACTCTGACGCGACCTA	This study
Q-R-1	Reverse primer	TGGTTAAGATCTCCGTAAGATGGG	This study
Q-R-2	Reverse primer	CCAAGTGGTTAAGATCTCCGTAAGA	This study
Q-R-3	Reverse primer	GGACATGACGGCGGAAAC	This study

2.10.2.3 EvaGreen® real-time PCR

In order to quantify the frequency of SNPs leading to benzimidazole resistance in populations of *B. cinerea*, different primer pairs were used in two separate reactions. In the first reaction, allele-specific primer pairs were used to determine the number of E198A copies (Q-E198A-AM/Q-R-2) or F200Y copies (F200Y/Q-R-3). In the second reaction, a species-specific primer pair (Q-Bc3-F/Q-Bc3-F) was used to determine the number of copies of the β-tubulin gene.

To create a standard curve, tenfold serial dilutions of genotype fungal DNA (ranging from 2.3×10^6 to 2.3×10^2 copies), which was extracted from BC-266.6 (E198A) or B-75-5 (F200Y), was tested in quadruplicate for each experimental run. Additionally, extracted DNA from isolates BC-266.6, BC-11.3, B-75-5 and ultrapure water were used as references in each run.

To create defined DNA pools with known frequency of resistance alleles, genomic DNA of BC-266.6 (E198A), BC-11.3 (wild-type) and B-75-5 (F200Y) were diluted to working concentrations of 4.7 x 10^5 copies μL^{-1}. DNA pools with a total copy number of 2.3 x 10^6 and known allele frequencies of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 1% were produced by diluting mutant allele DNA with wild-type DNA. Pooled DNA samples were allowed to equilibrate at room temperature for 1 h. Three technical replicates were used for each DNA pool with known frequency of resistance alleles.

To create DNA pools of field isolates from the experimental site near Saint Brice in August 2011, extracted DNA was diluted to working concentrations of 4.7 x 10^5 copies μL^{-1}. DNA of 17 to 22 isolates per plot were mixed to create DNA pools with a total copy number of 1.9 x 10^6. Pooled DNA samples were allowed to equilibrate at room temperature for 1 h. Two technical replicates were used for each DNA pool of field isolates.

Extracted DNA from infected berries of grapevine was adjusted to a concentration of 20 ng μL^{-1} genomic DNA. Each biological replicate was tested in duplicate.

For each real-time PCR reaction (20µl), 0.25 µM of each primer and template DNA (5 µL) were mixed with Fast Plus EvaGreen® Master Mix (high Rox) in a 96-well reaction plate and covered with adhesive film.

Real-time PCR assays were performed in a real-time thermocycler using the following protocol: 95°C, 5 min, 1 cycle; 95°C, 15 s, 60°C, 60 s, 40 cycles. Increase of fluorescent emission signal from the EvaGreen® dye was measured during the 60°C step with normalization of the signal by use of the ROX dye. Melting temperature of the amplicon was determined by subsequent melting curve steps at 95°C, 15 s and 60°C to 95°C, 60 s, in steps of 0.3°C s^{-1}.

Subsequently to determination of the copy number, the frequency of the resistance allele in the sample was calculated by Equation 2a. Relative standard deviation was used to describe the variation of calculated allele frequency according to Equation 2b.

(a) $$Allele\% = \frac{N_{allele}}{\bar{N}_{species}} * 100$$

(b) $$RSD\% = \frac{\sigma}{\bar{X}} * 100$$

Equation 2 *Allele%:* frequency of resistance allele; N_{allele}: copy number determined by allele-specific primer pair; $\overline{N}_{\text{species}}$ copy number determined by species-specific primer pair, RSD%: Relative standard deviation; σ: standard deviation; $\overline{X}$: mean resistance allele frequency.

2.11 DATA ANALYSIS

2.11.1 STATISTICAL ANALYSIS

For statistical analysis and graphical representation the statistical program IBM® SPSS® Statistics Version 20 (IBM Corp. Armonk, NY, USA) and MS® Office® Excel® 2010 (Microsoft Corp. Redmond, WA, USA) were used.

Normal distribution of data was confirmed by one sample Kolmogorov–Smirnov test ($p > 0.1$). Homogeneity of variances of the data set was tested by Levene's test ($p > 0.05$, KOEHLER *et al.* 2002). Fitness parameters of *B. cinerea* phenotypes were analyzed using one-factorial ANOVA and *a posteriori* Tukey's HSD test (KOEHLER *et al.* 2002). For comparison of two *B. cinerea* fungicide sensitivity groups, data was logarithmically transformed when necessary and analyzed by Student's *t* test ($p \leq 0.05$) for independent samples (KOEHLER *et al.* 2002). Percentage data of competitive ability experiments was transformed using arcsine square root to normalize data (MCDONALD, 2009). Data was analyzed by a univariate full-factorial general linear model (Typ III) and *a posteriori* Tukey's HSD test or Scheffé's test for normally distributed data or multiple Mann-Whitney-U tests for data without normal distribution at $p \leq 0.05$ (KOEHLER *et al.* 2002). The Pearson's Chi^2 test ($p \leq 0.05$) was used to compare survival rates of *B. cinerea* phenotypes (KOEHLER *et al.* 2002).

Dose-response relationship between mepanipyrim concentrations and mycelial growth of six *B. cinerea* isolates was determined by non-linear regression using the sequential quadratic programming estimation method. A four parameter model adapted from (STREIBIG, 1988) was used to calculate EC_{50} values (Equation 3a) and a formula adapted from (STREIBIG *et al.* 1995) was used to interpolate EC_{90} values (Equation 3b).

(a) $$Y = C + \frac{D-C}{1+e^{[b*(\ln(EC_{50})-\ln(X)]}}$$

(b) $$EC_{90} = EC_{50} * \left[\frac{(100-90)}{90}\right]^{\frac{1}{b}}$$

Equation 3 Y: relative mycelial growth; X: fungicide concentration; C: minimum asymptote; D: maximum asymptote; b: slope; $EC_{50/90}$: fungicide concentration, which reduces growth to 50 / 90 %.

To select a model for analysis of the frequency of fungicide-resistant *B. cinerea* phenotypes detected in field experiments three information criteria (-2 restricted log-likelihood, corrected Akaike's and Bayesian's) and graphical analysis of standardized residuals were used (FAHRMEIR, 2010). The chosen model was a univariat generalized linear model (Typ III) using experimental site,

date of monitoring, fungicide treatment, block and (maximum three-way) interactions as parameters. Parameters were connected normally and a Satterthwaite approximation for degrees of freedom was used. Wald Chi² statistics were calculated for model effects and *a priori* sequential Bonferroni tests ($p \leq 0.05$) were used for multiple comparisons (FAHRMEIR, 2010). Normal distribution, autocorrelation and homoscedasticity of variance of standardized residuals were determined graphically.

Disease incidence and severity of *B. cinerea* at experimental sites was analyzed by univariate full factorial general linear model (Type III) and *a posteriori* Tukey's HSD test ($p \leq 0.05$, KOEHLER *et al.* 2002). To test for correlation of percentage of fungicide-resistant phenotypes with disease incidence as well as severity Spearman's rank regression analysis was used (KOEHLER *et al.* 2002).

Real-time PCR fluorescence data was processed using the Applied Biosystems® StepOne™ Software v2.2.2 (Life Technologies Corp. Foster City, CA, USA). The threshold was set manually and the cycle reaching this point was called threshold cycle (C_t). For calculation of the copy number, a calibration curve was generated by plotting the C_t values against the logarithm of the copy number. Also, slope and coefficient of determination of the calibration curve and efficacy of the reaction was calculated. Melting temperature of the product was determined by dissociation of DNA during heating resulting in a reduction of the fluorescence signal. The first derivative of melting curve displays the maximum of fluorescence decline and indicates the melting temperature, at which 50% of DNA is dissociated (Handbook of ABI® StepOne™ Plus).

A validation of real-time PCR protocol was based on the determination of the level of detection and the level of quantification calculated according to IUPAC recommendations for analytical nomenclature (Equation 4).

(a) $$LOD = \bar{X}_{neg} + 3 * \sigma_{neg}$$

(b) $$LOQ = \bar{X}_{neg} + 10 * \sigma_{neg}$$

Equation 4 LOD: level of detection; LOQ: level of quantification; $\overline{X}_{neg}$: mean resistance allele frequency of wild-type template; σ_{neg}: standard deviation of wild-type template (INCZEDY, 1998).

Pearson linear regression analysis was used to test for correlations between known allele frequencies as well as percentage of resistant phenotypes and estimated allele frequencies. Normal distribution, autocorrelation of standardized residuals and homoscedasticity of variance was determined graphically. Paired sample Student's *t* test for normally distributed data or by Wilcoxon rank-sum test ($p \leq 0.05$) for data without normal distribution were used to identify differences between microbiological and molecular detection methods in treatments of field experiments. Differences in each plot between the resistance allele frequency and the percentage of fungicide-resistant phenotypes (set as test values) were analyzed by one sample Student's *t* test for normally distributed data or by Wilcoxon signed rank test ($p \leq 0.05$) for data without normal distribution (KOEHLER *et al.* 2002). The same anal-

yses were performed to show differences between microbiological and molecular detection methods within the berry inoculation experiment.

2.11.2 ANALYSIS OF SPATIAL AND TEMPORAL DISTRIBUTION

For spatial analysis of the field experiments in Bordeaux, collected fungicide-resistant phenotypes of *B. cinerea* were geo-referenced (WGS-1984) in a two dimensional data set using the geographic information system ESRI® ArcMap™ Editor 9.2 (ESRI Inc. Redlands, CA, USA). To test for complete spatial randomness, Moran's I significance test ($p \leq 0.05$) was performed using a dummy variable (0, 1) for absence or presence of fungicide-resistant phenotypes (MORAN, 1950).

To identify size of aggregation of benzimidazole-resistant phenotypes a Ripley's K analysis was performed with an edge correction by simulation of outer boundary values. Observed values were plotted against the radius of notional circles drawn around randomly chosen points. Also, the upper and lower 99% confidence envelopes under null hypothesis of complete spatial randomness were plotted in the graph. Observed values above the upper envelope indicate significant aggregation and those below the lower envelope indicate significant regularity (RIPLEY, 1977).

For further spatial analysis of benzimidazole-resistant phenotypes, binomial data (0, 1) had to be transformed to count values. A circular buffer zone of 2 m diameter was placed around each point and transformed point value was calculated according to Equation 5. Sequential ranks were assigned to transformed values of points. Ranks were multiplied by two for non-parametrical analysis (WINDER *et al.* 2008).

$$X_{trans} = \frac{\bar{X}_{buf} + X_a}{2}$$

Equation 5 X_{trans}: transformed value of point; X_a: dummy variable (0, 1) for absence or presence of fungicide-resistant phenotype of *Botrytis cinerea* at point P_a; $\overline{X}_{buf}$: mean value of points within the buffer of 2 m diameter placed on point P_a.

Spatial analysis of rank data was conducted using the software tool SADIE Shell Version 1.5.2 (IACR-Rothamsted, Harpenden, UK). SADIE stands for Spatial Analysis by Distance IndicEs. This tool is used for evaluation of two-dimensional distribution of geo-referenced data sets. The aggregation index (I_a) indicates the degree of aggregation. Values of $I_a = 1$indicate random distribution, while $I_a > 1$ indicates aggregation of observed counts into clusters. Due to hundreds of permutations a hypothesis test ($p_a \leq 0.05$) is possible (PERRY, 1995). Clustering index values were interpolated using Inverse Distance Weighting (IDW) method for graphical representation (PERRY *et al.* 2002). Also, clustering indices v_i and v_j, are calculated by SADIE, which indicate aggregations of similar ranks.

Gap clusters contain null values or small ranks ($v_j < -1$) and patch clusters contain high ranks ($v_i > 1$). In graphical representation a heuristic threshold of 1.5 is used. However, belonging to a cluster does not mean that there is a statistically significant ($p_i/p_j \leq 0.05$) aggregation (PERRY *et al.* 1999).

Spatiotemporal analysis was performed using the software tool Association Analysis Shell Version 1.5.2 (IACR-Rothamsted, Harpenden, UK). Interpolated SADIE cluster index values were assigned to point data of plants in the experimental field. Spatial correlation with adjustment for randomization was calculated between data sets of consecutive monitoring dates (PERRY and DIXON, 2002).

3 RESULTS

3.1 INFLUENCE OF RESISTANCE MANAGEMENT STRATEGIES ON POPULATION DYNAMICS OF *BOTRYTIS CINEREA* ISOLATES RESISTANT TO FUNGICIDES IN THREE VINEYARDS NEAR BORDEAUX

In order to study the influence of different resistance management strategies on the development of phenotypes of *B. cinerea* resistant to anti-microtubule and anilinopyrimidine fungicides within populations, a three year field trial at three vineyards near Bordeaux was initiated in 2009. Thiophanate-methyl (TM) and mepanipyrim (MP) were applied in three different resistance management strategies. A mixture of TM and MP was applied at end of flowering (mixture). Also, TM was applied at end of flowering and MP was applied at bunch closure (alternation). Additionally, one fungicide was applied in annual alternation at end of flowering. In 2009 and 2011, thiophanate-methyl was applied. In 2010, mepanipyrim was applied. Strategies were compared to one time solo application of TM and conventional fungicide treatments, where no benzimidazoles were applied. Monitoring procedures were conducted biannually at time of flowering (BBCH 65 - 68) and prior to harvest (BBCH 89). In total 1948, 1445 and 1665 isolates of *B. cinerea* were collected from the sites near Grezillac, Saint Brice and Loupes, respectively. Subsequently, frequencies of phenotypes resistant to fungicides in *B. cinerea* populations were determined. Discriminative concentrations of TM and MP were chosen according to EC_{50}- and EC_{90}-values of selected isolates (Appendix Table 7-4 and Figure 7-1).

3.1.1 DISEASE INCIDENCE AND DISEASE SEVERITY

In order to determine the efficacy of fungicide applications against *B. cinerea*, disease incidence expressed as percentage of bunches of berries infected and disease severity expressed as percentage of bunch area affected was measured prior to grapevine harvest at BBCH 89 in 2009 to 2011 at the three experimental sites near Bordeaux.

At the Loupes site no significant differences in percentages of infected bunches of berries as well as percentages of infected bunch area were detected in 2009 and in 2011 (8 - 21 % and 0.2 - 5.5 %). However, in 2010 alternation of TM and MP resulted in a significantly lower percentage of infected bunches of berries as well as percentage of infected bunch area (9 and 0.6 %) in comparison to the conventional fungicide treatment, where no fungicides to control *B. cinerea* were applied (20 and 1.8 %, Figure 3–1 c&f).

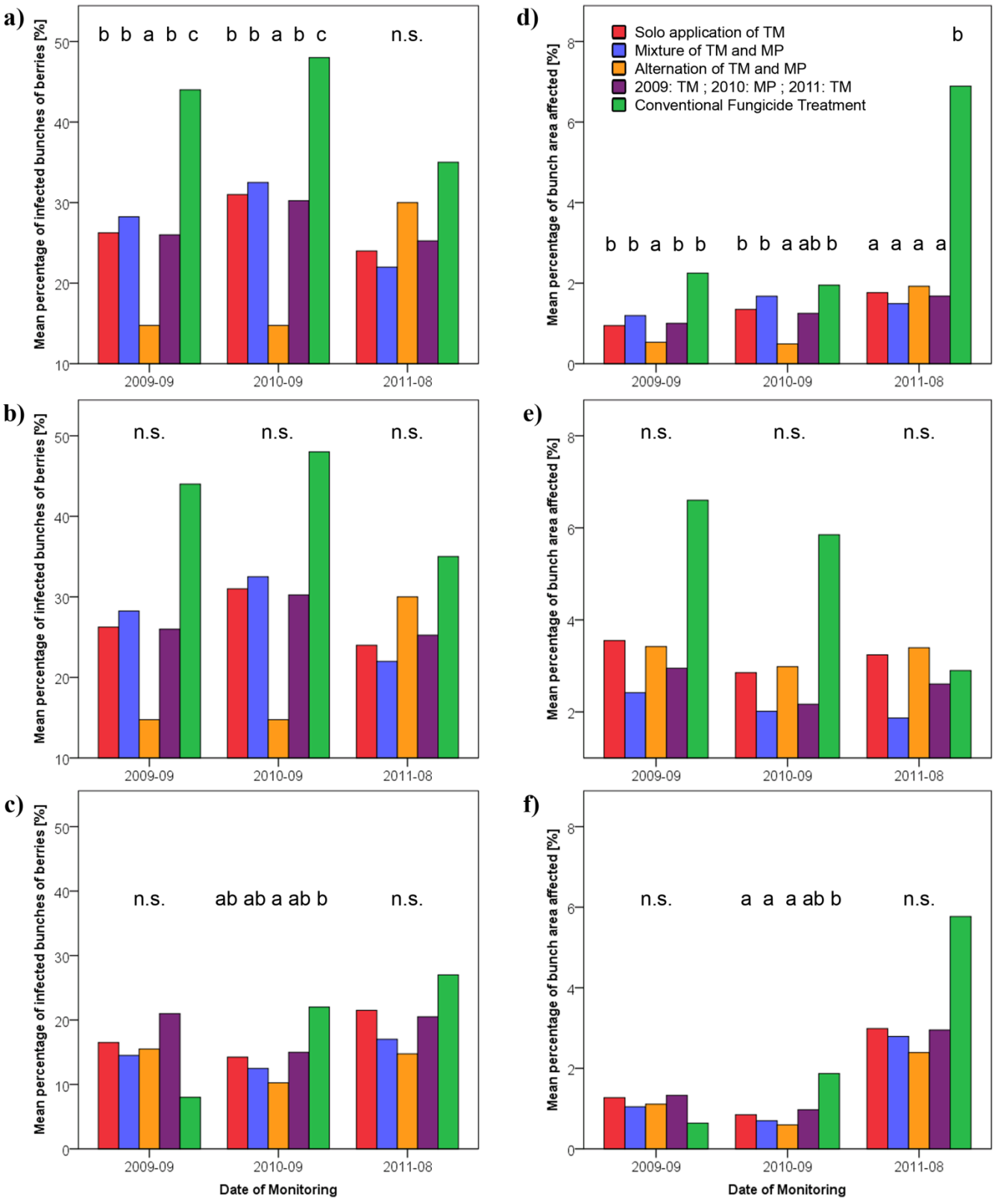

Figure 3-1 Effect of fungicide applications on disease incidence and disease severity caused by *Botrytis cinerea* on grapevine prior to harvest in 2009 to 2011 at three sites near Bordeaux (Grezillac: a&d, Saint Brice: b&e, Loupes: c&f). Disease incidence was expressed as infected bunches of berries (a–c) and disease severity was expressed as percentage of bunch area affected (d–f). At least 100 bunches of berries were measured in each of four repetitions per treatment. TM: Thiophanate-methyl; MP: Mepanipyrim; Conventional fungicide treatment: no fungicides to control *B. cinerea* were applied. Statistical analysis: identical letters show no significant difference (n.s.) between treatments according to Tukey's HSD test at p = 0.05.

At the Saint Brice site no significant differences in percentages of infected bunches of berries as well as percentage of infected bunch area were detected in 2009, 2010 and 2011 (11 - 24 % and 1.9 - 6.3 %, Figure 3–1 b&e). Additionally to the fungicide spray program of the resistance management strategies, cyprodinil and fludioxonil (Switch®) as well as iprodione (Rovral®) were applied in the farmer´s plots in 2009 and fenhexamid (Teldor®) was applied in all plots in 2010 (Table 7-2).

At the Grezillac site significantly lower percentages of infected bunches of berries as well as percentages of infected bunch area were measured in 2009 and 2010 for the alternation of TM and MP (14 - 15 % and 0.3 - 0.5 %) compared to the three fungicide spray programs (26 - 31 % and 1.0 - 1.9 %). The conventional fungicide treatment, where no fungicides to control *B. cinerea* were applied, resulted in a significantly higher percentage of infected bunches of berries (43 – 47 %) compared to all fungicide spray programs in 2009 and 2010. However, in 2011 there were no significant differences in percentages of infected bunches of berries were detected for all treatments. The conventional fungicide treatment in 2011 resulted in a significantly higher percentage of infected bunch area (7 %) compared to the four fungicide spray programs (0.4 - 1.8 %, Figure 3–1 a&d).

Relationships between percentages of fungicide-resistant phenotypes and percentages of infected bunches of berries or percentages of infected bunch area was determined by linear regression. No significant correlation with a coefficient of determination higher than 0.3 was detected (Appendix Table 7-9).

3.1.2 INCIDENCE OF PHENOTYPES RESISTANT TO ANTI-MICROTUBULE FUNGICIDES

Prior to application of fungicide spray programs, a monitoring was conducted in June 2009 to determine the sensitivity profile of the three experimental sites. 3 - 12 % of collected *B. cinerea* isolates showed a resistance to thiophanate-methyl (BenR1). No isolate with a multiple resistance to thiophanate-methyl and diethofencarb (BenR2) was found at the Grezillac and Loupes site. However, at the Saint Brice site 4 % of BenR2 isolates were detected.

After fungicide applications from June to August 2009 including thiophanate-methyl (TM), percentages of BenR1 and BenR2 isolates increased to 10 - 23 % and 5 - 16 % in comparison to the levels of resistance detected in June 2009. At all three experimental sites no significant differences in BenR1 and BenR2 isolate frequencies were observed between the four fungicide spray programs including TM. However, these treatments resulted in a significantly higher percentage of BenR1 and BenR2 isolates compared to the conventional fungicide treatments, where no TM was applied (0 % – 12 % and 0 – 1 %, Figure 3–2 a-c).

In June 2010 percentages of BenR1 isolates decreased to 6 - 17 % in plots treated with TM in 2009 for all experimental sites compared to those detected in September 2009. At the Grezillac site

decrease of BenR1 isolate frequencies was significant for plots treated with solo application of TM and alternation of TM and mepanipyrim (MP) in 2009. Also, at the Loupes site a significant decrease of BenR1 isolate frequencies was observed at mixture, alternation and annual alternation of TM and MP. In June 2010 at the Grezillac and Loupes site, frequencies of BenR1 and BenR2 isolates did not differ significantly between 2009 treated plots and the conventional fungicide treatments, where no TM was applied in 2009. Due to low isolation rate no statistics were calculated for the Saint Brice site.

After fungicide applications from June to August 2010 at the experimental sites near Grezillac and Loupes, percentages of BenR1 isolates increased significantly after solo application of TM, mixture and alternation of mepanipyrim (MP) and TM (29 - 64 %) compared to those detected in the monitoring in June 2009. In September 2010 no significant differences in BenR1 frequencies were observed between the three fungicide spray programs including TM in 2010. However, solo application of TM and alternation of TM and MP resulted in significantly higher frequencies of BenR1 isolates (29 -50 % and 44 - 64 %) compared to those detected in plots with conventional fungicide treatments and annual alternation, where no TM was applied in 2010 (6 - 8 % and 9 - 10 %, Figure 3–2 a&c). At the Saint Brice site a similar development of BenR1 isolate frequencies was observed, but it did not lead to significant differences between the four fungicide spray programs (Figure 3–2 b). However, all treatments including TM resulted in a significantly higher percentage of BenR1 isolates compared to the conventional fungicide treatment, where no TM was applied (6 %, Figure 3–2 a-c). In September 2010 frequencies of BenR2 isolates (0 % - 13 %) did not differ significantly between treatments at all three experimental sites.

Comparing monitoring date September 2010 and September 2009 at the experimental sites near Grezillac and Loupes, a significant increase of BenR1 isolate frequencies was detected in plots treated with a mixture or alternation of TM and MP. Solo application of TM and the conventional fungicide treatments resulted in a nonsignificant increase of BenR1 isolates. However, annual alternation of TM and MP resulted in a significant decrease of BenR1 frequencies from September 2009 to 2010. At the Saint Brice site a similar development of BenR1 isolate frequencies was observed, but it did not lead to significant differences between the monitoring dates.

In May 2011 percentages of BenR1 and BenR2 isolates decreased in most TM treated plots in 2010 for all experimental sites (0 - 37 % and 0 - 5%) compared to those detected in September 2010. Due to low isolation rates no statistics were calculated.

After fungicide applications from May to July 2011 including TM, percentages of BenR1 isolates increased to 30 - 66 %. In August 2011 no significant differences in BenR1 frequencies were observed for the four fungicide spray programs. However, all four spray programs including TM resulted in a significantly higher percentage of BenR1 isolates compared to conventional fungicide treatments, where no TM was applied at all three experimental sites (4 - 5 %, Figure 3–2 a-c).

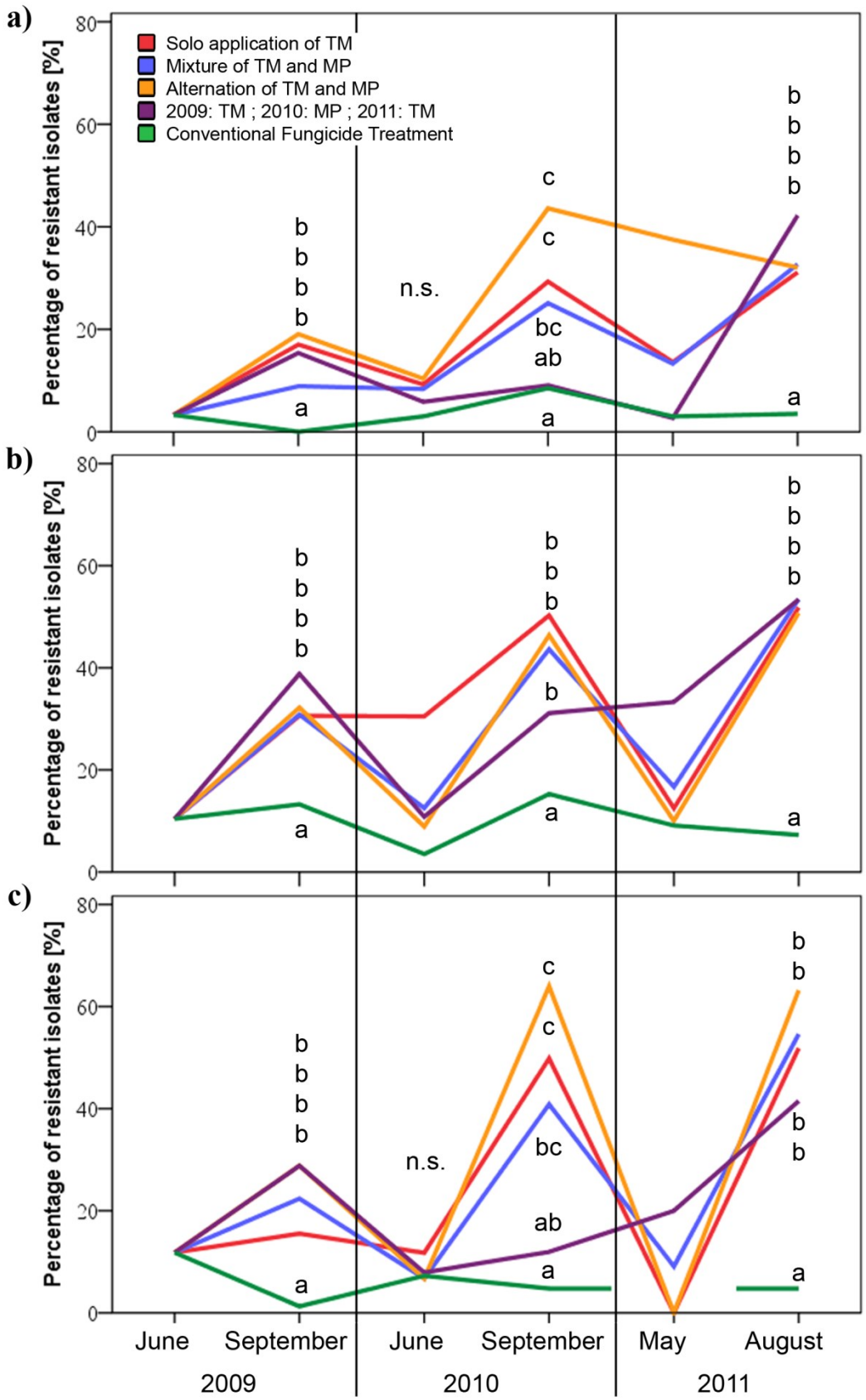

Figure 3-2 Effect of resistance management strategies on percentage of *Botrytis cinerea* isolates resistant to thiophanate-methyl (TM) collected from three sites near Bordeaux (a: Grezillac, b: Saint Brice, c: Loupes) in June 2009 to August 2011. 96 flowers or 22 berries were collected from each of four plots per treatment. MP: mepanipyrim. Statistical analysis: identical letters show no significant difference (n.s.) between treatments according to a generalized linear model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05. Samplings with low number of isolates were excluded from statistical analysis.

In August 2011 frequencies of BenR2 isolates (0 % - 13 %) did not differ significantly between treatments at the experimental sites near Saint Brice and Loupes. However, at the Grezillac site the annual alternation lead to a significantly higher percentage of BenR2 isolates compared to the other three fungicide spray programs and the conventional fungicide treatment.

Comparing monitoring date September 2010 and August 2011 at the experimental sites near Grezillac and Loupes, a significant increase of BenR1 isolate frequencies was detected in case of annual alternation of TM and MP. Comparing monitoring date September 2009 and August 2011, a significant increase of BenR1 isolate frequencies was observed in plots treated with solo application of TM, mixture and alternation of TM and MP at the Loupes site. Additionally, at the Grezillac site a significant increase was observed at mixture of TM and MP. For all other treatments at both sites and all treatments at Saint Brice site a non-significant increase from September 2009 to August 2011 was observed. Also, no significant differences in percentages of BenR2 isolates were observed comparing different dates of monitoring (Appendix Table 7-5 to 7–7).

3.1.3 INCIDENCE OF PHENOTYPES WITH A REDUCED SENSITIVITY TO ANILINOPYRIMIDINES

Prior to application of spray programs, a monitoring in June 2009 was conducted to determine the sensitivity profile of the experimental sites. 6 - 13 % of collected *B. cinerea* isolates showed a reduced sensitivity to MP (AniR). 0 - 4% showed a reduced sensitivity to MP and to TM (AniRBenR).

After fungicide application at the Grezillac site from June to August 2009, percentages of AniR isolates differed significantly between alternation of MP and TM (32 %) and all other treatments (8 - 12 %, Figure 3–3 a). At the Loupes site there were no significant differences in AniR frequencies between the four fungicide spray programs and conventional fungicide treatment (4 - 12 %), where no anilinopyrimidines were applied (Figure 3–3 c). At the Grezillac and Loupes site no significant differences in percentage of BenRAniR isolates were detected between the four fungicide spray programs and the conventional fungicide treatments (0 - 8 %), where no anilinopyrimidines were applied (Figure 3–3 a&c). However, at the Saint Brice site all four fungicide spray programs resulted in a significantly higher percentage of AniR isolates (13 - 24 %) compared to the conventional fungicide treatment, where cyprodinil and fludioxonil (Switch®) as well as iprodione (Rovral®) were applied (1 %, Figure 3–3 b). Additionally, the mixture of TM and MP resulted in a significantly higher percentage of BenRAniR (20 %) compared to the conventional fungicide treatment, where no anilinopyrimidines were applied (1 %, Figure 3–3 b).

Compared to September 2009, percentages of AniR and BenRAniR isolates remained stable at 7 - 17 % and 0 - 4 % in June 2010. At the Grezillac and Loupes site, frequencies of AniR and BenRAniR isolates did not differ significantly between plots treated in 2009 and the conventional fungicide treatments, where no anilinopyrimidines were applied in 2009.

After fungicide application from June to August 2010, percentages of AniR and BenRAniR isolates increased significantly for plots treated with alternation of TM and MP (28 % and 26 – 27 %) compared to those in June 2010. At the Loupes and Grezillac site, this treatment resulted in a significantly higher percentage of AniR and BenRAniR isolates in September 2010 compared to solo application of TM and the conventional fungicide treatment (9 - 11 %, Figure 3–3 a&c and Figure 3–4 a&c), where no anilinopyrimidines were applied. Additionally, solo application of MP in 2010 at the Grezillac site resulted in a significantly higher percentage of AniR isolates compared to the solo application of TM and the conventional fungicide treatment, where no anilinopyrimidines were applied (Figure 3–3 a). At the Saint Brice site no significant differences in BenRAniR and AniR frequencies were detected between the fungicide spray programs and the conventional treatment (7 - 14 %), where fehexamid (Teldor®) was applied in all plots (Figure 3–3 b and Figure 3–4 b).

Comparing the monitoring date September 2010 and September 2009 at the Grezillac site, percentages of AniR and BenRAniR isolates increased significantly within annual alternation of TM and MP. Additionally, alternation of TM and MP resulted in a significant increase of BenRAniR isolates at the Grezillac and Loupes site.

In May 2011 percentages of AniR and BenRAniR isolates remained stable at 0 - 25 % and 0 - 20 %. Due to low isolation rates no statistics were calculated.

At the Loupes site fungicide applications from May to July 2011 resulted in significantly higher percentages of AniR and BenRAniR at mixture and alternation of TM and MP (26 - 33 % and 18 - 29 %) compared to the conventional fungicide treatment (2 % and 0 %), where no anilinopyrimidines were applied (Figure 3–3 c and Figure 3–4 c). At the Saint Brice site a significantly higher percentage of AniR isolates was detected at alternation of TM and MP (40 %) compared to solo application and annual alternation of TM and MP (11 - 16 %), where no anilinopyrimidines were applied in 2011 (Figure 3–3 b). Additionally, alternation and mixture of TM and MP lead to a significantly higher percentage of BenRAniR isolates compared (17 - 20 %) to the conventional fungicide treatment (1 %), where no anilinopyrimidines were applied (Figure 3–4 b). At the Grezillac site no significant differences in percentages of AniR and BenRAniR isolates between treatments were detected (9 - 15 % and 4 - 8 % Figure 3–3 a and Figure 3–4 a).

Comparing the monitoring date August 2011 and September 2010 at the Grezillac and Loupes site, no significant differences were observed. However, at the Saint Brice site annual alternation of TM and MP resulted in a significant increase of percentages of AniR and BenRAniR isolates. Comparing the monitoring date August 2011 to September 2009, frequencies of AniR and BenRAniR increased significantly at the Saint Brice and Loupes site. Interestingly, the conventional fungicide treatment at the Saint Brice site, where fenhexamid (Teldor®) was applied in 2011 in all plots, resulted in a significant increase of AniR isolates in August 2011 compared to September 2009.

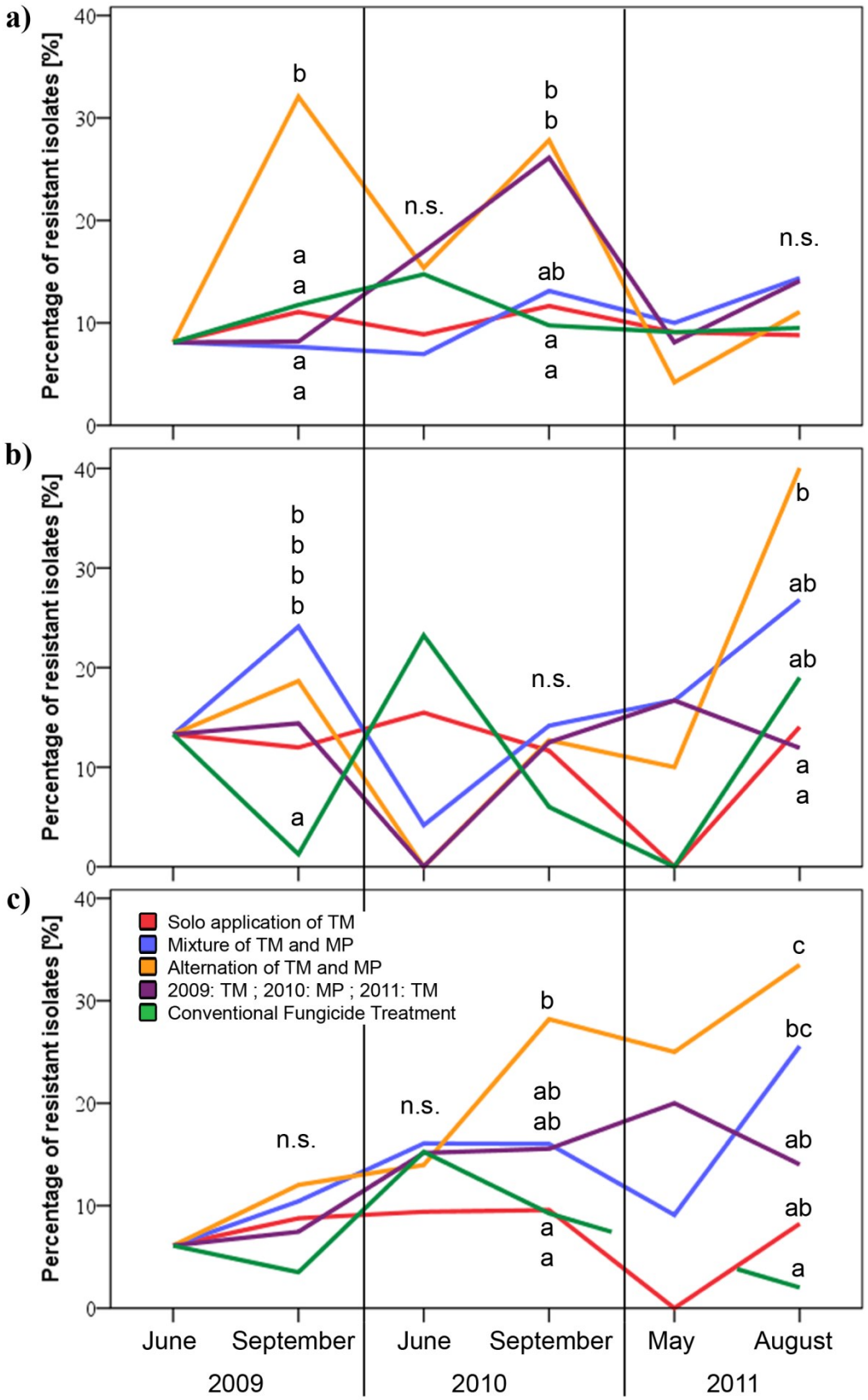

Figure 3-3 Effect of resistance management strategies on the percentage of *Botrytis cinerea* isolates with a reduced sensitivity to mepanipyrim (MP) collected from three experimental sites near Bordeaux (a: Grezillac, b: Saint Brice, c: Loupes) in June 2009 to August 2011. 96 flowers or 22 berries were collected from each of four plots per treatment. TM: thiophanate-methyl. Statistical analysis: identical letters show no significant difference (n.s.) between treatments according to a Generalized Linear Model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05. Samplings with low number of isolates were excluded from statistical analysis.

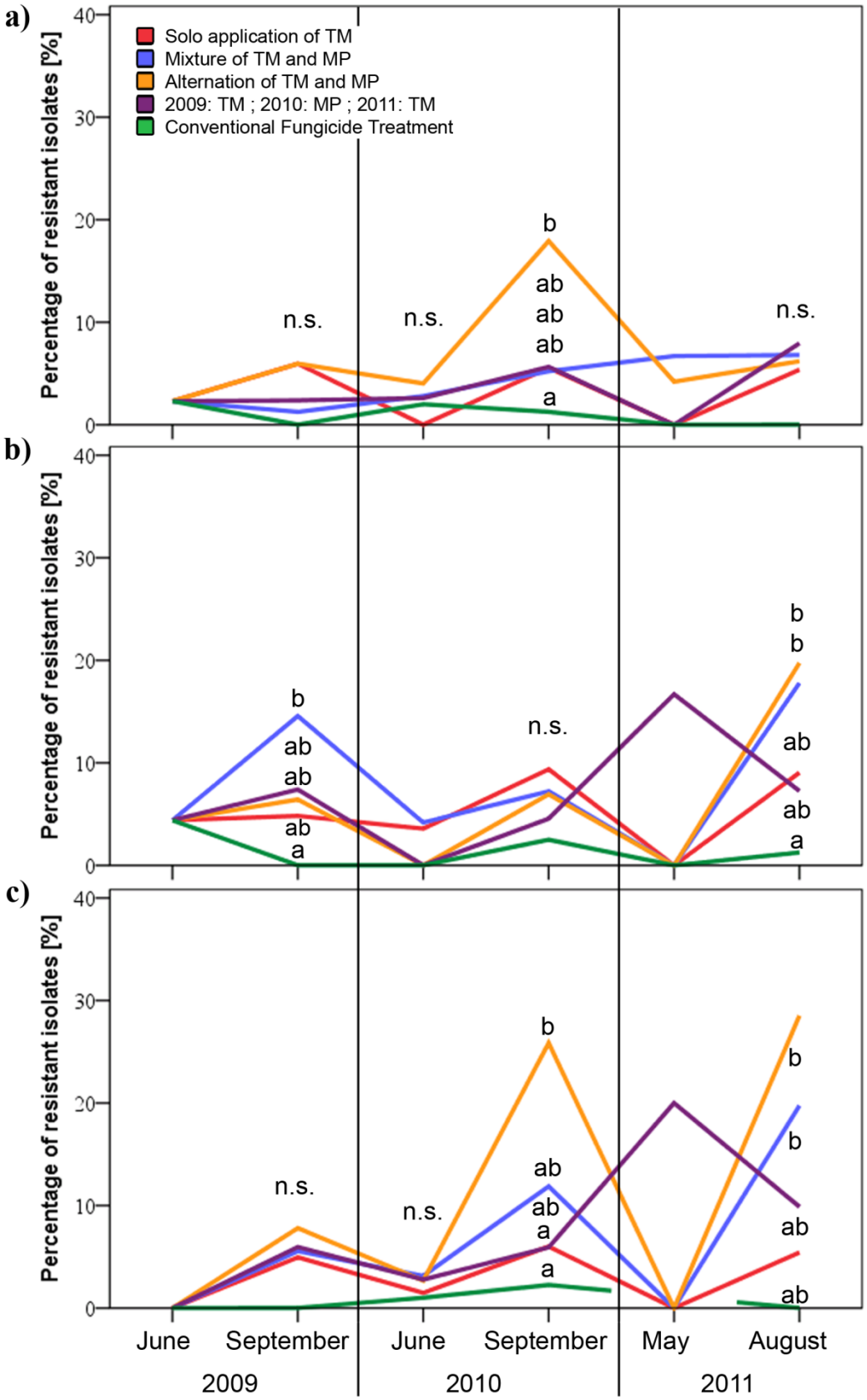

Figure 3-4 Effect of resistance management strategies on the percentage of *Botrytis cinerea* isolates with a resistance to thiophanate-methyl (TM) and a reduced sensitivity to mepanipyrim (MP) collected from three experimental sites near Bordeaux (a: Grezillac, b: Saint Brice, c: Loupes) in June 2009 to August 2011. 96 flowers or 22 berries were collected from each of four plots per treatment. Statistical analysis: identical letters show no significant difference between (n.s.) treatments according to a Generalized Linear Model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05. Samplings with low number of isolates were excluded from statistical analysis.

3.2 SPATIAL AND TEMPORAL DISTRIBUTION OF BENZIMIDAZOLE-RESISTANT ISOLATES OF *BOTRYTIS CINEREA*

The results of the preceding chapter showed a change in frequency of fungicide-resistant phenotypes in populations of *B. cinerea* due to fungicide applications and winter periods. In order to get further information about the spread between plots and the spatial persistence of resistant phenotypes, the degree and size of aggregation as well as its' orientation were analysed. For spatial analysis, incidence of isolates of *B. cinerea* was geo-referenced and different geo-statistical methods were applied (Moran's I, Ripley's K and SADIE). Subsequently, spatial data of successive monitoring dates were correlated for temporal analysis.

3.2.1 GREZILLAC

Significant aggregations of benzimidazole-resistant phenotypes (BenR) and phenotypes with a reduced sensitivity to anilinopyrimidines (AniR) were observed at monitoring dates prior to harvest according to Moran's I (Table 3-1). Fungicide-resistant phenotypes other than those mentioned above showed no significant aggregation at any date of monitoring (Appendix Table 7-12).

Table 3-1 Aggregation indexes for benzimidazole-resistant *Botrytis cinerea* isolates at Grezillac for six dates of monitoring: Moran`s I and indexes calculated by non-parametric SADIE analysis: I_a: aggregation index; v_i: patch cluster index; v_j: gap cluster index. Statistical analysis: p-values derived by hypothesis tests under H_0 of spatial randomness. Indices in boldface indicate significant spatial autocorrelation at $p \leq 0.05$.

Index/ p-value	Date of Monitoring					
	2009		2010		2011	
	June (n=92)	September (n=410)	June (n=340)	September (n=413)	May (n=292)	August (n=401)
Moran's I	-0.037	**0.111**	-0.016	**0.106**	0.005	**0.045**
p-value	0.424	0.000	0.496	0.000	0.837	0.000
I_a	0.96	**1.99**	1.02	**1.60**	1.44	**1.59**
p-value	0.618	0.001	0.398	0.019	0.058	0.024
v_i	0.66	**2.08**	1.39	**1.74**	1.30	**1.56**
p-value	0.927	0.000	0.064	0.014	0.123	0.033
v_j	-0.62	**-2.07**	-1.04	**-1.61**	**-1.64**	**-1.73**
p-value	0.964	0.001	0.338	0.018	0.026	0.014

Moran's I, aggregation index (I_a) and the patch clustering index (v_i) of SADIE analysis matched closely. All indices showed that BenR isolates were significantly aggregated at monitoring dates prior to harvest. However, declining p-values of I_a and v_i indicated a decrease of the degree of aggregation in successive years (Table 3-2).

By overlay analysis of the interpolated clustering index values and the field trial map, gap and patch clusters could be assigned to fungicide treatments. In June 2009, no significant aggregation (I_a=0.96) was observed due to low sample size. In September 2009, significant patch clusters (v_i=2.08) were observed in plots treated with a solo application of thiophanate-methyl (TM), alternation of TM and mepanipyrim (MP) and annual alternation TM and MP (TM in 2009). Ripley's K analysis identified significant aggregations with a diameter up to 3 m. Also, a big gap cluster was located in the intersection of these treatments in the north-eastern part of the trial field. A mixture of TM and MP treated plots showed either gap clusters or little patch clusters (Figure 3–5 b).

In June 2010, BenR isolates were not aggregated (I_a=1.02). In September 2010, plots treated with solo application of TM and alternation of TM and MP resulted in patch clusters (v_i=1.74) bigger than plot size, which was confirmed by Ripley's K analysis identifying significant aggregations with a diameter up to 30 m. Annual alternation of TM and MP (MP in 2010) resulted in gap or small patch clusters. Application of the mixture of TM and MP resulted in both big gap and patch clusters (Figure 3–5 d).

In May 2011, no significant patch clusters (I_a=1.44, v_i=1.30) were observed (Figure 3–5). In August 2011, patch clusters bigger than plot size, which was confirmed by Ripley's K analysis identifying significant aggregations with a diameter up to 34 m. Additionally, small patch clusters were observed in all fungicide treatments (Figure 3–5 f). In all three years no patch, but significant gap clusters (v_j=-1.61 to -2.07) were observed in conventionally treated plots, where no TM was applied, even though they were located east of the TM-treated plots, which corresponds to the main direction of the wind (Figure 3–5).

Table 3-2 Temporal analysis of spatial distributions of benzimidazole-resistant *Botrytis cinerea* isolates of six successive dates of monitoring at Grezillac. Spatial distributions were calculated by non-parametric SADIE analysis. Statistical analysis: asterisks indicate significant correlation between monitoring dates according to hypothesis test under H_0 of no association ($p \leq 0.05$).

Date of Monitoring		2009		2010		2011	
		June (n=92)	September (n=410)	June (n=340)	September (n=413)	May (n=292)	August (n=401)
2009	June	-	-	-	-	-	-
	Sept.	0.037	-	-	-	-	-
2010	June	0.024	**0.280***	-	-	-	-
	Sept.	-0.069	**0.299***	0.042	-	-	-
2011	May	0.081	0.038	0.037	**0.269***	-	-
	August	0.044	**0.552***	-0.022	**0.349***	**0.257***	-

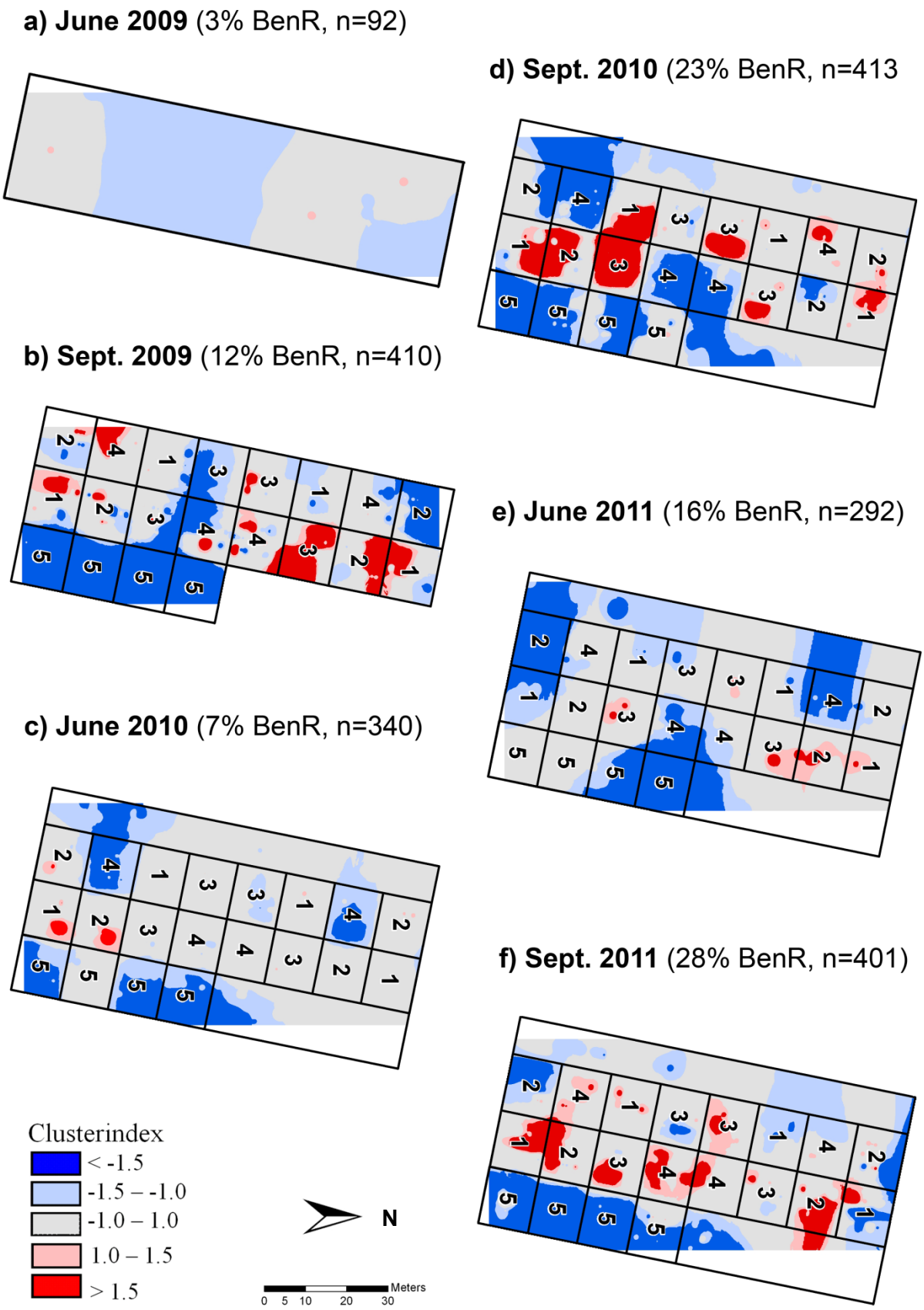

Figure 3-5 Effect of fungicide applications on the spatial distribution of benzimidazole-resistant *Botrytis cinerea* isolates expressed as interpolated cluster index values calculated by non-parametric SADIE analysis for six dates of monitoring (a – f) at Grezillac. Red shading indicates patchiness and blue shading indicates gaps. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

A comparison of cluster indices throughout the experimental period showed significant positive correlations for successive monitoring dates. Also, monitoring dates prior to harvest showed significant positive correlations with one another. The highest overall measure of association ($X = 0.552$) was calculated for correlation of monitoring dates September 2009 and August 2011. Due to a low number of isolates, no significant associations were observed for June 2009 (Table 3-2).

3.2.2 SAINT BRICE

Significant aggregations of BenR and AniR phenotypes were observed at most monitoring dates prior to harvest according to Moran's I. Additionally, aggregations of isolates with a reduced sensitivity to anilinopyrimidines and resistance to benzimidazoles (AniRBenR) were significant at monitoring conducted in September 2009. Fungicide-resistant phenotypes other than those mentioned above showed no significant aggregation at any date of monitoring (Appendix Table 7-12).

Moran's I, aggregation index (I_a) and the patch clustering index (v_i) of SADIE analysis matched closely. All indices showed that BenR isolates were significantly aggregated at monitoring dates prior to harvest. The monitoring conducted in June 2010 showed in a significant aggregation of BenR isolates (Table 3-3).

Table 3-3 Aggregation indexes for benzimidazole-resistant *Botrytis cinerea* isolates at Saint Brice for six dates of monitoring: Moran`s I and indexes calculated by non-parametric SADIE analysis: I_a: aggregation index; v_i: patch cluster index; v_j: gap cluster index. Statistical analysis: p-values derived by hypothesis tests under H_0 of spatial randomness. Indices in boldface indicate significant spatial autocorrelation at $p \leq 0.05$.

Index/ p-value	Date of Monitoring					
	2009		2010		2011	
	June (n=48)	September (n=375)	June (n=125)	September (n=426)	May (n=82)	August (n=389)
Moran's I	0.033	**0.015**	**0.215**	**0.028**	0.001	**0.057**
p-value	0.068	0.000	0.001	0.000	0.882	0.000
I_a	1.38	**2.01**	**1.37**	**2.42**	0.79	**2.85**
p-value	0.062	0.001	0.026	0.000	0.924	0.000
v_i	1.18	**2.25**	**1.85**	**2.84**	0.79	**3.07**
p-value	0.057	0.000	0.001	0.000	0.924	0.000
v_j	-1.41	**-2.04**	-1.25	**-2.32**	-0.77	**-2.77**
p-value	0.17	0.001	0.097	0.000	0.891	0.000

By overlay analysis of the interpolated clustering index values and the field trial map, gap and patch clusters could be assigned to fungicide treatments. In June 2009, no significant aggregation (I_a=1.38) was observed due to low sample size (Appendix Figure 7-9). In September 2009, significant patch and gap clusters (v_i=2.25; v_j=-2.04) were observed in all plots regardless of fungicide treatment (Figure 3–6 b). No patch cluster was bigger than a plot, which was confirmed by Ripley's K analysis identifying significant aggregations with a diameter up to 15 m.

In June 2010, a gap cluster (v_j=-1.25) was located in the northern part of the trial field. Only one patch cluster (v_i=1.85) was located in a plot treated with alternation of thiophanate-methyl and mepanipyrim (Figure 3–6 c). Ripley's K analysis identified significant aggregations with a diameter up to 24 m. In September 2010, one big patch cluster (v_i=2.84) including plots of all fungicide treatments was observed, which was confirmed by identification of significant aggregations with a diameter up to 86 m. Plots at the western edge of the site showed no patch clusters (Figure 3–6 d).

In May 2011, no significant aggregations were observed due to low sample size (Appendix Figure 7-8). In August 2011, a big patch cluster (v_i=3.07) was located in the middle of the trial field including plots of all fungicide treatments. Also, significant aggregations with a diameter up to 83 m were identified. The plots at the north-western as well as the north-eastern edge of the experimental site showed no patch clusters (Figure 3–6 f).

A comparison of cluster indices throughout the experimental period showed significant positive correlations between monitoring dates prior to harvest with one another. The highest overall measure of association (X = 0.573) was calculated for correlation of monitoring dates September 2010 and August 2011. Additionally, a significant association was observed for monitoring date June 2009 and June 2010. In general, monitoring dates at time of flowering showed an overall dissociation to monitoring dates prior to harvest and *vice versa*. Due to a low number of isolates, no significant associations were observed for monitoring date June 2010 and May 2011 (Table 3-4).

Table 3-4 Temporal analysis of spatial distributions of benzimidazole-resistant *Botrytis cinerea* isolates of six successive dates of monitoring at Saint Brice. Spatial distributions were calculated by non-parametric SADIE analysis. Statistical analysis: asterisks indicate significant correlation between monitoring dates according to hypothesis test under H_0 of no association ($p \leq 0.05$).

Date of Monitoring		2009		2010		2011	
		June (n=48)	September (n=375)	June (n=125)	September (n=426)	May (n=82)	August (n=389)
2009	June	-	-	-	-	-	-
	Sept.	-0.073	-	-	-	-	-
2010	June	**0.291***	0.123	-	-	-	-
	Sept.	-0.089	**0.324***	0.087	-	-	-
2011	May	0.033	0.170	0.208	-0.022	-	-
	August	-0.157	**0.452***	0.193	**0.573***	-0.056	-

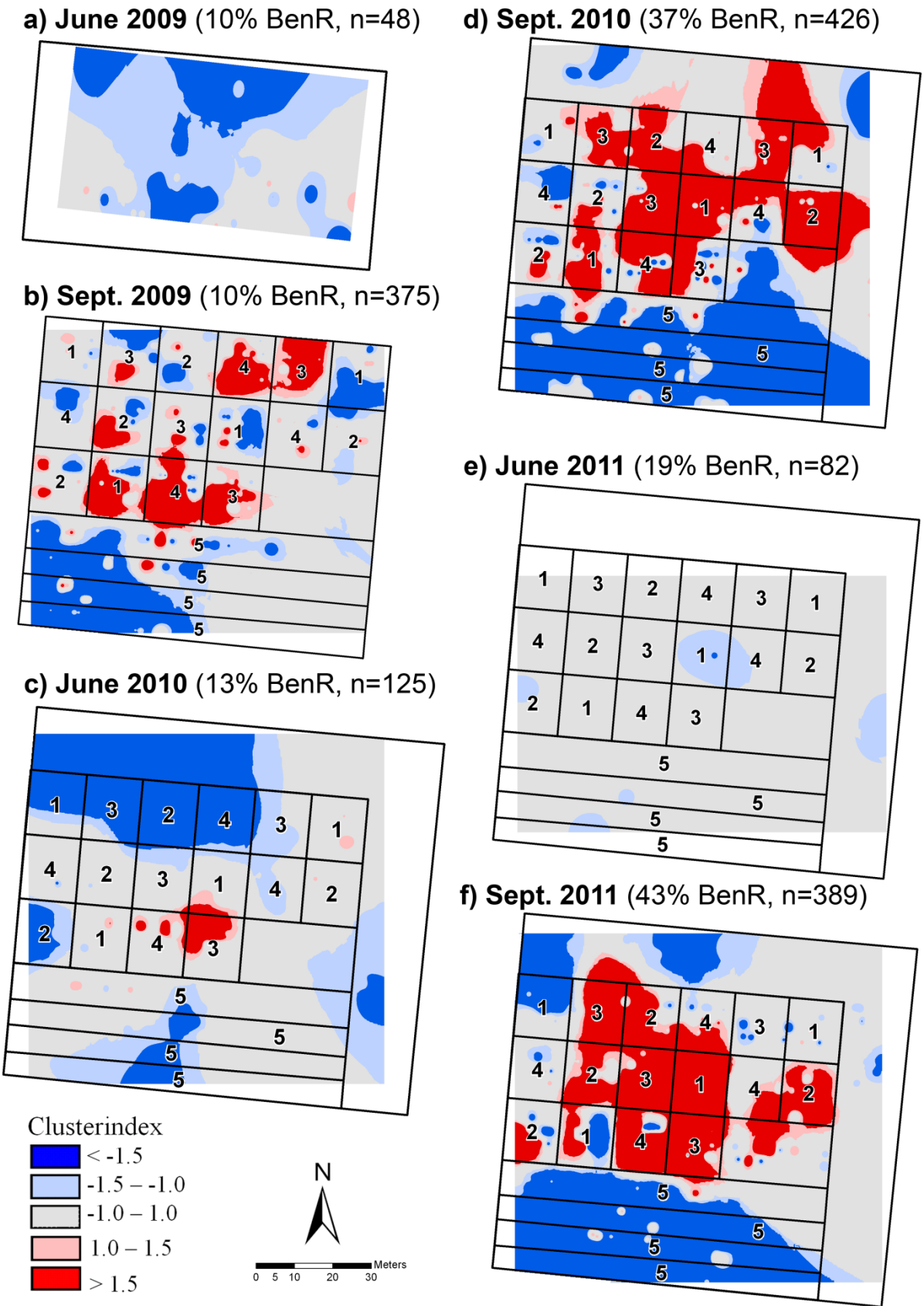

Figure 3-6 Effect of fungicide applications on the spatial distribution of benzimidazole-resistant *Botrytis cinerea* isolates expressed as interpolated cluster index values calculated by non-parametric SADIE analysis for six dates of monitoring (a – f) at Saint Brice. Red shading indicates patchiness and blue shading indicates gaps. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

3.2.3 LOUPES

BenR and AniR phenotypes showed a significant aggregation for monitoring dates prior to harvest as well as for monitoring date June 2010 according to Moran's I. Additionally, aggregations of AniRBenR phenotypes were significant at the monitorings conducted in September 2010 and August 2011. Fungicide-resistant phenotypes other than those mentioned above showed no significant aggregation at any date of monitoring (Appendix Table 7-12.)

Moran's I and the patch clustering index (v_i) of SADIE analysis matched closely. All indices showed that BenR isolates were significantly aggregated prior to harvest. Additionally, monitoring conducted in June 2010 disclosed a significant aggregation of BenR isolates (Table 3-5).

Table 3-5 Aggregation indexes for benzimidazole-resistant *Botrytis cinerea* isolates at Loupes for six dates of monitoring: Moran`s I and indexes calculated by non-parametric SADIE analysis: I_a: aggregation index; v_i: patch cluster index; v_j: gap cluster index. Statistical analysis: p-values derived by hypothesis tests under H_0 of spatial randomness. Indices in boldface indicate significant spatial autocorrelation at $p \leq 0.05$.

Index/ p-value	Date of Monitoring					
	2009		2010		2011	
	June (n=28)	September (n=379)	June (n=423)	September (n=408)	May (n=48)	August (n=359)
Moran's I	0.035	**0.039**	**0.077**	**0.191**	-0.011	**0.016**
p-value	0.308	0.016	0.000	0.000	0.736	0.007
I_a	0.75	1.43	**2.33**	**1.77**	1.20	1.26
p-value	0.735	0.071	0.009	0.034	0.197	0.215
v_i	0.74	**1.75**	**3.31**	**2.22**	1.47	**1.74**
p-value	0.736	0.041	0.000	0.009	0.079	0.032
v_j	-0.76	-1.20	**-2.17**	-1.44	-1.20	-1.15
p-value	0.681	0.256	0.001	0.103	0.275	0.291

By overlay analysis of the interpolated clustering index values and the field trial map, gap and patch clusters could be assigned to fungicide treatments. In June 2009, no significant aggregation (I_a=0.75) was observed due to a low sample size (Appendix Figure 7-9). In September 2009, patch clusters (v_i=1.75) were observed with significant aggregations showing a diameter up to 40 m in all northern plots regardless of fungicide treatment. In other parts of the trial field plots treated with different fungicide treatments showed patch as well as gap clusters (Figure 3–7 b).

In June 2010, the significant patch cluster (v_i=3.31) in the northern plots decreased to a diameter of up to 12 m. Additionally, a patch cluster appeared in the center and in the southern part of the trial field (Figure 3–7 c). In September 2010, SADIE analysis identified a significant patch cluster (v_i=2.22) including three plots at the northern edge with significant aggregations showing a diameter up to 26 m. In other parts of the trial field plots treated with different fungicide treatments showed patch as well as gap clusters regardless of fungicide treatment (Figure 3–7 d).

In May 2011, no significant aggregation (I_a=1.2 0) was observed due to low a sample size (Appendix Figure 3-7c). In August 2011, significant patch clusters (v_i=1.74) were observed in the northern as well as in the southern part of the trial field regardless of fungicide treatment. Ripley's K analysis identified significant aggregations showing a diameter up to 24 m. In general, patch clusters dominated most plots (Figure 3–7 f). In all three years no patch, but gap clusters were observed in conventionally treated plots located next to the trial field in the western direction. However, in conventionally treated plots located next to the trial field in eastern direction, which corresponds to the main direction of the wind, patch clusters were observed less frequently (Figure 3–7 b - f). However, the absence of patch clusters could be due to a low sample size in this region of the trial field (Appendix Figure 7-10).

A comparison of cluster indices throughout the experimental period showed highly significant positive correlations between monitoring dates prior to harvest to another. Additionally, monitoring date June 2010 showed significant associations to monitoring dates September 2010 and August 2011, even if the overall measure of association was low. The highest overall measure of association (X = 0.521) was calculated for the correlation of monitoring dates September 2010 and August 2011. Due to a low number of isolates, no significant associations were observed for monitoring date May 2011 (Table 3-6).

Table 3-6 Temporal analysis of spatial distributions of benzimidazole-resistant *Botrytis cinerea* isolates of six successive dates of monitoring at Loupes. Spatial distributions were calculated by non-parametric SADIE analysis. Statistical analysis: asterisks indicate significant correlation between monitoring dates according to hypothesis test under H_0 of no association ($p \leq 0.05$).

Date of Monitoring		2009		2010		2011	
		June (n=28)	September (n=379)	June (n=423)	September (n=408)	May (n=48)	August (n=359)
2009	June	-	-	-	-	-	-
	Sept.	-0.148	-	-	-	-	-
2010	June	-0.103	0.034	-	-	-	-
	Sept.	-0.186	**0.327***	**0.116***	-	-	-
2011	May	0.191	-0.045	0.134	0.151	-	-
	August	-0.071	**0.315***	**0.151***	**0.512***	0.039	-

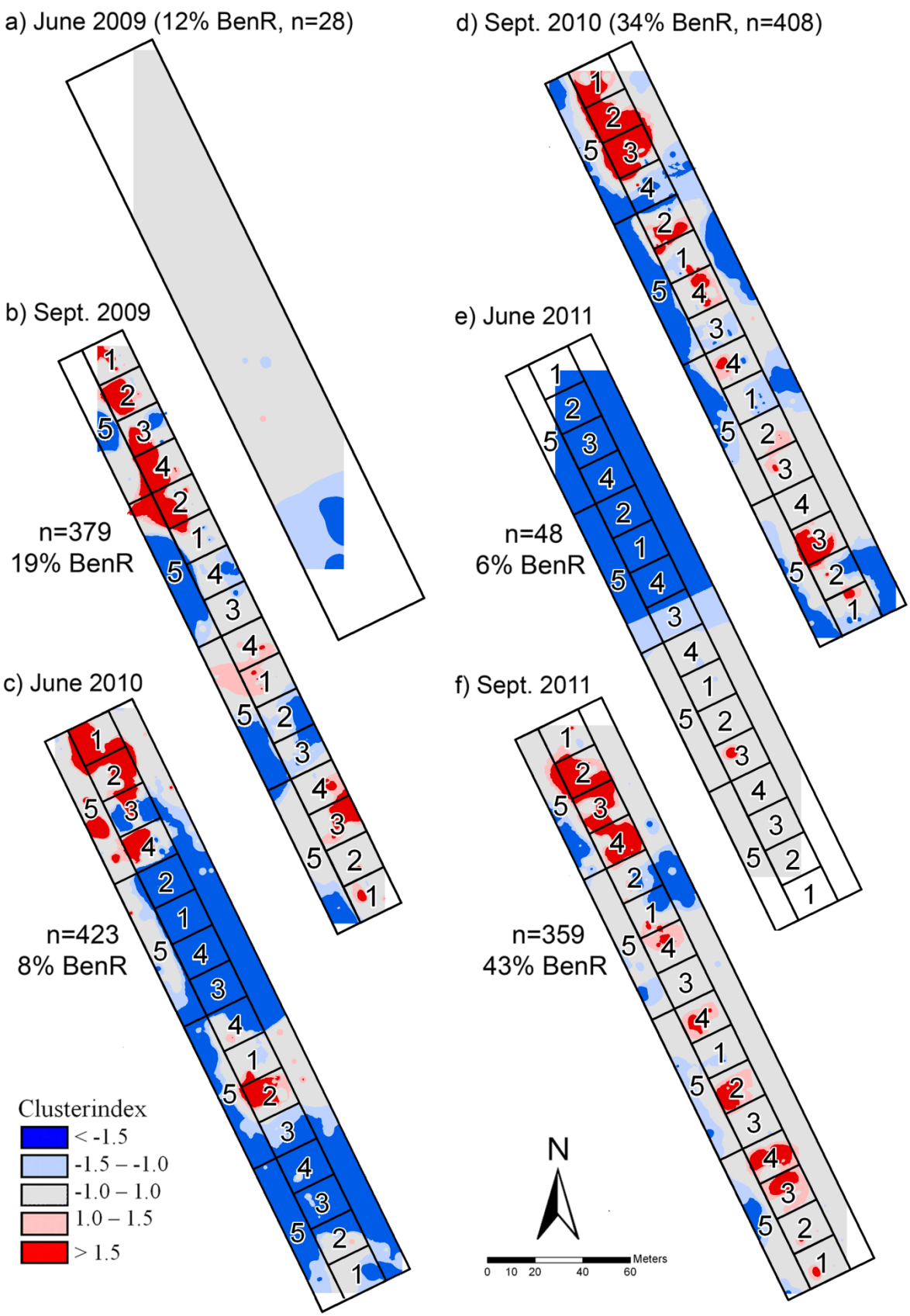

Figure 3-7 Effect of fungicide applications on the spatial distribution of benzimidazole-resistant *Botrytis cinerea* isolates expressed as interpolated cluster index values calculated by non-parametric SADIE analysis for six dates of monitoring (a – f) at Loupes. Red shading indicates patchiness and blue shading indicates gaps. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

3.3 FREQUENCY OF ALLELES CONFERRING BENZIMIDAZOLE RESISTANCE IN POPULATIONS OF *BOTRYTIS CINEREA*

Single nucleoid polymorphisms (SNPs) confer high levels of benzimidazole-resistance in *B. cinerea* (BenR1-phenotype). Some SNPs lead to a double resistance to benzimidazoles as well as N-phenyl-carbamates (BenR2-phenotype). The presence of those SNPs can have important implications on the use strategy of benzimidazoles. Therefore, methods were developed for detection and quantification of SNPs leading to benzimidazole resistance.

3.3.1 GENETIC CHARACTERIZATION OF BENZIMIDAZOLE-RESISTANT ISOLATES OF *B. CINEREA*

In order to detect BenR isolates prior to fungicide application, a monitoring was conducted in June 2009. In total, 16 of 174 *B. cinerea* isolates were resistant to benzimidazoles. Subsequently, two separate allele-specific PCRs (as-PCR) were performed in order to identify SNPs responsible for resistance and to verify DNA-extraction by amplification of β-tubulin gene fragments.

Amplification of DNA from all 16 BenR isolates and the two reference isolates resulted in a product of 381 bp length using primer pair Bc-F/Bc-R (Figure 3-8 a). However, 13 of 16 BenR isolates and reference isolate BC-266.6 amplified a product of 281 bp using primer pair BC-E198A/BC-R. No fragment was visible for the isolates B-75-2, B-75-5 and B-87-1 as well as for the reference isolate BC-11.3 (Figure 3-8 b). No fragment was visible for the water template in both reactions.

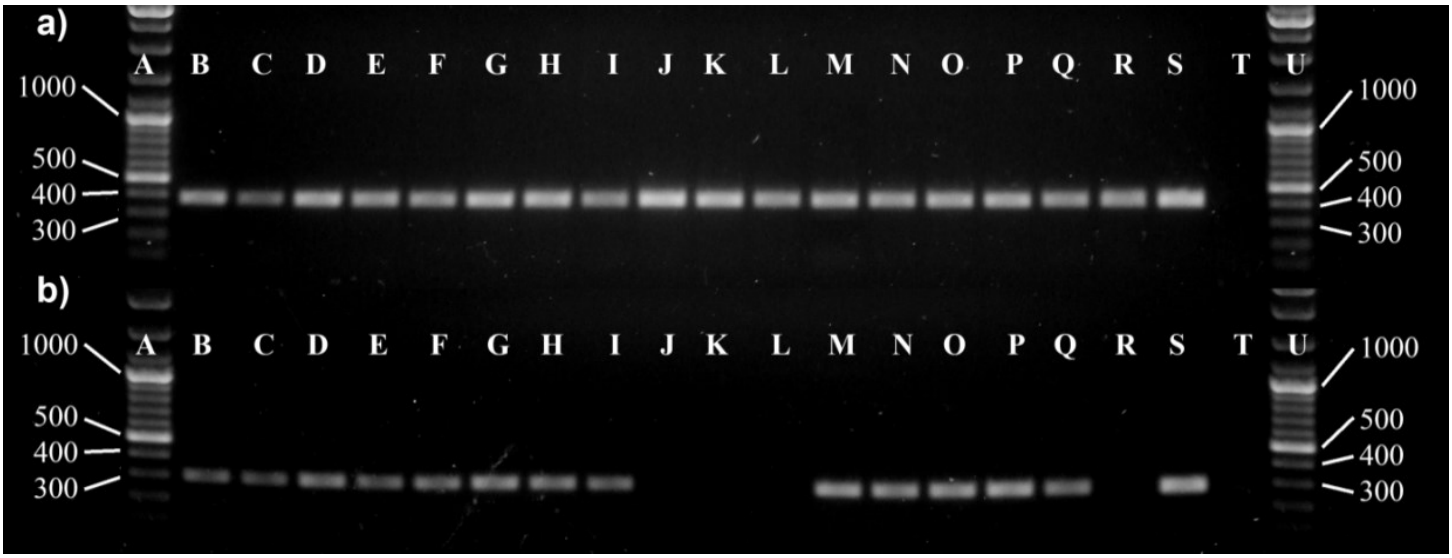

Figure 3-8 Presence of the E198A-mutation in 13 of 16 *Botrytis cinerea* isolates obtained in the monitoring conducted at three sites near Bordeaux in June 2009. Amplification of β-tubulin gene fragments using (a) primer pairs BC-F/BC-R to identify *B. cinerea* and (b) BC-E198A/BC-R to detect E198A-mutation. Tracks are as follows: B: A-69-3, C: A-69-1, D: A-107-1, E: A-107-4, F: A-126-3, G: B-13-4, H: B-13-3, I: B-42-2, J: B-75-2, K: B-75-5, L: B-87-1, M: C-67-3, N: C-81-3, O: C118-2, P: B-25-5, Q: C-141-2; R: BC-11.3; S: BC-266.6; T: water template, A&U: 100 bp ladder.

All three field isolates showed the BenR2 phenotype. Thus, primers pairs were designed to identify the unknown SNPs (Appendix Table 7-13). Field isolates B-75-2, B-75-5, B-87-1 were tested in a duplex as-PCR using the additional primer pair Bc2-F/Bc2-R to verify DNA-extraction by amplification of a β-tubulin gene fragment. Amplification of DNA from all three field isolates resulted in a product of 121 bp length using primer pair Q-F200Y/Q-R3. Additionally, all isolates except BC-266.6 yielded a product of 132 bp length using primer pair Q-WT-AM/Q-R1. Only reference isolate BC-266.6 amplified a product of 115 bp length using primer pair Q-E198A-AM/Q-R2. A product of 95 bp length was amplified for the three field isolates and the two reference isolates using primer pair Bc2-F/Bc3-R. No fragment was visible for the water template in any of the three reactions (Figure 3-9).

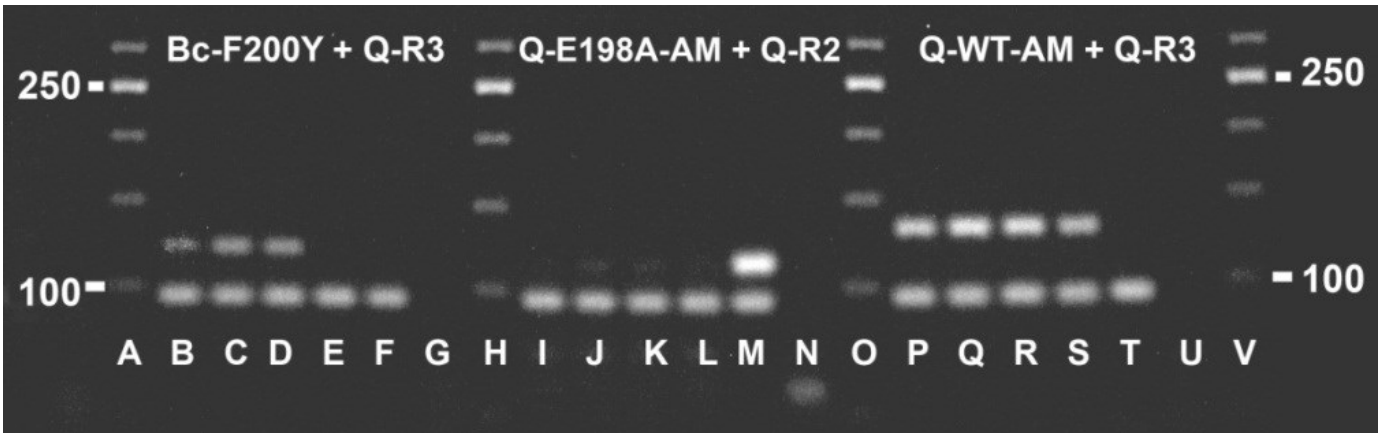

Figure 3-9 Presence of the F200Y-mutation in all three diethofencarb-resistant isolates of *Botrytis cinerea* obtained in the monitoring conducted in June 2009. Amplification of β-tubulin gene fragments using primer pairs Bc-F200Y/Q-R3, Q-E198A-AM/Q-R2 or Q-WT-AM/Q-R3 and additionally Bc2-F/Bc3-R to detect SNPs and to identify *B. cinerea*, respectively. Tracks: B,I,P: B-75-2, C,J,Q: B-75-5, D,K,R: B-87-1, E,L,S: B-11.3, F,M,T: B-266.6, G,N,U: water template, A,H,O,V: ladder.

3.3.2 VALIDATION OF REAL-TIME PCR PROTOCOL FOR RESISTANCE ALLELES

In order to quantify the resistance alleles in populations of *B. cinerea*, a real-time as-PCR protocol was developed. Mismatch-primers were used to identify the SNPs and a suitable reaction buffer was chosen (Appendix Table 7-14). For validation of the real-time as-PCR protocol, resistance allele frequencies were measured and compared to known allele frequencies in DNA pools of defined populations as well as berries inoculated with mixed isolate suspensions of *B. cinerea*.

3.3.2.1 Resistance alleles in defined populations

Resistance allele frequencies were measured in two separate real-time as-PCRs. In the first reaction, allele-specific primer pairs were used to determine the number of E198A or F200Y copies. In the second reaction, a species-specific primer pair Bc1-F/Bc1-R was used to determine the number of copies of the β-tubulin gene. Subsequently, resistance allele frequency was calculated.

Standard curves for quantification of E198A, F200Y allele and total copy number of the β-tubulin gene showed slopes of -3.30, -3.17 and -3.45, respectively. Efficacies of 101 %, 105 % and 94 % were derived from these slopes. Standard curves showed coefficients of determination of 0.997, 0.995 and 0.999, respectively.

A comparison of the expected E198A allele frequency with the measured E198A allele frequency showed a positive correlation with a slope of 0.934 and coefficient of determination of 0.991. This indicated that the method is valid over a wide range of allele frequencies (Table 3-7). Standard deviation of the threshold cycle number (C_t) did not exceed 0.2 cycles (Appendix Table 7-15). The wild-type template showed a significant amplification with a C_t of 29.2 resulting in a theoretical E198A allele frequency of 0.15 %. However, it`s melting temperature peak was at 78.6 ± 0.1°C compared to 79.0 ± 0.2°C of pure E198A template. Primer dimers (if present) showed two weak melting temperature peaks at 75 ± 2°C. Level of detection (LOD) and quantification (LOQ) were estimated as 0.18 % and 0.25 % E198A allele frequency (Table 3-7). To exclude false positive samples, only samples with an E198A allele frequency higher than 0.25 % were considered as positive in following experiments.

Table 3-7 Validation of the allele-specific real-time PCR protocol by correlation of expected and measured E198A or F200Y allele frequency in DNA pools of defined *Botrytis cinerea* populations. RSD: relative standard deviation; LOD: level of detection; LOQ: level of quantification. Coefficient of determination (R^2) was calculated by Pearson's linear regression analysis.

Expected resistance allele frequency [%]	n	Measured resistance allele frequencies			
		E198A allele frequency		F200Y allele frequency	
		Mean [%]	RSD [%]	Mean [%]	RSD [%]
0	2	0.15	6.5	0.002	31.2
1	2/3	0.97	0.8	0.7	27.7
5	3	3.6	8.3	4.1	16.2
10	3	7.8	8.2	11.0	13.5
20	2	18.5	6.6	18.7	20.9
30	3	28.5	4.1	28.7	10.3
40	3	35.1	2.4	45.1	7.3
50	3	47.7	1.6	48.4	5.9
60	3	58.1	1.6	62.7	10.7
70	3	65.9	3.0	71.4	4.5
80	3	77.8	6.2	84.5	3.1
90	3	88.0	3.5	91.4	2.8
100	3	97.5	4.4	102.2	4.4
LOD	-	0.18		0.005	
LOQ	-	0.25		0.01	
R^2	34/35	0.991		0.985	

A comparison of expected F200Y allele frequency to measured F200Y allele frequency showed a positive correlation with a slope of 1.013 and a coefficient of determination of 0.985. This indicated that the method is valid over a wide range of allele frequencies (Table 3-7). Standard deviation of C_t-values did not exceed 0.4 cycles (Appendix Table 7-15). The melting curve analysis of pure F200Y template showed one melting temperature peak at 79.9 ± 0.2°C. The wild-type template as well as water control showed formation of primer dimers with two weak melting temperature peaks at 77 ± 2°C. A C_t of 36.7 was measured, which resulted in a theoretical F200Y allele frequency of 0.002 % for pure wild-type template. Level of detection and quantification were estimated as 0.005 % and 0.01 % F200Y allele frequency, respectively (Table 3-7). Therefore, only samples with a F200Y allele frequency higher than 0.01 % were considered as positive in following experiments.

3.3.2.2 E198A allele frequency in inoculated berries

The results of the preceding chapter showed a validation of the real-time as-PCR protocol using pools of extracted DNA from *B. cinerea*. In order to validate the protocol for *B. cinerea* in tissue of grapevine, berries were inoculated with mixed isolate suspensions. After incubation, the percentage of benzimidazole-resistant (BenR1) conidia washed off from berries prior to DNA-extraction was compared to the E198A allele frequency detected by real-time as-PCR protocol.

Standard curves for the quantification of the E198A and the total copy number of the β-tubulin gene showed efficacies of 102 % and 93 %, which were derived from slopes of -3.27 and -3.52. Standard curves showed coefficients of determination of 0.995 and 0.997.

Table 3-8 Validation of the allele-specific real-time PCR protocol for *Botrytis cinerea* in berries of grapevine by comparison of the measured mutant E198A allele frequency to the percentage of benzimidazole-resistant (BenR1) conidia determined by fungicide sensitivity assay. Berries were inoculated with mixed isolate suspensions of *B. cinerea*. R: benzimidazole-resistant conidia. S: benzimidazole-sensitive conidia. LOQ: Level of quantification (0.25 %). Statistical analysis: significant difference between methods according to Student's *t* or Wilcoxon rank-sum test for paired samples ($p \leq 0.05$).

Mixed isolate Inoculum	n	Percentage of BenR1 conidia [%]		Measured E198A frequency [%]		p-value
		Mean	SD	Mean	SD	
100% R	2	100.0	0.0	102.6	4.5	1.000
100% S	2	0.0	0.0	<LOQ	-	-
9% R : 91% S	3	0.2	0.4	12.7	1.8	**0.006**
Mock inoculation	2	-	-	<LOQ	-	-

For berries inoculated with benzimidazole sensitive conidial suspension (100% S), no BenR1 conidia as well as an E198A allele frequency lower than the level of quantification (LOQ) was detected. Inoculation of berries with BenR1 conidial suspension (100% R) resulted in suspensions containing only BenR1 conidia. Furthermore, quantification of the E198A allele showed a frequency of about

100 %. Mock inoculation caused no disease symptoms and as-PCR did not lead to amplification of *Botrytis* specific DNA fragments confirmed by melting curve analysis (Table 3-8).

Interestingly, inoculation with a mixed isolate suspension (9% R : 91% S) resulted in a significantly lower percentage of BenR1 conidia (0.2 %) compared to the E198A allele frequency (12.7 %). Comparing the initial resistance frequency of 9.0 % to the E198A allele frequency (12.7 %) no significant differences were detected. In contrast, comparison of the initial frequency of 9.0 % to the percentage of BenR1 conidia (0.2 %) showed a significant difference (Table 3-8).

3.3.3 QUANTIFICATION OF RESISTANCE ALLELES IN FIELD POPULATIONS OF *B. CINEREA*

The results in chapter 3.1.2 showed an increase in *B. cinerea* phenotype frequency resistant to anti-microtubule fungicides for all resistance management strategies tested. In order to study the influence of resistance management strategies on the frequency of single nucleotide polymorphisms (SNPs) conferring benzimidazole resistance, DNA was extracted from 408 *B. cinerea* isolates collected from 20 plots of the Saint Brice site in August 2011. The extracted DNA from each plot was pooled, it`s resistance allele frequency was measured by real-time as-PCR and compared to the percentage of phenotypes resistant to benzimidazoles.

Standard curves for quantification of the E198A, the F200Y allele and the total copy number of the β-tubulin gene showed efficacies of 108 %, 105 % and 95 %, which were derived from slopes of -3.13, -3.15 and -3.44, respectively. Standard curves showed coefficients of determination of 0.997, 0.998 and 0.999, respectively.

In the plots B-T5-R3 and B-T5-R4, no isolates resistant to thiophanate-methyl and sensitive to diethofencarb (BenR1) were found. In agreement with these results, E198A allele frequencies lower than the level of quantification (LOQ) were detected. Similar results were obtained for F200Y allele frequencies of the plots B-T5-R1, B-T5-R2 and B-T5-R4, where no isolate resistant to thiophanate-methyl and diethofencarb (BenR2) was found (Table 3-9). A correlation of resistant phenotype frequencies with resistance allele frequencies showed a coefficient of determination of 0.963 for E198A and BenR1 as well as 0.870 for F200Y and BenR2 frequency. Resistance allele frequencies deviated from the resistance phenotype frequency rarely by more than 3 %. However, the plots B-T1-R1, B-T2-R3 and B-T3-R1 showed a significantly lower E198A allele frequency compared to the BenR1 phenotype frequency. In addition, the plots B-T1-R4, B-T2-R1 and B-T4-R4 showed a significantly lower F200Y allele frequency compared to the BenR2 phenotype frequency (Table 3-9). No significant differences between the two detection methods were found comparing means of four repetitions of each fungicide application.

Table 3-9 Real-time allele specific PCRs and fungicide sensitivity assays showed similar results when testing field populations of *Botrytis cinerea* collected at the Saint Brice site in August 2011. Measured E198A and F200Y resistance allele frequencies in pooled DNA samples of each plot were compared to percentages of phenotypes resistant to anti-microtubule fungicides: BenR1: thiophanate-methyl resistant; BenR2: diethofencarb-resistant; n: number of isolates collected from each plot; LOQ: Level of quantification (E198A: 0.25 %, F200Y: 0.01). Statistical analysis: asterisk indicates significant difference between percentage of resistant phenotypes and resistance allele frequency of each plot according to one sample Student's *t* test or by Wilcoxon signed rank test ($p \leq 0.05$).

Plot	n	Percentage of BenR1 isolates [%]	E198A allele frequency [%]		Percentage of BenR2 isolates [%]	F200Y allele frequency [%]	
			Mean	SD		Mean	SD
B-T1-R1	17	**23.5**	**16.1***	1.9	0.0	<LOQ	-
B-T1-R2	20	85.0	79.3	7.4	5.0	5.6	3.2
B-T1-R3	22	40.9	42.8	2.0	14.3	12.8	2.5
B-T1-R4	17	52.9	55.2	3.4	**17.6**	**10.0***	0.1
B-T2-R1	17	62.5	59.6	5.4	**25.0**	**20.7***	0.4
B-T2-R2	22	45.5	49.8	3.6	9.1	10.4	1.3
B-T2-R3	19	**26.3**	**20.4***	1.3	10.5	12.0	0.5
B-T2-R4	20	45.0	42.7	3.2	15.0	14.0	3.7
B-T3-R1	20	**40.0**	**33.2***	2.1	10.0	12.5	0.8
B-T3-R2	21	52.4	47.1	4.9	4.8	2.7	2.6
B-T3-R3	22	54.5	53.0	2.9	8.5	8.2	0.1
B-T3-R4	22	22.7	22.6	1.4	9.1	9.1	1.1
B-T4-R1	20	42.4	45.7	3.4	17.6	15.7	1.4
B-T4-R2	20	20.0	22.8	3.2	20.0	24.0	4.0
B-T4-R3	22	54.5	52.1	1.7	18.2	18.0	0.4
B-T4-R4	22	31.8	30.2	3.7	**9.1**	**5.9***	0.2
B-T5-R1	20	10.0	13.3	3.6	0.0	<LOQ	-
B-T5-R2	22	4.5	5.5	0.3	0.0	<LOQ	-
B-T5-R3	21	0.0	<LOQ	-	9.1	8.1	2.0
B-T5-R4	22	0.0	<LOQ	-	0.0	<LOQ	-

3.4 FITNESS OF BENZIMIDAZOLE-RESISTANT ISOLATES OF *BOTRYTIS CINEREA*

The results of chapter 3.1.2 showed a change in frequency of anti-microtubule fungicide-resistant phenotypes in populations of *B. cinerea* after winter periods. This development could be an indicator for the difference in fitness of the benzimidazole-resistant compared to the -sensitive portion of the population. Therefore, frost tolerance and various fitness parameters of different fungicide-resistant phenotypes were investigated.

3.4.1 EFFECT OF FROST ON VITALITY OF PHENOTYPES RESISTANT TO DIFFERENT FUNGICIDE CLASSES

Frost tolerance of fungicide-resistant phenotypes was determined by freezing of 20 to 30 isolates of *B. cinerea* per phenotype (Appendix Table 7-16). Subsequent assessment of vital mycelia showed no significant difference between survival rates (75.9 to 94.7 %) of six phenotypes tested (Table 3-10).

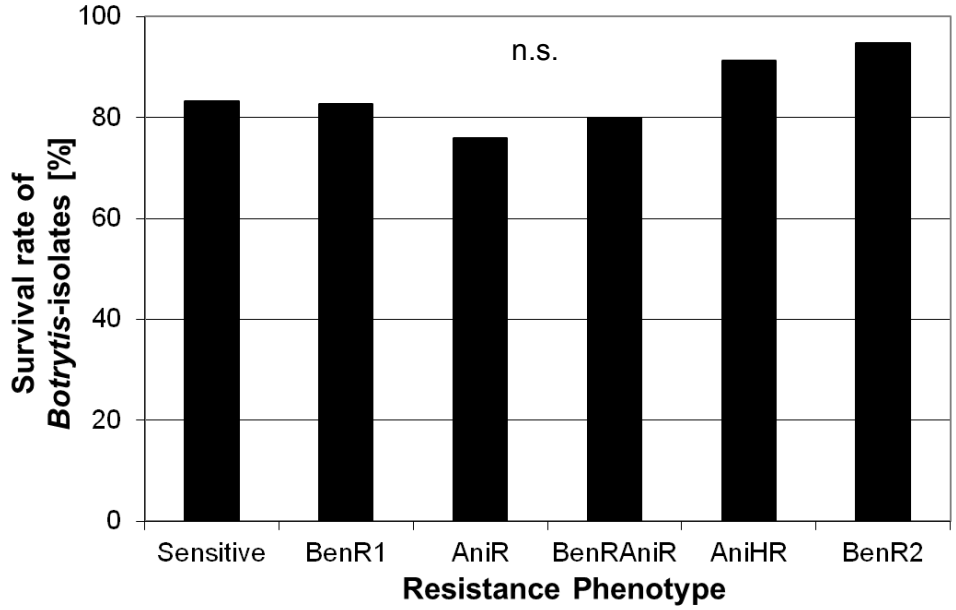

Figure 3-10 Survival rate of six different phenotypes of *Botrytis cinerea* after freezing at -20°C. Isolates were randomly chosen from a monitoring conducted at three experimental sites near Bordeaux in September 2010 (n=20 – 30). BenR1: resistant to benzimidazoles (MBC); AniR: reduced sensitivity to anilinopyrimidines (Ani); AniHR: Ani resistant; BenR2: MBC- and diethofencarb-resistant; BenRAniR: MBC-resistant and a reduced sensitivity to Ani; BenRAniHR: MBC- and Ani-resistant. Statistical analysis: no significant difference (n.s.) comparing survival rates of phenotypes according to Pearson's Chi^2 test at $p = 0.05$.

3.4.2 BENZIMIDAZOLE-SENSITIVE AND -RESISTANT ISOLATES AT FAVOURABLE AND UNFAVOURABLE DEVELOPMENT CONDITIONS

Additionally, different parasitic and saprophytic fitness parameters were tested. According to chapter 3.3.1 and 3.4.2.1 the E198A genotype was the most important single nucleotide polymorphism conferring resistance to benzimidazoles (MBC). Therefore, fitness parameters of a group of MBC-sensitive isolates were compared to that of a group of MBC-resistant isolates of the E198A genotype. Fitness costs associated with resistance to fungicides might be more costly under conditions that are suboptimal for a fungus. Therefore, fitness parameters and competitive ability of the two fungicide sensitivity groups were compared at favourable and unfavourable development conditions.

3.4.2.1 Genetic characterization

In order to identify MBC-resistant *B. cinerea* isolates showing the E198A genotype for the fitness experiments, twelve benzimidazole-resistant field isolates collected from five German vineyards in 2007 were tested in a duplex allele-specific PCR.

Amplification of DNA from all twelve field isolates of *B. cinerea* and the reference isolate BC-11.3 resulted in a product of 381 bp length using primer pair Bc-F/Bc-R. Additionally, all twelve field isolates amplified a product of 281 bp length using primer pair Bc-E198A/Bc-R. No fragment was visible for the water template (Figure 3-11).

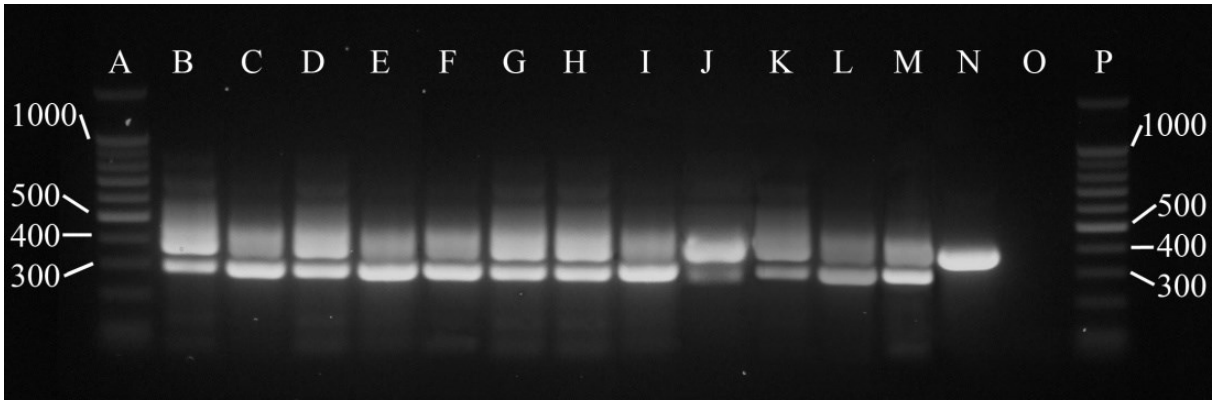

Figure 3-11 Presence of the E198A-mutation in all twelve *Botrytis cinerea* isolates detected by duplex allele-specific PCR. Isolates were collected from five German vineyards in 2007 (Table 2-2). Amplification of β-tubulin gene fragments using primer pairs BC-F/BC-R and BC-E198A/BC-R to identify *B. cinerea* and to detect E198A-mutation, respectively.

3.4.2.2 Fitness parameters

Mycelial growth, spore production, spore germination and aggressiveness on detached leaves of grapevine of ten sensitive (wild-type, BenS) and ten isolates resistant to benzimidazoles showing the E198A genotype (BenR1) were measured under favourable and unfavourable development conditions for the fungus.

Under favourable development conditions for the fungus, fitness parameters did not differ significantly between the two fungicide sensitivity groups. BenS isolates displayed a mycelial growth of 38.7 mm, a germination rate of 94.1 %, a spore production of 1,550 spores per mm² and a lesion size of 211 mm^2. Similar results were obtained for the group of BenR1 isolates, which showed a mycelial growth of 39.4 mm, a germination rate of 94.4 %, a spore production of 1,620 spores per mm² and a mean lesion size of 213 mm^2, respectively (Table 3-10).

Under unfavourable development conditions for the fungus, fitness parameters differed between sensitive and resistant isolates. The mean mycelial growth of BenR1 isolates was 37.8 mm,

hence significantly lower than that of the BenS isolates, which was 41.7 mm (Table 3-10). While BenR1 isolates produced in average only 111 spores per mm², BenS isolates yielded in average 276 spores per mm². The mean lesion size of BenS isolates was 165 mm^2, hence significantly higher than that of BenR1 isolates, which was 56 mm^2. However, there was no significant difference in spore germination between BenR1 and BenS isolates (94.6 % and 95.8 %, Table 3-10). Under unfavourable development conditions, significant differences in fitness parameters were obtained even though a great variability was obtained within the same fungicide sensitivity groups. Isolate Rech4, V5-3-1, V5-5-2, V6-3-3 and V6-3-5 of the BenR1 sensitivity group showed a significantly lower mycelial growth, spore production and lesion size compared to most BenS isolates (Appendix Table 7-18).

Table 3-10 Comparison of fitness parameters of ten benzimidazole-sensitive to ten -resistant isolates of *Botrytis cinerea* under favourable and unfavourable development conditions for the fungus. Fitness parameters tested: mycelial growth, spore production, spore germination and lesion size on leaves of grapevine. Statistical analysis: p-values in boldface indicate significant difference between fungicide sensitivity groups according to Student`s *t*-test ($p \leq 0.05$).

	Fitness parameter	N	Sensitive group		Resistant group		p-value
			Mean	SEM	Mean	SEM	
Favourable development conditions	Mycelial growth [mm]	10	38.7	5.8	39.4	4.3	0.772
	Spore production [conidia mm^{-1}]	10	1550	497	1620	519	0.837
	Spore germination [%]	10	94.1	1.8	94.4	1.1	0.890
	Lesion size [mm²]	10	211	35	213	49	0.775
Unfavourable development conditions	Mycelial growth [mm]	10	41.7	1.8	37.8	1.3	**0.031**
	Spore production [conidia mm^{-1}]	10	276	67	111	28	**0.037**
	Spore germination [%]	10	95.9	0.4	94.6	1.1	0.790
	Lesion size [mm²]	10	165	77	56	38	**0.012**

Separation of effects of temperature and nutrient availability on mycelial growth showed a significantly higher mycelial growth of BenS isolates on $CDA_{10\%}$ incubated at 6°C. Grown on PDA at 6°C, BenS isolates showed a non-significantly higher mycelial growth. BenR isolates showed a non-significantly higher mycelial growth on $CDA_{10\%}$ or PDA at 21°C (Appendix Table 7-20).

3.4.2.3 Competitive ability

In the preceding chapter isolates of *B. cinerea* resistant or sensitive to benzimidazoles were tested separately. In the competitive experiments, population dynamics of the two fungicide sensitivity groups were assessed on either grapevine plants or autoclaved leaf discs under favourable and unfavourable development conditions as well as with and without fungicide application.

Under favourable development conditions percentages of BenR conidia did rarely change on autoclaved leaf discs. After two generations, initial resistance frequencies of 90.1, 48.1 and 16.4 % resulted in final resistance frequencies of 79.4, 46.4 and 13.6 %, respectively (Figure 3-12 a). Similar results were obtained on grapevine plants without fungicide application (Figure 3-12 c).

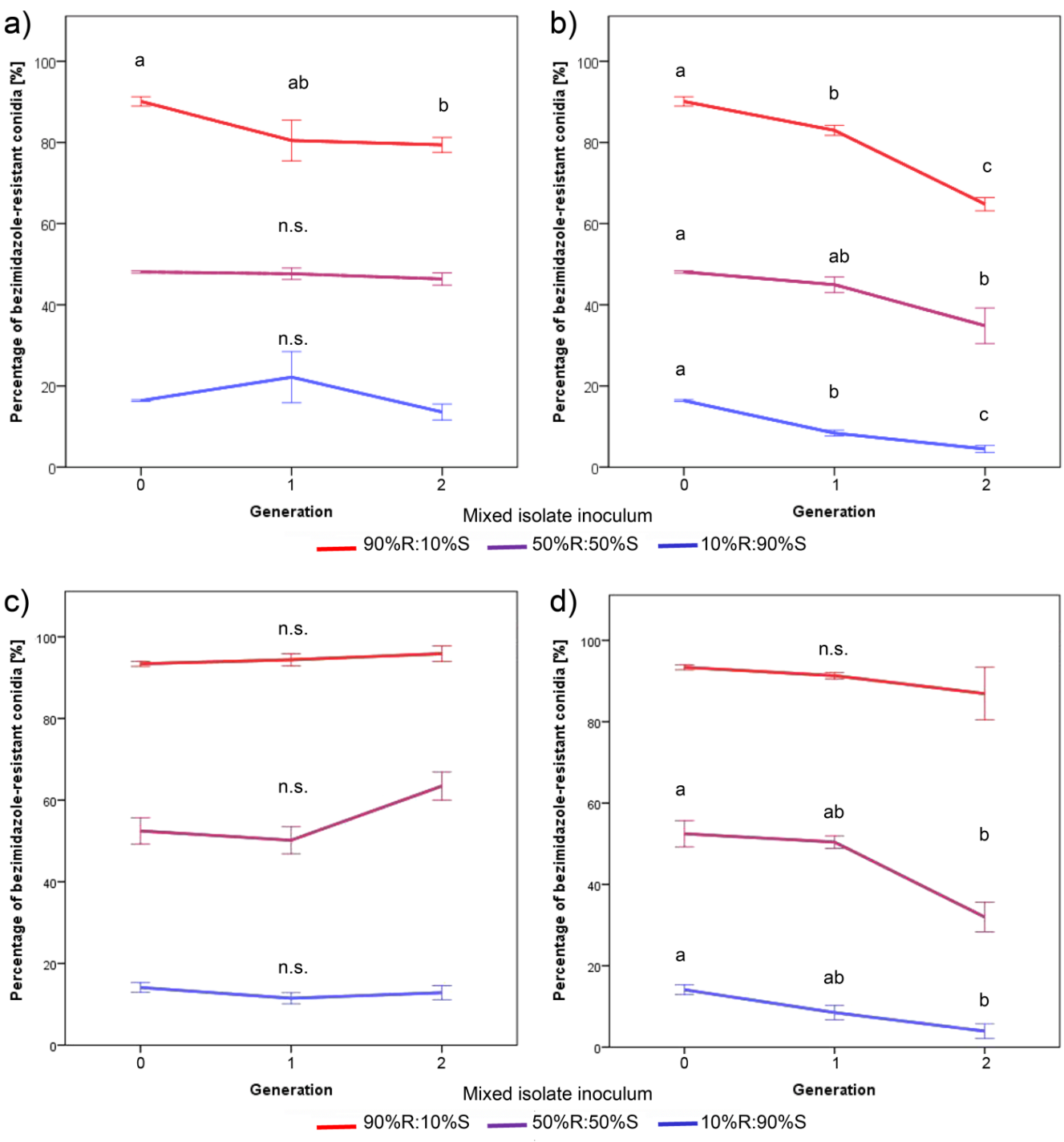

Figure 3-12 Effect of incubating temperatures of 21°C (a, c) or 6°C (b, d) on population dynamics of three ratios of benzimidazole-sensitive (S) and -resistant (R) isolates of *Botrytis cinerea* inoculated on autoclaved leaf discs (a, b) or two month-old plants of grapevine (c, d) for two subsequent generations. Error bars show standard error of the mean (n = 3 - 5). Statistical analysis: same letters indicate no significant difference (n.s.) between generations according to Tukey's HSD or Scheffé test at p = 0.05.

Under unfavourable development conditions a shift in frequencies of benzimidazole-resistant conidia was observed. The percentage of resistant conidia decreased significantly from 90.1 % to 83.0 % after one to 64.8 % after two generations. Initial resistance frequency of 48.1 % decreased non-significantly to 45.0 % after one and significantly to 34.8 % after two generations. The initial resistance frequency of 14.1 % decreased significantly to 8.4 % after one to 4.5 % after two generations (Figure 3-12 b). Similar results were obtained on grapevine plants without fungicide application (Figure 3-12 d). This effect was even more pronounced regarding a mixture of the BenR isolate V5-3-1 and the strong sensitive competitor isolate Rech1. The percentage of resistant conidia decreased significantly from initial frequencies to less than 1 % after one generation at 6°C on autoclaved leaf discs (Appendix Table 7-20).

Application of thiophanate-methyl under favourable or unfavourable development conditions resulted in a significant increase from initial resistance frequencies of 52.5, 14.1 and 1.6 % to 98 - 100 % of resistant conidia after one generation. All samples showed a resistance frequency of 100 % after two generations (Figure 3-13 a&b).

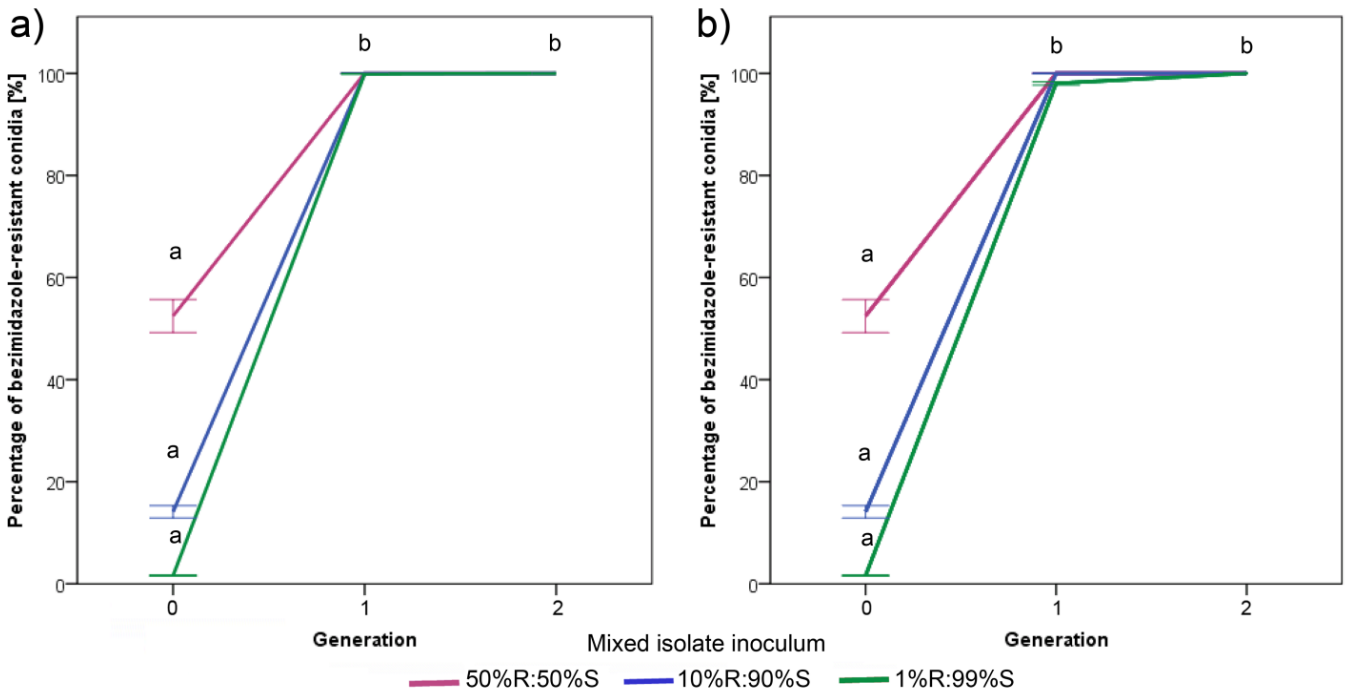

Figure 3-13 Effect of thiophanate-methyl application and incubating temperatures of 21°C (a) or 6°C (b) on population dynamics of three ratios of benzimidazole-sensitive (S) and -resistant (R) isolates of *Botrytis cinerea* inoculated on two month-old plants of grapevine for two subsequent generations. Error bars show standard error of the mean. Statistical analysis: same letters indicate no significant difference (n.s.) between generations according to multiple Mann-Whitney U tests at p = 0.05 (n=5).

4 DISCUSSION

The first fungicides with a specific mode of action, such as benzimidazoles (Methyl Benzimidazole Carbamates: MBCs) and carboxamides, were discovered in the 1960s and early 1970s. During the late 1970s and early 1980s compounds with new modes of action, such as dicarboximides, phenylamides and sterol biosynthesis inhibitors, entered the market (KUCK *et al.* 2007). These 'single-site' fungicides control fungal plant pathogens more effectively compared to the non-systemic 'multi-site' fungicides, which inhibit simultaneously a range of enzymes and cellular structures, such as copper and sulfur based mixtures (MCCALLAN *et al.* 1949). However, the specific modes of action of 'single-site' fungicides resulted in the selection of resistant strains in fungal populations leading to the withdrawal of registration of MBCs to control *Venturia inaequalis* on apples and *Botrytis cinerea* on grapevine (KIEBACHER and HOFFMANN, 1980; HOFFMANN and LOECHER, 1979). The resistance development of *B. cinerea* to dicarboximides or that of *Phytophthora infestans* to phenylamines was of limited importance due to the reduced fitness of resistant strains (HSIANG, 1991; KADISH and COHEN, 1988). Because of these observations and the need for collaboration of crop protection companies, the Fungicide Resistance Action Committee (FRAC) was formed in 1981 to discuss resistance problems and formulate plans for cooperative efforts to avoid or manage resistance to fungicide. FRAC provides guidelines on the use of fungicides to assess and manage the build-up of resistance within fungal populations (RUSSELL, 2006). The following tools are recommended by European and Mediterranean Plant Protection Organization (EPPO) Standard on Resistance Risk Analysis (LAVADINHO, 2002):

- Use of good plant protection practice and recommendations for individual crops
- Adaption of application frequency, timing and dose rate
- Use of mixtures/alternations of fungicides from different cross-resistance groups
- Use of fungicides, which show a negatively correlated cross-resistance pattern

The gained experiences in resistance management were used at the introduction of anilinopyrimidines as well as fenhexamid in the mid to late 1990s to control *B. cinerea* (HILBER and SCHUEPP, 1996; SUTY, PONTZEN and STENZEL, 1999). E.g. the anilinopyrimidine fungicide cyprodinil was introduced to the market as a pre-packed mixture with fludioxonil (FORSTER and STAUB, 1996). In 2004, boscalid entered the market either as a solo product or also as a pre-packed mixture with pyraclostrobin. Additionally, the number of treatments per season was limited (STAMMLER and SPEAKMAN, 2006; MYRESIOTIS *et al.* 2008).

In the present study different resistance management strategies were tested to slow down the build-up of resistance of *B. cinerea* towards MBC fungicides in grapevine. This is difficult, because *B. cinerea* has a high tendency to become resistant to frequently applied systemic fungicides, due to a

high number of generations per year, a high number of progeny, a wide host range and a high genetic variability within the population (BRENT and HOLLOMON, 2007). Additionally, MBCs are fungicides at risk, because MBC-resistant strains show a monogenetic resistance type with high resistance levels leading to a disruptive selection process towards MBC-resistant strains of *B. cinerea* (LEROUX *et al.* 2002). Also, grapevine is a perennial plant, so that fungicide-resistant strains selected by fungicide applications can survive the winter in old rachis, tendrils, leafs and cane debris and make up most of the primary inoculum in the following season (SEYB, 2004).

Three-year field trials were initiated in three vineyards near Bordeaux in 2009. In order to evaluate a suitable resistance management strategy, three spray programs were tested:

- Mixture: The mixture of thiophanate-methyl, a MBC fungicide, with mepanipyrim, an anilinopyrimidine fungicide, was applied at end of flowering in all three years.
- Alternation: Thiophanate-methyl was applied at the end of flowering and mepanipyrim was applied at bunch closure in all three years.
- Annual alternation: Solo application of the fungicide at end of flowering. In 2009 and 2011, thiophanate-methyl was applied and in 2010, mepanipyrim was applied.

Strategies were compared to the one time solo application of thiophanate-methyl in all three years (unrestricted use) and to the conventional fungicide treatments, where no MBCs were applied. Monitoring procedures were conducted biannually at time of flowering and prior to harvest. Subsequently, frequencies of phenotypes resistant to fungicides in *B. cinerea* populations were determined.

In June 2009 the sensitivity profile, which is the baseline sensitivity for an existing fungicide at a specific location, showed a frequency of MBC-resistant isolates of 3 – 12 %. Most MBC-resistant isolates showed the BenR1 phenotype caused by the E198A mutation. Similar results were obtained by LEROCH *et al.* (2010) and DERPMANN *et al.* (2010) in German vineyards, where no MBCs were applied in vineyards in the last 30 years. Interestingly, studies of *B. cinerea* strains, which were not exposed to MBCs, showed a frequency of MBC-resistant strains of 6% in a mycological collection (SCHUEPP and LAUBER, 1977). Also, a *B. cinerea* population collected from untreated vines in Germany showed a frequency of MBC-resistant strains of about 10^{-4} (FEHRMANN, 1976). This data suggest a moderate frequency of naturally occurring MBC-resistant strains. In France, regional discrepancies are visible due to different use histories of MBCs to control *B. cinerea* or *Pseudopeziza tracheiphila* (LEROUX *et al.* 1985). The mixture of carbendazim with diethofencarb was registered from the end of the 1980s to 2007 in France (Service Régional de la Protection des Végétaux, personal communication). In 2008, frequencies of MBC-resistant strains of 0 – 22 % were observed in seven vine-growing regions as well as 52 % in the Champagne region (Biorizon, personal communication).

In the present study, three of 174 *B. cinerea* isolates collected from the Saint Brice site showed a BenR2 phenotype, which shows a multiple resistance to MBCs and diethofencarb, a compound with

a negative cross-resistance pattern. Such low frequencies were also detected in other monitoring procedures conducted in the Champagne region in the last years (PETIT *et al.* 2010; LEROUX *et al.* 2002). A reduced fitness of BenR2 strains was suggested by LEROUX *et al.* (1999) due to the decreased frequency of this phenotype in periods, where the mixture of carbendazim with diethofencarb was not applied. In the present study, the percentages of BenR2 isolates did not increase significantly from 2009 to 2011 in plots treated with thiophanate-methyl (in average 9%).

Genetic characterization of 16 MBC-resistant isolates of *B. cinerea* showed, that 13 isolates carried the E198A mutation indicating that this was the most important single nucleotide polymorphism (SNP). Also, all twelve MBC-resistant isolates collected from German vineyards in 2007 carried this SNP. According to MA *et al.* (2005) this SNP is the dominant SNP leading to MBC-resistance in field isolates of various fungal plant pathogens. The three isolates, which showed the BenR2 phenotype, carried the F200Y mutation first described by YARDEN and KATAN (1993).

In all three years the application of spray programs including thiophanate-methyl resulted in a significant increase of the frequency of BenR1 isolates compared to the conventional fungicide treatment, where no MBCs were applied. In September 2009, all strategies tested resulted in similar BenR1 isolate frequencies compared to the unrestricted use (16 to 23 %). In September 2010, the application of mepanipyrim as part of the annual alternation resulted in a significantly lower percentage of BenR1 isolates (9 – 26 %) compared to the percentage of BenR1 isolates selected by the mixture or alternation of thiophanate-methyl and mepanipyrim as well as the unrestricted use of thiophanate-methyl (25 – 51 %). However, this effect was compensated after the second application of thiophanate-methyl in 2011. At the end of the study, all tested resistance management strategies resulted in similar frequencies of BenR1 isolates compared to the unrestricted use (30 to 65 %). Thus, the tested resistance management strategies could not slow down the build-up of resistance to MBCs in *B. cinerea* populations. Correspondingly, the build-up of resistance to MBCs in *Cercospora beticola* populations could not be slowed down by the mixture of maneb with benomyl at a reduced dose (KARAOGLANIDIS *et al.* 2003). The selection pressure of an one time solo treatment of thiophanate-methyl resulted in an average increase of 12 – 22 % in 2009. Such an increase per treatment could explain the observed frequencies of 61 – 96 % of MBC-resistant isolates in 43 German vineyards with up to twenty treatments including MBCs in 1971 to 1974 (HOFFMANN and LOECHER, 1979). A decrease of fungicide performance was observed at frequencies of MBC-resistant strains in the population of more than 50% (LEROUX *et al.* 1985). However, the dry summer in 2011 led to a low infection pressure resulting in no significant differences in disease incidence and severity compared to the conventional fungicide treatment, where no fungicides to control *B. cinerea* were applied in two of three sites.

From October 2009 to May 2010, when no selection pressure through fungicides was present, a significant decrease of the frequency of BenR1-phenotypes of 11 % in average was observed at two sites in June 2010. Due to the low isolation rate in May 2011, this observation could not be verified.

Such a decrease over the winter period was not mentioned in literature for MBC-resistant strains. The monitoring procedures were conducted biannually at the time of flowering and prior to harvest. A difference in *Botrytis* populations colonizing flowers compared to those colonizing bunches of berries could explain the observed decrease. WALKER *et al.* (2011) described the presence of varying proportions of *B. pseudocinerea* (*vacuma* Group I) and *B. cinerea* (*vacuma* and *transposa* Group II) within a vegetation period. *B. pseudocinerea* isolates were only found on leaves and floral caps. Additionally, *vacuma* Group II isolate frequency reached its maximum on senescing floral caps and decreased significantly on leaves and berries until harvest. Rarely, *vacuma* Group II strains were isolated from overwintering canes (GIRAUD *et al.* 1997; MARTINEZ *et al.* 2005; FOURNIER *et al.* 2005). Additionally, BenR1 and BenR2 phenotype frequency within the *vacuma* groups was lower compared to that of the *transposa* group (GIRAUD *et al.* 1999; MARTINEZ *et al.* 2005). In the present study, the decrease in frequency of BenR1 isolates collected from flower-associated populations compared to that of the preceding berry-associated populations could result from a difference in the frequency of *vacuma* and *transposa* groups dependent on the colonized plant organs. However, the frequency of BenR1 isolates in flower- and berry-associated populations did not differ significantly in conventionally treated plots, where no MBCs were applied. Additionally, the frequency of BenR1 isolates remained stable in flower-associated populations in June 2010 compared to that in berry-associated populations in September 2010 for plots, which received a solo application of mepanipyrim in 2010 as part of the annual alternation. Thus, a difference in *B. cinerea* population structure colonizing plant organs of grapevine is not the main factor determining the dynamics of the frequency of MBC-resistant isolates.

In average, the winter period of 2009-10 was 2°C colder compared to the 30 year mean (Appendix Table 7-10 and 7–11). Thus, a reduced cold tolerance of MBC-resistant isolates could reduce the fraction of resistant isolates in the next vegetation period, as observed for fenhexamid-resistant isolates of *B. cinerea* (SUTY *et al.* 1999; BILLARD *et al.* 2012). However, in the present study no difference in cold tolerance was detected comparing various fungicide-resistant phenotypes. This result is in agreement with that of HSIANG *et al.* (1992), who detected no significant differences in the viability of sclerotia of MBC-sensitive and –resistant isolates of *B. cinerea*. Therefore, the overwintering population on wood was probably not influenced by low temperatures. Similar observations were made by BEEVER, LARACY and PAK (1989) who reported no change in MBC-resistant isolate frequency in wood-associated populations of *B. cinerea* over the winter period.

PAK *et al.* (1990) reported that the frequency of dicarboximide-resistant isolates in overwintering populations on wood closely mirrored that in berry-associated populations of *B. cinerea* at harvest, suggesting that the inoculum was largely originating from within the plot. In the present study, the spatial stability of aggregations of MBC-resistant isolates was investigated by geo-statistical methods. Only one significant aggregation of MBC-resistant isolates, which was stable regardless of the time of monitoring, was detected in the northern part of the trial site Loupes. At all other sites, no significant

spatio-temporal correlation was observed for aggregations of MBC-resistant isolates comparing berry- to subsequent flower-associated populations of *B. cinerea*. Even spatial randomness was detected for the flower-associated population at Grezillac site in 2010. These results indicate a mixture of the inoculum originating from the wood-associated population of *B. cinerea*, which is persistent over the winter period, with other sources of inoculum produced inside or outside the vineyard. A recent study of WALKER *et al.* (2013b) showed that the population of *B. cinerea* on grapevine is structured into three clusters, which probably undergo genetic recombination from surrounding locations during that time. This process leads to higher genetic diversity in flower-associated populations of *B. cinerea*. In the present study, MBC-resistant isolates were also detected in the surrounding untreated plots. Frequencies of MBC-resistant isolates did increase slowly over time at Saint Brice site, however not significantly. This could be an indicator for a mixture of the populations within the trial field and the surrounding plots. *Vice versa*, an influence of the untreated surrounding plots on the trial fields cannot be excluded. Dicarboximide- and anilinopyrimidine-resistant isolates of *B. cinerea* as well as strobilurin-resistant ascospores of *Mycosphaerella graminicola* were dispersed from treated plots leading to an increase of the frequency of resistant isolates in surrounding plots, especially those which corresponded to the main orientation of the wind (GULLINO, ALOI and GARIBALDI, 1989; BAROFFIO *et al.* 2003; FRAAIJE *et al.* 2005). Comparing berry-associated populations of *B. cinerea* observed in 2009 with those in 2010, the solo application of mepanipyrim in 2010 as part of the annual alternation resulted in an average decrease of about 10 % while surrounding plots treated with thiophanate-methyl resulted in an average increase of about 20 % at two sites. Thus, mixing of *Botrytis* populations by migration from surrounding plots is not the most important factor explaining the observed results.

A lack of spatiotemporal correlation was also observed for dicarboximide-resistant strains of *Monilinia fructicola* in stone fruits. This indicated a poor persistence of resistant strains at specific locations, which can be interpreted as an indicator of a difference in fitness of the resistant strains (ELMER, GAUNT and FRAMPTON, 1998). But no fitness costs associated with resistance to MBCs were detected (AKUTSU *et al.* 1988; BEEVER, LARACY and PAK, 1989; STAUB *et al.* 1991; HSIANG and CHASTAGNER, 1991; ELAD, YUNIS and KATAN, 1992). However, fitness costs associated with resistance to fungicides can be more pronounced under conditions that are suboptimal for a fungal species (BROWN *et al.* 2006). Therefore, fitness parameters of MBC-resistant isolates were compared to that of MBC-sensitive isolates at favourable and unfavourable development conditions, such as lower temperature and low nutrient availability. A group of ten MBC-sensitive and a group of ten MBC-resistant isolates of the E198A genotype were used in these experiments to represent the biological diversity of *B. cinerea*. At favourable development conditions (21°C) no significant differences were observed as described by the authors mentioned above. Interestingly, the MBC-resistant isolates showed a significantly slower mycelial growth, a smaller lesion size on inoculated grapevine leaves, as well as a significantly lower number of progenies at unfavourable development conditions (6°C). Ac-

cording to AKAGI *et al.* (1995) the E198A-mutation in the β-tubulin gene alters the binding site to carbendazim by change of an ethyl sized pocket of the protein. As described for benomyl-resistant strains of *Schizosaccharomyces pombe*, pleiotropic effects of other mutations on the tubulin genes leading to altered microtubule architecture result in a reduced development at low temperature (ROY and FANTES, 1982). Possibly, the E198A-mutation on the β-tubulin gene is also associated with reduced development at sub-optimal conditions due to temperature and nutrition. Correspondingly, isogenic strains carrying fenhexamid-resistance alleles grew more slowly compared to the wild-type strain and displayed variations in the production of sclerotia and conidia especially at low temperatures and poor nutritional conditions (BILLARD *et al.* 2012).

In order to corroborate this hypothesis for MBC-resistant strains, the intraspecific competition of the two sensitivity groups was tested on grapevine plants and autoclaved leaf discs in the present study. Competitive assays conducted by other researchers focused on comparing pairs of fungicide-resistant isolates with a difference in aggressiveness. In such studies, the stronger competitor became prevalent regardless of its fungicide sensitivity, which was interpreted as an indicator for no fitness costs of resistance to fungicides (BARDAS *et al.* 2008; KARAOGLANIDIS *et al.* 2011). In the present study, such an experimental setup was tested using one strong MBC-sensitive isolate in mixture with one less competitive MBC-resistant isolate. The frequency of the sensitive competitor increased slowly at 21°C whereas at 6°C it made up more than 90% of the population after one generation. However, such an experimental design was unsuitable for detecting small fitness costs, especially for a fungal pathogen like *Botrytis cinerea* with its' very high genetic variation (GIRAUD *et al.* 1997; YOURMAN, JEFFERS and DEAN, 2000). Therefore, a mixture of ten isolates for each sensitivity group was used to compensate for the genetic variation in the present study. Competitive assays conducted at 6°C confirmed the detected differences in conidial production resulting in an average decrease of the MBC-resistant portion of 6% to 8% per generation without selection pressure. In contrast, no significant decrease within two generations was observed at an optimal temperature of 21°C. However, a thiophanate-methyl application prior to inoculation lead to an increase from 1% to more than 95% of MBC-resistant isolates regardless of the incubation temperature. Such an increase can be explained by the negative selection-pressure of the fungicide application on the MBC-sensitive isolates in the population in combination with the high spray coverage in experiments with single plants. These results are in compliance with the observed increase of MBC-resistant isolates caused by the thiophanate-methyl applications with a lower spray coverage in the field experiments.

A decrease of the frequency of MBC-resistant isolates of *B. cinerea* was also observed in long-term monitoring procedures in situations were no MBCs were applied. ISHII *et al.* (1992) reported less than 10 % of MBC-resistant isolates 15 years after discontinuation of benzimidazoles. Correspondingly, a decrease to about 10% was observed in German vineyards 30 years after seldom use or discontinuation of MBCs (LEROCH *et al.* 2010; DERPMANN *et al.* 2010). However, these studies did

not show the dynamics of the decrease, which leaves room for speculation on the underlying evolutionary factors. Recently, the Institut national de la recherche agronomique (INRA) published the results of a long-term monitoring conducted for different fungicide-resistant phenotypes of *Botrytis cinerea* from 1985 to 2011 in vineyards in the Champagne region (WALKER *et al.* 2013a; Figure 4-1). This data shows a plateau-phase at about 90% of BenR1 strain frequency from 1985 until 1988. Subsequently, the BenR2 strains became prevalent due to the selection pressure of the mixture of carbendazim with diethofencarb. Until 1998 the sum of both strains made up more than 90% of the population in changing ratios depending on the fungicidal selection pressure of the mixture. From 1999 to 2002 the percentage of BenR1 strains stagnated at 80% and that of BenR2 strains fell to less than 5% due to the rare application of the mixture. This observation shows the lower fitness of BenR2 strains compared to that of BenR1 and sensitive strains, which was also observed by ISHII *et al.* (1992) as well as ZIOGAS and GIRGIS (1993). Interestingly, the frequency of BenR1 strains decreased from about 80% in 2002 to about 20% in 2011 after very rare or no application of the mixture (LEROUX *et al.* 2013a). Such a linear decrease of about 7% per year is similar to the observed decrease in the present studies' laboratory as well as field experiments. Without selection pressure of MBCs, small fitness costs associated with resistance to MBCs might have reduced the percentage of MBC-resistant isolates within the primary inoculum, when the fungus was confronted with reduced nutrient availability and low temperatures. This led to a decrease of the MBC-resistant fraction in the consecutive berry-associated populations, if no MBCs were applied during the season.

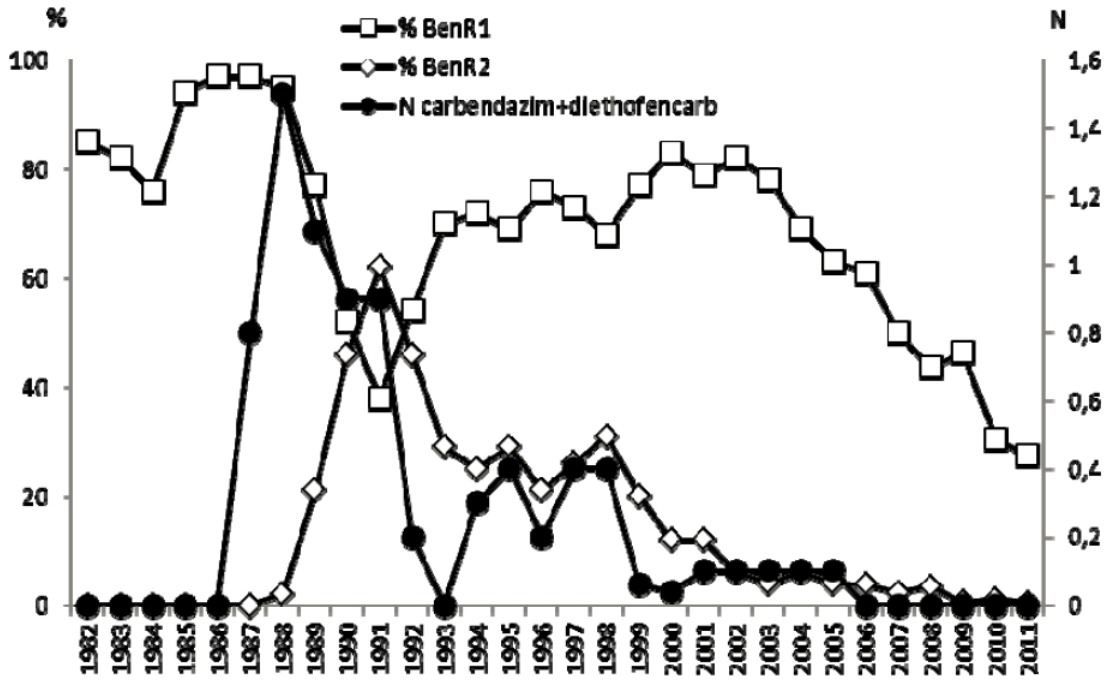

Figure 4-1 Evolution of *Botrytis cinerea* resistance to anti-microtubule agents (mean frequency of BenR1 and BenR2 strains) in Champagne vineyards, according to fungicidal selection pressure (mixture of carbendazim with diethofencarb; N as mean number of sprays per season) according to WALKER *et al.* (2013a).

Over time this difference in fitness could have led to a linear decrease resulting in frequency of MBC-resistant strains of less than 10% of the population as observed in German and French vineyards (LEROCH *et al.* 2010; DERPMANN *et al.* 2010; WALKER *et al.* 2013a, Biorizon, personal communication). WALKER *et al.* (2013b) reached to a similar conclusion comparing the population structure of *B. cinerea* from grapevine and surrounding locations. First estimation of resistance cost from field data of the BenR1-phenotype was in most cases similar to that of dicarboximide-resistant strains (ImiR1-phenotype) due to a negative selection rate from September to next June (i.e. winter-period) and the resulting gene flow from surrounding locations during that time.

An alternative hypothesis is a directional selection, which favors MBC-sensitive strains (BenS). This could have resulted in the observed linear decrease of the frequency of BenR1 strains, as well. Such a positive selection of MBC-sensitive strains could emanate from the active ingredient zoxamide, which is a benzamide fungicide. The compound binds to the β-tubulin of oomycetes as well as to that of some ascomycetes (YOUNG *et al.* 2005). Zoxamide has been registered since 2004 in the European Union to control *Plasmopara viticola* and other oomycetes (EU Pesticide Database). Different mixtures of zoxamide with copper or mancozeb are available in France since 2005 and in Germany since 2006 (e-PHY database; ANONYM, 2013). Zoxamide exhibits a negatively correlated cross-resistance towards MBCs in *B. cinerea* with BenS strains showing higher resistance to zoxamide (resistance factor > 20) compared to BenR1 strains (YOUNG *et al.* 2005). The cross-resistance pattern of zoxamide is similar to that of the N-phenyl-carbamate (NPC) diethofencarb, which effectively controlled BenR1 strains on cucumber as solo application (FUJIMURA *et al.* 1990). However, BenR2 strains were detected a few years after introduction of the mixture of MBCs with NPCs (LEROUX *et al.* 1999). BenR2 strains are resistant to zoxamide, as well (MALANDRAKIS *et al.* 2011). The risk of positive selection of BenR2 strains is probably reduced by solo application of zoxamide. In intraspecific competition with the more competitive BenR1 strains after MBC fungicide application or with BenS strains after zoxamide application, the frequency of BenR2 isolates might not increase as strongly as that after application of the mixture of MBC with NPC fungicides. At the trial site near Grezillac, zoxamide was applied once in 2010, which did not result in an increase of BenR2 isolate frequency. Conversely, a significant difference in resistance development to MBCs was observed at Gezillac site (15%, 32%, 34%) compared to the other two trial sites (24-33%, 43-46% and 52-53%) for the berry-associated populations of *B. cinerea* treated with thiophanate-methyl in 2009, 2010 and 2011. However, such differences could have arisen from the different sensitivity profiles of the three locations (3% at Grezillac site, 10-12% at other sites).

In the present study, mepanipyrim was applied as a partner compound in mixture or alternation, thus a build-up of different phenotypes resistant to anilinopyrimidine in *B. cinerea* populations was monitored, as well. The results showed stable frequencies of isolates with a resistance to anilinopyrimides (AniR1), which ranged from 0 – 7%. Similarly, BAROFFIO *et al.* (2003) detected frequen-

cies of 0 – 8% of AniR1 strains in plots treated once per year with anilinopyrimides. Additionally, isolates with a reduced sensitivity to anilinopyrimidines (AniR) were detected in the present study. The discriminatory dose used was similar to that used by LEROCH *et al.* (2010) to identify multi-drug resistant (MDR) phenotypes as described by KRETSCHMER *et al.* (2009) as well as LEROUX *et al.* (2013). In the present study, AniR isolates of *B. cinerea* were not characterized by further fungicide sensitivity assays (e.g. using tolnaftate) or molecular techniques as used by the authors mentioned above. In mepanipyrim-treated plots AniR isolate frequencies ranged from 10 – 30%. Similar frequencies of MDR strains were detected in Germany and France (LEROCH *et al.* 2010; WALKER *et al.* 2013a). Also, a combined resistance phenotype (BenRAniR) was detected at frequencies of about 10% in mepanipyrim-treated plots. In all analyzed isolates, the resistance to MBCs was caused by the E198A or the F200Y mutation. The mixture of thiophanate-methyl with mepanipyrim led to lower AniR as well as BenRAniR frequencies compared to those detected at alternation of both fungicides at two of three sites. Such an observation indicates a reduced positive selection of MDR strains through use of mixtures of anilinopyrimidine with MBC fungicides. However, no solo application of mepanipyrim was included in the trial to be compared to the mixture and alternation with MBC fungicides. A reduced positive selection of MDR strains can be explained by low resistance factors (RF) of MDR strains to the MBC fungicide carbendazim (RF < 3.5) compared to those of all other fungicide classes used to control *B. cinerea* (RF > 10, KRETSCHMER *et al.* 2009; LEROUX *et al.* 2013). However, a novel clade of *B. cinerea* (Group S) was detected in strawberry fields. These strains show a stronger variant of the MDR1 phenotype (MDR1h), which is caused by a deletion in the transcription factor gene *mrr1*. Up until now these strains are limited to German strawberry fields, but dispersal to vineyards cannot be excluded, which can cause a threat to fungicide performance in the future (LEROCH *et al.* 2013).

Evolution of multi-drug as well as target-site resistance in fungal pathogens to a variety of fungicide classes cannot be avoided due to large-scale growing of monocultures and the strong selection pressure of 'single-site' fungicides. Therefore, suitable anti-resistance strategies were developed in the last decades (EDIN and TORRIANI, 2012). Phenylamides (PA), which control solely oomycetes, were first used in 1977. In 1980, serious loss of control by the PA-fungicide metalaxyl occurred in downy mildew of cucumbers *(Pseudoperonospora cubensis*) in Israel and Greece and potato late blight (*Phytophthora infestans*) in Holland and Ireland. Isolates of the fungus were shown to be highly resistant to metalaxyl in sprayed plant and detached leaf assays (GEORGOPOULOS and GRIGORIU, 1981; DAVIDSE *et al.* 1981). Resistance to metalaxyl was observed in the following years in grape downy mildew (*P. viticola*) in France and South Africa as well as in tobacco blue mold (*Peronospora tabacina*) in Central America. Subsequently, resistance problems became apparent in other crop pathogens (DAGGETT, GOTZ and THERRIEN, 1993; REUVENI *et al.* 1985). The development of resistance to PA in *P. infestans* was correlated with unrestricted use of metalaxyl, as in Holland, and had not occurred in countries, where only mixtures with macozeb were applied, such as in the UK (STAUB *et al.*

1991). These observations led to a recommendation of the PA Working Group of FRAC founded in 1982 (since 1999 Expert Group), which includes using only mixtures with multi-site inhibitors, avoiding curative use and limiting the number of sprays per season (SCHWINN, STAUB and DAVIDSE, 1995). However, different mixtures, in particular with 'multi-site' inhibitors, did not significantly delay the selection of PA-resistant *P. viticola* isolates in French vineyards (LEROUX, 2000). Several new oomycete-active fungicides, such as zoxamide, were registered and have broadened the options for diversified spray programs, so that PA fungicides are used up until today (HOLLOMON, 2010).

Also, Carboxylic Acid Amides (CAA) fungicides are oomycete-active. The first active ingredient of this class was dimethomorph, which was introduced in the late 1980s. New fungicides of this chemical class were introduced until today (iprovalicarb, flumorph, mandipropamid and most recently valifenalate, GISI, 2012). No resistance to CAAs has been detected for *P. infestans* and *Bremia lactucae* (COHEN and GISI, 2007). However, CAA-resistant isolates of *P. viticola* were already reported in 1994, two years after the introduction of dimethomorph in France. Since then resistance has increased gradually (CHABANE *et al.* 1996; GESSLER *et al.* 2011). The CAA Working Group of FRAC recommends using CAAs only in mixtures with 'multi-site' inhibitors or other effective non cross-resistant mixing partners, a maximum of four treatments during one season may contain one CAA fungicide, which should be used in a preventive manner.

Sterol Biosynthesis Inhibitors (SBIs) are a large class of fungicides, which include triazoles and imidazoles. SBIs were first introduced in the mid-1970s and since then over 30 different SBIs have been used in crop protection. Declines in sensitivity to SBIs were reported in the 1980s for a number of pathogens, such as barley powdery mildew (*Blumeria graminis*), cucurbit powdery mildew (*Podosphaera fusca*), septoria leaf blotch (*Mycosphaerella* spp.) and leaf scald (*Rhynchosporium secalis*) on different cereals (*V. inaequalis*, WOLFE and FLETCHER, 1981; HUGGENBERGER *et al.* 1984) as well as apple scab on apple (STANIS and JONES, 1985). However, resistance development was relatively slow and it was depended on the intensity and exclusivity of SBI treatments. Additionally, the resistance factors of SBI-resistant strains increased during prolonged selection pressure, due to the combination of several point mutations on the *CYP51* gene and possibly overexpression of efflux transporters (quantitative resistance type, BRUNNER *et al.* 2008; LEROUX and WALKER, 2011). The introduction of new azole fungicides (e.g. prothioconazole), which exhibit much lower resistance factors in various pathogens, has largely overcome the decrease in efficacy of older products (HOLLOMON, 2012). The SBI Working Group of FRAC, which was founded in 1987, recommends avoidance of repeated solo applications, use of mixtures or rotation with effective non-cross-resistant fungicides and an avoidance of reduced doses. It has been suggested by COOLS and FRAAIJE (2008), that alternation of different azoles could be used to select against particular mutations on the *CYP51* gene, thereby posing the pathogen population an evolutionary conundrum. SBIs have continued to provide good control of most target pathogens after more than 30 years of widespread use. However, the diversity of

azole compounds will be reduced under the new European pesticide regulation (EC) 1107/2009 due to the categorization as potential endocrine disruptors (BRENT, 2012).

Quinone outside Inhibitors (QoIs) were introduced in the late 1990s for the control of a wide range of crop pathogens. Within two or more years, failure of control was observed for wheat powdery mildew (*B. graminis* f.sp. *tritici*), black sigatoka disease of bananas (*Mycosphaerella fijiensis* var. *difformis*), wheat leaf spot (*M. graminicola*) and grape downy mildew (*P. viticola*). Failures of control were associated with the presence of highly resistant strains in Germany and soon after in other countries (CHIN *et al.* 2001; FRAAIJE *et al.* 2002; SIEROTZKI *et al.* 2000; CHEN *et al.* 2007). QoI resistance is of the qualitative type, originating mainly from single mutations in the cytochrome *b* gene (GISI *et al.* 2002). Fitness penalties have been observed in QoI-resistant strains of *P. viticola* (GENET *et al.* 2006, SIEROTZKI *et al.* 2008). However, fitness penalties were not apparent in the case of *B. graminis* or *M. graminicola* (GISI *et al.* 2002, WAARD *et al.* 2006). General recommendations are made by the QoI Working Group of FRAC to limit the number of QoI applications, whether applied solo or in mixture with non-QoI fungicides. These compounds should provide satisfactory disease control when used alone on the target disease. Also, it is advised to use a rotation of applications with effective fungicides from other non-cross-resistant classes in a block application schedule specific for certain crops.

Resistance to benzimidazoles (Methyl Benzimidazole Carbamates: MBCs) was first detected in cucurbit powdery mildew (*P. fusca*) in the USA in 1969, after 2 years of its introduction. Other reports of resistance to MBC fungicides in various pathogens like *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *V. inaequalis*, *Cercospora* spp. and *M. fijiensis* and other *Erysiphales* soon followed (SCHROEDER and PROVVIDENTI, 1969; SZKOLNIK and GILPATRICK, 1969; BOLLEN and SCHOLTEN, 1971; FULLERTON *et al.* 1984; GEORGOPOULOS *et al.* 1973). *R. secalis* (barley scald) or *Oculimacula* spp. (eyespot on wheat) took more than ten years to develop detectable MBC-resistance (LOCKE, 1986; KENDALL *et al.* 1993). In some situations and regions, the use of mixtures or alternations with non-MBC fungicides was encouraged, although this was often done too late. In the USA, MBC fungicides were used to control *Cercospora* leaf spots of peanuts. Resistance problems soon occurred due to unrestricted use of benomyl. However in Texas, a mixture of benomyl with macozeb was used from the start leading to no severe development of resistance over many years (DELP, 1980; SMITH, 1988). The application of MBC fungicides to control the black sigatoka disease of banana (*M. fijiensis*) or pear scab (*Venturia nashicola*) was stopped due to high resistance frequencies. Subsequently, MBC-resistance frequencies declined slowly, because resistant strains were presumably less fit than sensitive ones and an effective re-entry in mixture or alternation with non-benzimidazole fungicides became possible (ISHII *et al.* 1985; ROMERO and SUTTON, 1998). The MBC Expert Forum of FRAC has no specific recommendations for MBC fungicides. Valid strategies are alternations or mixtures with non-cross-resistant fungicide having different sites of action. The number of treatments per season should

not exceed that indicated on the product label. Also, curative treatments must be reserved for special situations where no alternatives are available.

With the evidence of fitness costs associated with MBC-resistance in *Botrytis cinerea* presented in this study as well as the decline of resistance frequency presented by various authors (WALKER *et al.* 2013a, DERPMANN *et al.* 2010; LEROCH *et al.* 2010), a re-registration of thiophanate-methyl to control *Botrytis cinerea* could be possible. Thiophanate-methyl is registered in the EU until 2016 and the mixing compound mepanipyrim until 2014 (Directive 91/414/EEC, Annex I). Despite the medium to high resistance risk, thiophanate-methyl is registered solo or in mixture with iprodione or epoxiconazole against *S. sclerotiorum*, *C. beticola*, *Erysiphe betae* and *Ramularia beticola*. If thiophanate-methyl should be used to control *B. cinerea* in vineyards, then a strict resistance management strategy must be used as outlined in the following paragraphs.

A pre-packed mixture of thiophanate-methyl with mepanipyrim must be used in order to prevent large yield losses, if one component in the fungicide mixture suddenly fails (LODOVICA-GULLINO *et al.* 2012). A mixture is also preferred, because it can provide a wider spectrum of disease control. E.g. the mixture of thiophanate-methyl and an anilinopyrimidine could control downy mildew (*Erysiphe necator*) better than an anilinopyrimidine alone (Nisso Chemical Europe, personal communication). Additionally, MBC as well as anilinopyrimidine fungicides can control *Pseudopeziza tracheiphila* and *Guignardia bidwellii*, the causal agents of rotbrenner and black rot. Thus, the number of annual fungicide applications required in grapevine is minimized (STREETING *et al.* 1999; HEYE *et al.* 1994). Also, the mixture of thiophanate-methyl with mepanipyrim should be applied at full doses to prevent the selection of MDR phenotypes.

The number of treatments of thiophanate-methyl in mixture with mepanipyrim should be limited to one application per year. This application should be done as a protective treatment in a situation with low disease pressure. In grapevine, the mixture of thiophanate-methyl with mepanipyrim is recommended to be applied as the first treatment at end of flowering (BBCH 68). In low disease pressure situations the two effective systemic fungicides can protect the pollinated flower, thus no further fungicide applications to control *B. cinerea* are required. In regions with favorable weather conditions for *B. cinerea* infestations, additional applications with fungicides from other non-cross-resistant classes are required at bunch closure (BBCH 77) or verasation (BBCH 83). The fungicides, which are registered nowadays to control *B. cinerea*, are applied at lower dose rates compared to thiophanate-methyl. Lower dose rates lead to lower residual concentrations of the active ingredient(s) in the plant, which is in compliance with Regulation (EC) No 396/2005. The maximum residual levels (MRLs), which are the upper legal levels of a concentration for pesticide residues in or on food or feed, are 0.01 mg kg^{-1} and 3 mg kg^{-1} for thiophanate-methyl in table and wine grapes, respectively. Consequently, a single application of the mixture of thiophanate-methyl with mepanipyrim at BBCH 68 will not lead to residual concentrations in or on grapes higher than the MRLs.

In the present study, the solo application of mepanipyrim as part of the annual alternation led to a decrease in the frequency of MBC-resistant isolates in 2010. Thus, the mixture of thiophanate-methyl with mepanipyrim should be applied once every second or third year. Fungicide with different modes of action must be used to control *B. cinerea* in the intervening period. In addition, the sub-population resistant to MBC fungicides can be further reduced by the application of zoxamide, which is registered to control *P. viticola*. It has a secondary effect against *B. cinerea*, which results in a directional selection favoring MBC-sensitive strains (MALANDRAKIS *et al.* 2011). Such a resistance management strategy for anti-microtubule fungicides would primarily control *E. necator* and *P. viticola*, the two most important foliar diseases of grapevine, and secondarily control *B. cinerea* as well as *P. tracheiphila* and *G. bidwellii*.

However, it is not advisable to apply the mixture of thiophanate-methyl with mepanipyrim in grapevine-growing regions with a high percentage of MBC-resistant isolates (e.g. in the Champagne region). Thus, recommendations for use of MBC fungicides to control *B. cinerea* must be based on consistent, large-scale monitoring programs performed during the commercial use. The analyses required by EPPO (LAVADINHO, 2001) could be conducted by using pooled samples of *B. cinerea* and the high-throughput allele-specific real-time PCR developed in the present study. This way, the evolution of the major alleles responsible for benzimidazole-resistance can be monitored at population level and the success of the proposed resistance management strategy can be evaluated (BRENT and HOLLOMON, 2007).

It is likely that the integrated use of fungicides will remain one of the most important control strategies in the foreseeable future. However, all 'single-site' fungicides, which target key proteins, have an intrinsic risk of resistance development by fungal pathogens (HOLLOMON, 2012). Recently, phenotypes resistant to fungicides of the second generation of Succinate Dehyhrogenase Inhibitors (SDHIs), which are the latest fungicides registered to control *B. cinerea*, were detected (WEBER, 2011; LEROCH *et al.* 2013). Some mutations leading to SDHI-resistance show a positive cross-resistance pattern for all fungicides of this class (AVENOT and MICHAILIDES, 2010; VELOUKAS *et al.* 2013; LEROUX and WALKER, 2013). Hence, newly developed active ingredients of this fungicide class are confronted with medium to high frequencies of resistant strains at time of introduction to the market. The worst-case scenario in terms of resistance development of *B. cinerea* to SDHIs and other fungicides can be observed in American and German strawberry fields. High frequencies of phenotypes showing multiple target-site and/or multi-drug resistance with high resistance factors (MDR1h) were detected (LEROCH *et al.* 2013; FERNÁNDEZ-ORTUÑO *et al.* 2012). In such a situation, only old 'multi-site' fungicides can be applied for resistance management. Interestingly, many isolates with a multiple target-site resistance also showed a target-site resistance to benzimidazoles, probably of the BenR1 phenotype. Hence, a solo application of diethofencarb, which exhibit a negative selection pressure to BenR1 strains, could control phenotypes with multiple target-site resistances including

benzimidazole-resistance. A similar use pattern of thiophanate-methyl and diethofencarb could be applied as described for thiophanate-methyl and zoxamide in the present study. This way, the negative cross-resistance pattern of benzimidazole-resistant strains as well as the low resistance factors exhibited by MDR strains towards MBC-fungicides can be utilized for an overall resistance management strategy for all chemical classes registered to control *B. cinerea*.

Agricultural production must be increased in the next decades to meet the food and feed demands as well as the need for renewable resources of a growing human population reaching 9 billion in 2050 (GODFRAY *et al.* 2010). Therefore, genetic resources of plants must be used by breeders to increase yield (TESTER and LANGRIDGE, 2010) and crop protection must be used to safeguard the crop productivity, which relies heavily on pesticides (OERKE and DEHNE, 2004). However, pathogens are able to overcome diverse modes of action of pesticides by build-up of resistance. Therefore, research and development must focus on discovering new modes of action of pesticides as well as conserving existing chemicals (HOLLOMON, 2012). Beside classical breeding of pathogen-resistant plant varieties, transgenic plants can be used to increase the resistance of crops to fungal pathogens, e.g. by overexpressing essential genes of the pathogen leading to host induced gene silencing (NOWARA *et al.* 2010). All these tool are needed in the 'arms race' against the adaption of fungal pathogens to the selection pressure exhibited by mankind's innovations (HOLLOMON and BRENT, 2009). Thus, a registration of the mixture of thiophanate-methyl with mepanipyrim would contribute to the diversity of modes of action leading to a prolongation of the lifespan of newly introduced tools to control *B. cinerea* in grapevine in the future.

5 SUMMARY

Gray mold caused by the fungus *Botrytis cinerea* is an economically important disease in grapevine. The pathogen has an intrinsic risk to develop resistance to frequently applied systemic fungicides. Only a few years after introduction of benzimidazole fungicides like thiophanate-methyl, resistant strains of *B. cinerea* appeared frequently in European vineyards. Since the discontinuation of the use of benzimidazoles in 1975, a strong decrease of the frequency of benzimidazole-resistant isolates was detected. Since development of resistance to all registered fungicides is reported, implementation of anti-resistance strategies with frequent change of active ingredients with different mode of actions is necessary.

In the present study, the influence of different resistance management strategies on population dynamics of *B. cinerea* isolates resistant to benzimidazole and anilinopyrimidine fungicides was investigated. A field trial at three vineyards near Bordeaux was initiated in 2009. The tested strategies were a mixture and an alternation of thiophanate-methyl (TM) and mepanipyrim (MP). Also, an annual alternation of fungicides in solo applications (TM: 2009 and 2011, MP: 2010) was tested. Strategies were compared to a solo application of TM (unrestricted use) and conventional fungicide treatments, where no TM was applied. Disease incidence and severity as well as the frequencies of *B. cinerea* phenotypes resistant to TM (BenR1, BenR2) and MP (AniR1, AniR) were determined in monitoring procedures conducted prior (May or June) and subsequent to fungicide applications (August or September). The results of the three-year field trial can be summarized for the three trial sites as follows:

- In 2009 and 2010, all tested strategies reduced disease incidence and severity of *B. cinerea* compared to the conventional fungicide treatments, where no fungicides to control *B. cinerea* were applied. Disease control was most effective for alternation of TM and MP.
- No significant correlation between percentages of fungicide-resistant phenotypes and disease incidence or severity was detected.
- The baseline sensitivity in June 2009 was lower than 10% for BenR1 and AniR and lower than 5% for BenR2 and AniR1 phenotypes.
- In all three years, spray programs including TM resulted in significantly higher BenR1 isolate frequencies compared to those detected in conventionally treated plots, where no TM was applied.
- In September 2009, all strategies tested resulted in similar BenR1 isolate frequencies compared to the unrestricted use (23%).

- In September 2010, solo application of MP led to significantly lower BenR1 frequency (16%) compared to the spray programs including TM in 2010 (39%).
- At the end of the study in August 2011, no significant differences in BenR1 frequency were detected between the strategies tested and the unrestricted use of TM at high levels (47%). Also, no significant differences in BenR2 as well as AniR1 isolate frequencies were detected between the spray programs and the conventional fungicide treatment.
- The build-up of resistance to benzimidazoles in the course of three years was significantly higher at Saint Brice and Loupes site (from 11% to 53%) compared to that at Grezillac site (from 3% to 35%), where zoxamide was applied in 2010.
- Over the winter period 2009/10, where no selection pressure through fungicides was present, a decrease of BenR1 isolate frequency to 11% was detected. Due to the low isolation rate in May 2011, this observation could not be verified.
- The positive spatio-temporal correlation of benzimidazole-resistant isolates in populations colonizing flowers compared to that colonizing bunches of berries demonstrate the effect of short-distance dispersal of the conidia.
- The negative spatio-temporal correlation of benzimidazole-resistant isolates in populations colonizing bunches of berries compared to the consecutive population colonizing flowers as well as the temporal instability of aggregations of benzimidazole-resistant isolates observed after fungicide application indicated a fitness penalty of resistant isolates and a mixture of phenotypes in the primary inoculum after the winter-period 2009/10.
- Isolates with a reduced sensitivity to anilinopyrimidines (AniR) were detected, which could belong to the recently described MDR phenotypes. However, no further studies were conducted to characterize these phenotypes.
- AniR isolates were detected at frequencies of 10 – 30 %. However, results of the three trial sites were inconsistent. In general, applications of MP or other anilinopyrimides resulted in a higher AniR isolate frequency compared to treatments where no fungicides of this chemical group were applied.

Different single nucleotide polymorphisms (SNP) in the β-tubulin gene of *B. cinerea* confer resistance to benzimidazole fungicides. In the present study, SNPs were detected by means of an allele-specific polymerase chain reaction assay (as-PCR):

- Twelve of 123 isolates collected from five German vineyards in September 2007 were resistant to benzimidazoles. All twelve resistant isolates, which showed the BenR1 phenotype, carried the E198A-SNP.
- In June 2009, 16 of 174 isolates collected from three sites near Bordeaux were resistant to benzimidazoles. 13 isolates, which showed the BenR1 phenotype, carried the E198A-SNP. Three isolates, which showed the BenR2 phenotype, carried the F200Y-SNP.

For population analysis of *B. cinerea*, an EvaGreen® real-time as-PCR (as-qPCR) has been developed for quantitative detection of E198A and F200Y allele frequency:

- Efficacy of as-qPCR reactions ranged from 94 to 105% and the coefficient of determination of standard curves was higher than 0.995.
- For validation of the as-qPCR methods known allele frequencies in pooled DNA samples were compared to measured E198A and F200Y allele frequencies. Linear regression analysis showed a positive correlation (slope of 0.93 and 1.01) with coefficients of determination of 0.991 and 0.985. The level of detection (LOD) and quantification (LOQ) were estimated as 0.18% and 0.25% for E198A and 0.005% and 0.01% F200Y for allele frequency.
- As regards to field populations collected in August 2011, most BenR1 phenotypes showed the E198-SNP and most BenR2 phenotypes showed the F200Y-SNP. Thus, these two SNPs are the most important mutations leading to benzimidazole resistance in *B. cinerea*.

The development of resistance to fungicides in a population is largely dependent on the fitness of the resistant portion. Therefore, a variety of fitness parameters of ten BenR1 isolates, which carry the E198A-SNP, and ten benzimidazole-sensitive isolates (BenS) were compared under favourable and unfavourable development conditions with and without thiophanate-methyl application:

- No significant difference in frost tolerance was observed between the sensitive and resistant phenotypes tested.
- At favourable conditions, no significant differences in the tested fitness parameters were detected between the two sensitivity groups. However at unfavourable conditions, mycelium growth, spore production and lesion size on detached leaves of grapevine inoculated with BenS isolates were significantly higher compared to that of BenR1 isolates.
- At favourable conditions, no significant difference in the frequency of BenR1 conidia was detected after inoculation of autoclaved leaf discs or grapevine plants with three ratios of BenS and BenR1 isolates after two successive generations. However at unfavourable conditions, a significant decrease in the frequency of BenR1 conidia of about 7% per generation was observed.
- One application of thiophanate-methyl prior to inoculation of grapevine plants resulted in BenR1 conidia frequencies of 98 – 100% regardless of the initial frequency and the developmental conditions.

The results of the field trial as well as the laboratory experiments can explain the observed decrease of the frequency of benzimidazole-resistant isolates in German and French vineyards. Without selection pressure of benzimidazoles, small fitness costs associated with resistance could have reduced the percentage of resistant isolates within the primary inoculum, when the fungus was confronted with reduced nutrient availability and low temperatures. This led to a decrease of the resistant fraction in the consecutive berry-associated populations, if no benzimidazoles were applied during the

season. Over time this difference in fitness led to a linear decrease resulting the low frequency of BenR1 strains as observed in the long term monitoring conducted by INRA (LEROUX *et al.* 2013a). With the evidence of fitness costs associated with benzimidazole-resistance in *B. cinerea* presented in this study, a re-registration of thiophanate-methyl to control *B. cinerea* could be possible under the precondition of the following recommendations in order to manage the build-up of resistance:

- A pre-packed mixture of thiophanate-methyl with mepanipyrim must be used.
- The number of treatments of the mixture should be limited to one protective application per year at the end of flowering (BBCH 68).
- The mixture should be applied every second or third year. Different fungicide classes must be used to control *B. cinerea* in the intervening period.
- The sub-population of *B. cinerea* resistant to benzimidazole fungicides can be reduced by the application of zoxamide, which controls primarily *Plasmopara viticola* and secondarily exhibits a directional selection pressure favoring benzimidazole-sensitive strains of *B. cinerea*.
- It is not advisable to apply the mixture in grapevine-growing regions with a high percentage of benzimidazole-resistant isolates (e.g. in the Champagne region). Thus, recommendations for the use of benzimidazole fungicides to control *B. cinerea* must be based on consistent, large-scale monitoring programs performed during the commercial use.

A registration of the mixture of thiophanate-methyl with mepanipyrim would contribute to the diversity of modes of action controlling *B. cinerea*. Due to the emergence and development of resistance to 'single-site' fungicides of all chemical classes, a resistance management combining all tools available in an integrated pest management will be needed. Thus, a registration of the mixture of thiophanate-methyl with mepanipyrim would lead to a prolongation of the lifespan of newly introduced tools to control *B. cinerea* in grapevine in the future.

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7 APPENDIX

7.1 CHEMICAL TREATMENTS AT VINEYARDS NEAR BORDEAUX

Table 7-1 Chemical treatments at the vineyard near Grezillac from 2009 to 2011. Use, active ingredient(s), chemical group, mode of action and cross-resistance group (FRAC-code) are assigned to products applied. Treatments in boldface were applied solely in the surrounding farmer's plots.

Year	Date	Use	Product	Active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2009	10. Feb.	Herbicide	Surflan	Oryzalin	-	-	-
	25. Apr.	Fungicide	Sirbel	Folpet	Phthalimides	Unknown	M4
				Iprovalicarbe	CAA fungicides	Cellulose synthesis	F5
		Fungicide	Prosper	Spiroxamine	Morpholines	SBI (Class II)	G2
	8. Mai.	Fungicide	Valiant Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
				Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
		Fungicide	Milord	Tebuconazole	Triazoles	DMI (SBI class I)	G1
				Spiroxamine	Morpholines	SBI (Class II)	G2
	19. Mai.	Fungicide	Valiant Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	33
				Folpet	Phthalimides	Multi-site	M4
				Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
		Fungicide	Milord	Tebuconazole	Triazoles	DMI (SBI class I)	G1
				Spiroxamine	Morpholines	SBI (Class II)	G2
	29. Mai.	Fungicide	Cabrio Ultra	Folpet	Phthalimides	Multi-site	M4
				Pyraclostrobin	Strobilurines	QoI	C3
	9. Jun.	Fungicide	Mikal	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Prosper	Spiroxamine	Morpholines	SBI (Class II)	G2
	20. Jun.	Fungicide	Mikal	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Prosper	Spiroxamine	Morpholines	SBI (Class II)	G2
	1. Jul.	Fungicide	Cabrio Ultra	Folpet	Phthalimides	Multi-site	M4
				Pyraclostrobin	Strobilurines	QoI	C3
	18. Jul.	Herbicide	Roundup	Glyphosate	-	-	-
	28. Jul.	Fungicide	Folpan	Folpet	Phthalimides	Multi-site	M4
	8. Aug.	Fungicide	Folpan	Folpet	Phthalimides	Multi-site	M4
2010	17. Apr.	Fungicide	Artimon	Mancozeb	Dithiocarbamates	Multi-site	M3
				Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide	Microthiol	Sulfur	Inorganics	Multi-site	M2
	7. Mai.	Fungicide	Electis	Mancozeb	Dithiocarbamates	Multi-site	M3
				Zoxamide	Benzamides	Beta-tubulin	B3
		Fungicide	Kumulus	Sulfur	Inorganics	Multi-site	M2
	25. Mai.	Fungicide	Amalfi	Folpet	Phthalimides	Multi-site	M4
				Benalaxyl	Phenylamides	RNA synthesis	A1
		Fungicide	Antene	Tetraconazole	Triazoles	DMI (SBI class I)	G1
	7. Jun.	Fungicide	Mildicut	Cyazofamid	Cyano-imidazoles	QiI	C4
		Fungicide	Abilis	Triadimenol	Triazoles	DMI (SBI class I)	G1
	23. Jun.	Fungicide	Pergado F	Folpet	Phthalimides	Multi-site	M4
		Fungicide	Pepite	Mandipropamid	CAA fungicides	Cellulose synthesis	F5
		Fungicide	Corail	Tebuconazole	Triazoles	DMI (SBI class 1)	G1
	15. Jul.	Fungicide	Sidecar	Mancozeb	Dithiocarbamates	Multi-site	M3
				Benalaxyl	Phenylamides	RNA synthesis	A1
		Fungicide	Flint	Trifloxystrobin	Strobilurines	QoI	C3
	5. Aug.	Fungicide	Aviso Cup DF	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Metiram	Dithiocarbamates	Multi-site	M3

Continuation of Table 7–1 Chemical treatments at the vineyard near Grezillac from 2009 to 2011.

Year	Date	Use	Product	active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2011	13. Apr.	Fungicide	Artimon	Mancozeb	Dithiocarbamates	Multi-site	M3
				Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide	Soufre	Sulfur	Inorganics	Multi-site	M2
	5. Mai.	Fungicide	Hidalgo Star	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Consist	Trifloxystrobin	Strobilurines	QoI	C3
	17. Mai.	Fungicide	Amalfi	Folpet	Phthalimides	Multi-site	M4
				Benalaxyl	Phenylamides	RNA synthesis	A1
		Fungicide	Stikine	Tebuconazol	Triazoles	DMI (SBI class 1)	G1
	9. Jun.	Fungicide	Enervin	Metiram	Dithiocarbamates	Multi-site	M3
				Ametoctradin	triazolo-pyrimidylamines	QxI	C8
		Fungicide	Mayandra	Tebuconazole	Triazoles	DMI (SBI class 1)	G1
	28. Jun.	Fungicide	Valiant Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
				Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
		Fungicide	Legend	Quinoxyfen	azanapthalenes	Signal transduction (?)	E1
	27. Jul.	Fungicide	Sygant S	Folpet	Phthalimides	Multi-site	M4
				Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)

Table 7-2 Chemical treatments at the vineyard near Saint Brice from 2009 to 2011. Use, active ingredient(s), chemical group, mode of action and cross-resistance group (FRAC-code) are assigned to products applied. Treatments in boldface were applied solely in the surrounding farmer's plots.

Year	Date	Use	Product	Active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2009	8. Apr.	Herbicide	Emir	Oxyfluorfene	-	-	-
				Propyzamide	-	-	-
	1. Mai.	Fungicide	Polyram	Metiram	Dithiocarbamates	Multi-site	M3
	6. Mai.	Fungicide	Addax	Mancozeb	Dithiocarbamates	Multi-site	M3
	16. Mai.	Fungicide	Addax	Mancozeb	Dithiocarbamates	Multi-site	M3
	30. Mai.	Fungicide	Sirbel	Folpet	Phthalimides	Multi-site	U1 (33)
				Iprovalicarbe	CAA fungicides	Cellulose synthesis	F5
		Fungicide	Score	Difenoconazole	Triazoles	DMI (SBI class 1)	G1
	12. Jun.	Fungicide	Cabrio Star	Folpet	Phthalimides	Multi-site	M4
				Pyraclostrobin	Strobilurines	QoI	C3
	12. Jun.	Insecticide	Split Protech	Deltamethrine	-	-	-
	29. Jun.	Fungicide	Sirbel	Folpet	Phthalimides	Multi-site	M4
				Iprovalicarbe	CAA fungicides	Cellulose synthesis	F5
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	15. Jul.	Fungicide	Escadril	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Folpet	Phthalimides	Multi-site	M4
	31. Jul.	Fungicide	Badger	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Mancozeb	Dithiocarbamates	Multi-site	M3
	17. Jun.	**Fungicide**	**Switch**	**Cyprodinil**	**Anilinopyrimidines**	**Methionine biosynthesis**	**D1**
				Fludioxonil	**Phenylpyrroles**	**Signal transduction (?)**	**E2**
	1. Aug.	**Fungicide**	**Rovral**	**Iprodione**	**Dicarboximides**	**Signal transduction (?)**	**E3**

Continuation of Table 7–2 Chemical treatments at the vineyard near Saint Brice from 2009 to 2011.

Year	Date	Use	Product	active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2010	20. Apr.	Fungicide	Polyram DF	Metiram	Dithiocarbamates	Multi-site	M3
	30. Apr.	Fungicide	Ridgold F	Folpet	Phthalimides	Multi-site	M4
				Metalaxyl-M	Phenylamides	RNA synthesis	A1
		Fungicide	Soufre	Sulfur	Inorganics	Multi-site	M2
	15. Mai.	Fungicide	Rigold F	Folpet	Phthalimides	Multi-site	M4
				Metalaxyl-M	Phenylamides	RNA synthesis	A1
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	1. Jun.	Fungicide	Cabrio Top	Metiram	Dithiocarbamates	Multi-site	M3
				Pyraclostrobin	Strobilurines	QoI	C3
	18. Jun.	Fungicide	Sirbel DU	Folpet	Phthalimides	Multi-site	M4
				Iprovalicarb	CAA fungicides	Cellulose synthesis	F5
		Fungicide	Score	Difenoconazole	Triazoles	DMI (SBI class 1)	G1
	4. Jul.	Fungicide	Escadril	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Folpet	Phthalimides	Phthalimide	M4
	24. Jul.	Fungicide	Badger	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Mancozeb	Dithiocarbamates	Multi-site	M3
	1. Jul.	Fungicide	Teldor	Fenhexamid	Hydroxyanilides	SBI (Class III)	G3
2011	26. Apr.	Fungicide	Mancotec	Mancozeb	Dithiocarbamates	Multi-site	M3
	10. Mai.	Fungicide	Rigold F	Folpet	Phthalimides	Multi-site	M4
				Metalaxyl-M	Phenylamides	RNA synthesis	A1
		Fungicide	Hoggar	Spiroxamine	Morpholines	SBI (Class II)	G2
	28. Mai.	Fungicide	Cabrio Star	Folpet	Phthalimides	Multi-site	M4
				Pyraclostrobin	Strobilurines	QoI	C3
		Fungicide	**Switch**	**Cyprodinil**	**Anilinopyrimidines**	**Methionine biosynthesis**	**D1**
		Fungicide		**Fludioxonil**	**Phenylpyrroles**	**Signal transduction (?)**	**E2**
	11. Jun.	Fungicide	Rigold F	Folpet	Phthalimides	Multi-site	M4
				Metalaxyl-M	Phenylamides	RNA synthesis	A1
		Fungicide	Score	Difenoconazole	Triazoles	DMI (SBI class 1)	G1
	27. Jun.	Fungicide	Escadril	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Soufre	Sulfur	Inorganics	Multi-site	M2
	28. Jul.	**Fungicide**	**Rovral**	**Iprodione**	**Dicarboximides**	**Signal transduction (?)**	**E3**

Table 7-3 Chemical treatments at the vineyard near Loupes from 2009 to 2011. Use, active ingredient(s), chemical group, mode of action and cross-resistance group (FRAC-code) are assigned to products applied. Treatments in boldface were applied solely in the surrounding farmer's plots.

Year	Date	Use	Product	Active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2009	22. Feb.	Herbicide	Surflan	Oryzalin	-	-	-
	2. Mai.	Fungicide	Option Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
			Actiol	Sulfur	Inorganics	Multi-site	M2
	16. Mai.	Fungicide	Option Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	28. Mai.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Metiram	Dithiocarbamates	Multi-site	M3
	12. Jun.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Atonium	Myclobutanil	Triazoles	DMI (SBI class 1)	G1
	26. Jun.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Atonium	Myclobutanil	Triazoles	DMI (SBI class 1)	G1
	9. Jul.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Atomium	Myclobutanil	Triazoles	DMI (SBI class 1)	G1
	24. Jul.	Fungicide	Selva	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Copper	Inorganics	Multi-site	M1
	7. Aug.	Fungicide	Escardil	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Folpet	Phthalimides	Multi-site	M4
	8. Aug.	**Fungicide**	**Sekoya**	**Fluazinam**	**2-6-dinitro anilines**	**Oxidative phosphorylation**	**C5**
2010	17. Apr.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Soufre	Sulfur	Inorganics	Multi-site	M2
	26. Apr.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	11. Mai.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	25. Mai.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Corail	Tebuconazole	Triazoles	DMI (SBI class 1)	G1
	4. Jun.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Corail	Tebuconazole	Triazoles	DMI (SBI class 1)	G1
	15. Jun.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Legend	Quinoxyfen	azanapthalenes	Signal transduction (?)	E1
	29. Jun.	Fungicide	Option Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
		Fungicide		Folpet	Phthalimides	Multi-site	M4
		Fungicide	Legend	Quinoxyfen	azanapthalenes	Signal transduction (?)	E1
	22. Jul.	Fungicide	Bord. mixture	Copper	Inorganics	Multi-site	M1
		Fungicide	Thiovit Gold	Sulfur	Inorganics	Multi-site	M2
	6. Aug.	Fungicide	Escardil	Cymoxanil	Cyanoacetamide-oximes	Unknown	U1 (27)
				Folpet	Phthalimides	Multi-site	M4
	16. Aug.	**Fungicide**	**Sekoya**	**Fluazinam**	**2-6-dinitro anilines**	**Oxidative phosphorylation**	**C5**

Continuation of Table 7–3 Chemical treatments at the vineyard near Loupes from 2009 to 2011.

Year	Date	Use	Product	active ingredient(s)	Additional information on fungicides		
					Chemical group	Mode of action	FRAC-Code
2011	30. Apr.	Fungicide	Mikal Flash	Fosetyl-Al	Ethyl phosphonate	Unknown	U1 (33)
				Folpet	Phthalimide	Multi-site	M4
		Fungicide	Soufre	Sulfur	Inorganics	Multi-site	M2
	13. Mai.	Fungicide	Slogan	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Metiram	Dithiocarbamates	Multi-site	M3
		Fungicide	Hoggar	Spiroxamine	Morpholines	SBI (Class II)	G2
	26. Mai.	Fungicide	Mikal Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Score	Difenoconazole	Triazoles	DMI (SBI class 1)	G1
	9. Jun.	Fungicide	Mikal Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Score	Difenoconazole	Triazoles	DMI (SBI class 1)	G1
		Fungicide	**Sekoya**	**Fluazinam**	**2-6-dinitro anilines**	**Oxidative phosphorylation**	**C5**
	23. Jun.	Fungicide	Option Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Atemi 10	Cyproconazole	Triazoles	DMI (SBI class 1)	G1
	6. Jul.	Fungicide	Option Flash	Fosetyl-Al	Ethyl phosphonates	Unknown	U1 (33)
				Folpet	Phthalimides	Multi-site	M4
		Fungicide	Actiol	Sulfur	Inorganics	Multi-site	M2
	22. Jul.	Fungicide	Dithane	Mancozeb	Dithiocarbamates	Multi-site	M3
			Actiol	Sulfur	Inorganics	Multi-site	M2
	23. Jul.	**Fungicide**	**Rovral**	**Iprodione**	**Dicarboximides**	**Signal transduction (?)**	**E3**

7.2 DETERMINATION OF DISCRIMINATIVE CONCENTRATIONS OF ANILINOPYRIMIDINES

Table 7-4 Determination of EC_{50} and EC_{90} values of mepanipyrim and respective regression coefficients of determination of six isolates of *Botrytis cinerea*

Isolate	EC_{50} [ppm]	EC_{90} [ppm]	R^2
B-T2-R1-4	2,93	6,656	0,967
B-T4-R2-10	221,8	-	0,758
B-T4-R2-20	0,04	0,08	0,99
C-T2-R3-5	0,03	0,07	0,992
C-T2-R3-7	2,08	5,38	0,984
C-T2-R3-22	2,11	5,04	0,959

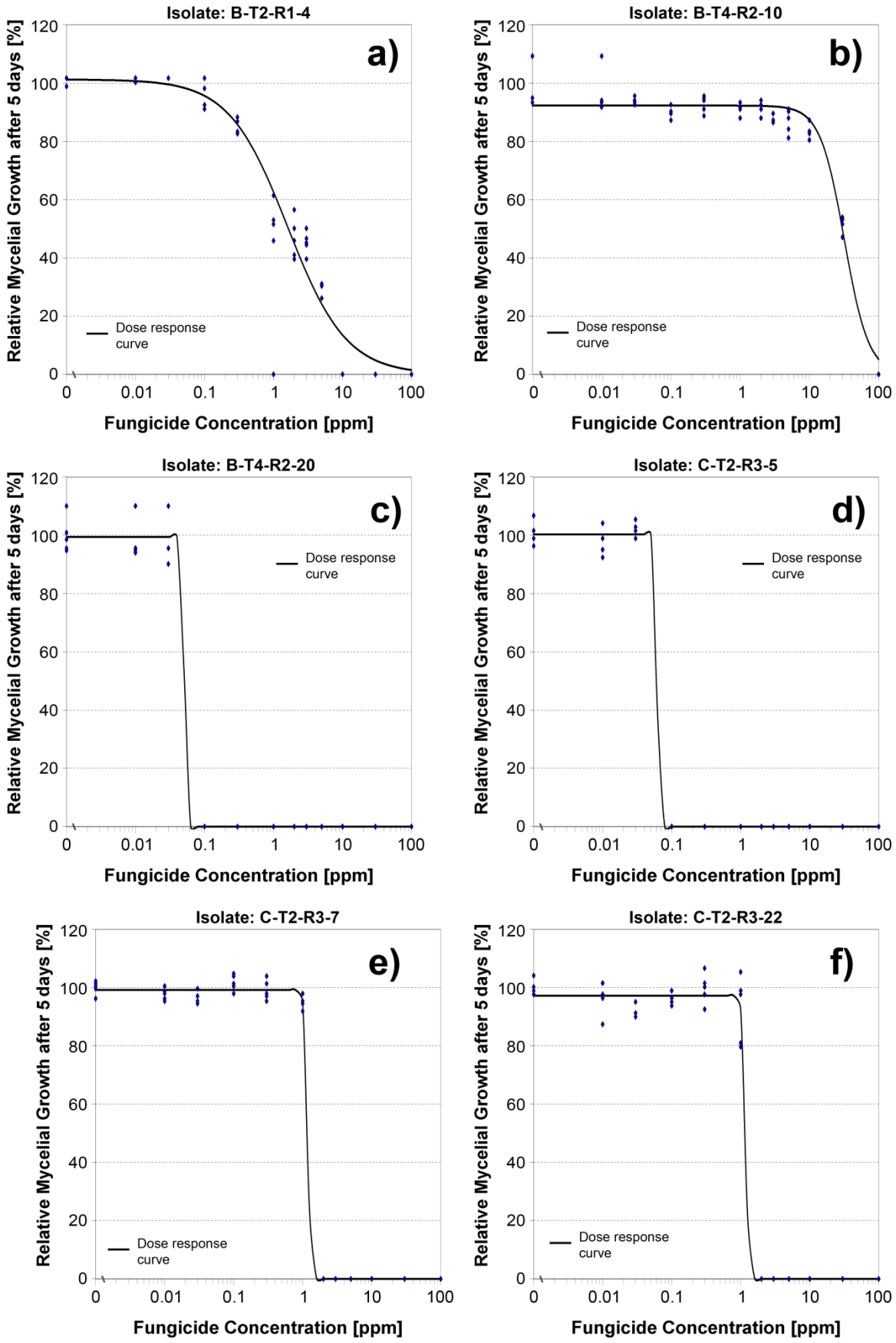

Figure 7-1 Dose-response-curves of the mycelial growth of six isolates of *Botrytis cinerea* (a-f) tested against a range of mepanipyrim concentrations

7.3 INFLUENCE OF RESISTANCE MANAGEMENT STRATEGIES ON POPULATIONS OF *B. CINEREA*

Table 7-5 Mean percentage of phenotypes of *Botrytis cinerea* resistant to fungicides. Isolates were collected from the experimental site located near Grezilac from 2009 to 2010. 20 samples of berries or 96 samples of flowers were collected from four plots per treatment. If sporulation of gained isolates did not appear on medium amended with fungicides, then isolates were considered as sensitive. In the conventional fungicide treatment samples were taken from farmers plot. Statistical analysis between treatments: identical letters show no significant difference between treatments according to a generalized linear model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05.

			Percentage of *Botrytis* isolates resistant to thiophanate-methyl [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			3,3 a		
	September	410	17,1 B b	9,2 B ab	19,5 B bc	15,3 B ab	0 A a
2010	June	340	9,2 A ab	7,9 A ab	10,7 A ab	5,7 A a	3,5 A a
	September	413	28,9 C b	25 BC bc	44,4 C d	9,1 AB a	8,2 A a
2011	May	292	13,6 AB ab	13,3 AB abc	37,5 B cd	2,7 A a	3 A a
	August	401	30,9 B b	34,1 B c	32,9 B cd	44,6 B b	3,5 A a
			Percentage of *Botrytis* isolates with a reduced sensitivity to mepanipyrim [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			8,1 a		
	September	410	11 A ab	7,9 A a	31,7 B b	8,2 A a	11,8 A a
2010	June	340	9,2 A ab	6,3 A a	16,1 A ab	17,1 A ab	15,1 A a
	September	413	10,8 AB c	13,2 ABC a	28,4 C b	26,1 BC b	9,4 A a
2011	May	292	9,1 A abc	10 A a	4,2 A a	8,1 A a	9,1 A a
	August	401	8,6 A c	14,6 A a	11,4 A ab	13,5 A ab	9,4 A a
			Percentage of *Botrytis* isolates resistant to mepanipyrim [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			0 a		
	September	410	2,4 A a	1,3 A a	6,1 A a	2,4 A a	2,4 A a
2010	June	340	1.3 A a	1,9 A a	0 A a	0 A a	2,3 A a
	September	413	0 A a	0 A a	1,2 A a	0 A a	0 A a
2011	May	292	4,5 A a	0 A a	0 A a	0 A a	0 A a
	August	401	0 A a	1,2 A a	1,3 A a	0 A a	0 A a
			Percentage of *Botrytis* isolates with a reduced sensitivity to mepanipyrim and a resistance to thiophanate-methyl [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			0 a		
	September	410	6,1 A a	1,3 A a	6,1 A ab	2,4 A a	0 A a
2010	June	340	0 A a	1,6 A a	3,6 A a	2,9 A a	2,3 A a
	September	413	4,8 AB a	5,3 AB a	18,5 B b	5,7 AB a	1,2 A a
2011	May	292	0 A a	6,7 A a	4,2 A a	0 A a	0 A a
	August	401	4,9 A a	7,3 A a	6,3 A ab	8,1 A a	0 A a
			Percentage of *Botrytis* isolates with a resistance to mepanipyrim and thiophanate-methyl [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			0 a		
	September	410	0 A a	0 A a	0 A a	0 A a	0 A a
2010	June	340	0 A a	1,6 A a	0 A a	0 A a	0 A a
	September	413	0 A a	0 A a	0 A a	0 A a	0 A a
2011	May	292	0 A a	0 A a	0 A a	0 A a	0 A a
	August	401	0 A a	0 A a	0 A a	0 A a	0 A a
			Percentage of *Botrytis* isolates with a resistance to diethofencarb and thiophanate-methyl [%]				
Time of Monitoring			TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	92			0 a		
	September	410	8,5 AB a	6,6 AB a	9,8 B b	12,9 B b	0 A a
2010	June	340	1,5 A a	1,6 A a	5,4 A ab	1,4 A ab	2,3 A a
	September	413	2,4 A a	0 A a	2,5 A ab	0 A a	1,2 A a
2011	May	292	4,5 A a	0 A a	0 A ab	2,7 A ab	0 A a
	August	401	4,9 AB a	0 A a	6,3 AB ab	13,5 B b	1,2 A a

Table 7-6 Mean percentage of phenotypes of *Botrytis cinerea* resistant to fungicides. Isolates were collected from the experimental site located near Saint Brice from 2009 to 2010. 20 samples of berries or 96 samples of flowers were collected from four plots per treatment. If sporulation of gained isolates did not appear on medium amended with fungicides, then isolates were considered as sensitive. In the conventional fungicide treatment samples were taken from farmers plot. Statistical analysis between treatments: identical letters show no significant difference between treatments according to a generalized linear model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05.

Percentage of isolates resistant to thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			10,4 a		
	September	375	31,3 B bc	31,3 B b	31,9 B bc	38,8 B b	12,7 A a
2010	June	125	33,3 A bc	16,7 A ab	8,7 A a	9,7 A a	5,7 A a
	September	426	50,6 B bc	43,5 B b	46,5 B bc	31 B b	13,7 A a
2011	May	82	12,5 A ab	16,7 A ab	10 A a	33,3 A ab	9,1 A a
	August	389	53,5 B c	53,2 B b	50,6 B c	53,1 B b	5,3 A a
Percentage of isolates with a reduced sensitivity to mepanipyrim[%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			13,3 a		
	September	375	13,3 B a	23,8 B a	19,7 B ab	15,3 B a	1,3 A a
2010	June	125	16,7 AB a	11,1 AB a	0 A a	0 A a	22,9 B b
	September	426	11,8 A a	14,1 A a	12,8 A a	12,6 A a	7,3 A ab
2011	May	82	0 A a	16,7 A a	10 A ab	16,7 A a	0 A ab
	August	389	15,5 A a	26 AB a	40 B b	11,1 A a	21,3 AB b
Percentage of isolates resistant to mepanipyrim [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			0 a		
	September	375	0 A a	1,6 A a	0 A a	1,4 A a	0 A a
2010	June	125	5,6 A a	0 A a	0 A a	0 A a	0 A a
	September	426	4,7 A a	4,7 A a	7 A a	3,4 A a	4,8 A a
2011	May	82	0 A a	0 A a	0 A a	0 A a	0 A a
	August	389	1,4 A a	0 A a	4,7 A a	1,2 A a	2,7 A a
Percentage of isolates with a reduced sensitivity to mepanipyrim and a resistance to thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			4,4 a		
	September	375	5 AB a	13,4 B a	7,2 AB a	7,5 AB a	0 A a
2010	June	125	5,6 A a	5,6 A a	0 A a	0 A a	0 A a
	September	426	9,4 A a	7,1 A a	7 A a	4,6 A a	2,4 A a
2011	May	82	0 A a	0 A a	0 A a	16,7 A a	0 A a
	August	389	9,9 AB a	16,9 B a	20 B a	6,2 AB a	1,3 A a
Percentage of isolates with a resistance to mepanipyrim and thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			0 a		
	September	375	0 A a	0 A a	0 A a	0 A a	0 A a
2010	June	125	0 A a	0 A a	0 A a	0 A a	0 A a
	September	426	0 A a	1,2 A a	0 A a	0 A a	0 A a
2011	May	82	0 A a	0 A a	0 A a	0 A a	0 A a
	August	389	0 A a	0 A a	0 A a	0 A a	0 A a
Percentage of isolates with a resistance to diethofencarb and thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	48			4,4 a		
	September	375	7,5 AB a	11,9 AB a	13 B a	11,3 AB a	1,3 A a
2010	June	125	0 A a	0 A a	4,3 A a	3,2 A a	2,9 A a
	September	426	5,9 A a	12,9 A a	8,1 A a	11,5 A a	7,3 A a
2011	May	82	0 A a	0 A a	0 A a	0 A a	0 A a
	August	389	8,5 AB a	14,3 AB a	7,1 AB a	16 B a	2,7 A a

Table 7-7 Mean percentage of phenotypes of *Botrytis cinerea* resistant to fungicides. Isolates were collected from the experimental site located near Loupes from 2009 to 2010. 20 samples of berries or 96 samples of flowers were collected from four plots per treatment. If sporulation of gained isolates did not appear on medium amended with fungicides, then isolates were considered as sensitive. In the conventional fungicide treatment samples were taken from farmers plot. Statistical analysis between treatments: identical letters show no significant difference between treatments according to a generalized linear model with sequential Bonferroni adjustment for multiple comparisons at p = 0.05

Percentage of isolates resistant to thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	12				
	September	379	16,7 B a	20,3 B b	28,8 B b	28,6 B bc	1,2 A a
2010	June	423	12,1 A a	6,2 A a	6,7 A a	7,7 A a	7,1 A a
	September	408	50 BC b	39,7 B bc	63,6 C c	12 A ab	5,3 A a
2011	May	58	0 A ab	9,1 A ab	0 A ab	20 A abc	0 A a
	August	359	52,9 B b	56,6 B c	65,5 B c	46 B c	5 A a
Percentage of isolates with a reduced sensitivity to mepanipyrim[%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	6				
	September	379	9 A a	10,1 A a	12,3 A ab	7,8 A a	3,7 A a
2010	June	423	8,8 A a	15,4 A a	13,3 A a	15,4 A a	14,3 A a
	September	408	9,5 A a	15,1 AB a	28,6 B bc	15,7 AB a	8,4 A a
2011	May	58	0 A a	9,1 A a	25 A abc	20 A a	0 A a
	August	359	8,8 AB a	26,4 BC a	32,8 C c	14,3 AB a	1,7 A a
Percentage of isolates resistant to mepanipyrim [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	0				
	September	379	2,6 A a	0 A a	0 A a	0 A a	0 A a
2010	June	423	1,1 A a	0 A a	0 A a	0 A a	3,6 A a
	September	408	0 A a	0 A a	0 A a	0 A a	0 A a
2011	May	58	0 A a	0 A a	0 A a	20 A a	0 A a
	August	359	0 A a	1,9 A a	1,7 A a	1,6 A a	0 A a
Percentage of isolates with a reduced sensitivity to mepanipyrim and a resistance to thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	0				
	September	379	5,1 A a	4,3 A ab	8,2 A ab	6,5 A a	0 A a
2010	June	423	1,1 A a	3,1 A a	2,9 A a	2,6 A a	1,2 A a
	September	408	6 A a	11 AB b	26 B bc	6 A a	2,3 A a
2011	May	58	0 A a	0 A ab	0 A abc	20 A a	0 A a
	August	359	5,9 AB a	20,8 BC b	27,6 C c	11,1 ABC a	0 A a
Percentage of isolates with a resistance to mepanipyrim and thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	0				
	September	379	0 A a	0 A a	0 A a	0 A a	0 A a
2010	June	423	0 A a	0 A a	0 A a	0 A a	0 A a
	September	408	0 A a	0 A a	0 A a	0 A a	0 A a
2011	May	58	0 A a	0 A a	0 A a	0 A a	0 A a
	August	359	0 A a	0 A a	0 A a	0 A a	0 A a
Percentage of isolates with a resistance to diethofencarb and thiophanate-methyl [%]							
			Treatment				
Time of Monitoring			solo TOPSIN	mixture TOPSIN	alternation TOPSIN	annual alternation	Conventional fungicide
Year	Month	n		+ JAPICA	& JAPICA	TOPSIN or JAPICA	treatment
2009	June	28	0				
	September	379	12,8 B a	13 B a	13,7 B a	23,4 B b	1,2 A a
2010	June	423	2,2 A a	1,5 A a	2,9 A a	3,8 A a	2,4 A a
	September	408	3,6 A a	6,8 A a	5,2 A a	2,4 A a	0 A a
2011	May	58	0 A a	0 A a	0 A a	0 A ab	0 A a
	August	359	5,9 A a	13,2 A a	8,6 A a	4,8 A a	1,7 A a

Table 7-8 Mean disease incidence and disease severity caused by *Botrytis cinerea* on grapevine prior to harvest in 2009 to 2011 at three sites near Bordeaux. Disease incidence was expressed as infected bunches of berries (a–c) and disease severity was expressed as percentage of bunch area affected (d–f). At least 100 bunches of berries were measured in each of four repetitions per treatment. TM: Thiophanate-methyl. MP: Mepanipyrim. Conventional fungicide treatment: no fungicides to control *B. cinerea* were applied.

	Location	Monitoring	Solo TOPSIN	Mixture TOPSIN + JAPICA	Alternation TOPSIN & JAPICA	2009: TOPSIN, 2010: JAPICA, 2011: TOPSIN	Conventional fungicide treatment
Percentage of infected bunches of berries [%]	Grezillac	2009-09	26,3	28,3	14,8	26	44
		2010-09	31	32,5	14,8	30,3	48
		2011-08	24	22	30	25,3	35
	S. Brice	2009-09	18	14,3	19,3	19,3	25
		2010-09	13	12,3	15,3	14,5	24
		2011-08	14,5	10,5	15,5	14,8	13
	Loupes	2009-09	16,5	14,5	15,5	21	8
		2010-09	14,3	12,5	10,3	15	22
		2011-08	21,5	17	14,8	20,5	27
Percentage of infected bunch area [%]	Grezillac	2009-09	0,95	1,19	0,53	1	2,25
		2010-09	1,35	1,68	0,49	1,25	1,95
		2011-08	1,76	1,49	1,92	1,68	6,89
	S. Brice	2009-09	3,55	2,42	3,42	2,95	6,6
		2010-09	2,85	2,02	2,98	2,17	5,85
		2011-08	3,24	1,87	3,4	2,61	2,9
	Loupes	2009-09	1,27	1,05	1,11	1,33	0,64
		2010-09	0,85	0,7	0,6	0,97	1,87
		2011-08	2,99	2,79	2,39	2,95	5,77

Table 7-9 Pearson correlation index calculated for percentage of isolates resistant to fungicides and disease incidence as well as disease severity of *Botrytis cinerea* on grapevine prior to harvest in 2009 to 2011 at three sites near Bordeaux. Disease incidence was expressed as infected bunches of berries and disease severity was expressed as percentage of bunch area affected. Abbreviations of phenotypes resistant to fungicides: BenR: benzimidazole resistant; AniR: isolates with a reduced sensitivity to anilinopyrimidines; BenRAniR: isolates with a reduced sensitivity to anilinopyrimidines as well as a resistance to benzimidazoles.

BenR	Percentage of infected bunches		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,082	0,138	0,008
Saint Brice	0,003	0,008	0,039
Loupes	0,075	0,206	0,068

AniR	Percentage of infected bunches		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,277	0,161	0,035
Saint Brice	0,028	0,001	0,044
Loupes	0,003	0,034	0,052

BenRAniR	Percentage of infected bunches		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,021	0,248	0,018
Saint Brice	0,019	0	0,123
Loupes	0	0,088	0,065

BenR	Percentage of bunch area affected		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,048	0,086	0,104
Saint Brice	0,006	0,09	0,023
Loupes	0,044	0,229	0,001

AniR	Percentage of bunch area affected		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,28	0,182	0,25
Saint Brice	0,032	0,011	0,035
Loupes	0,001	0,01	0,02

BenRAniR	Percentage of bunch area affected		
	Coefficient of determination (R^2)		
	2009	2010	2011
Grezillac	0,047	0,198	0,018
Saint Brice	0,054	0,006	0,158
Loupes	0,002	0,079	0,019

7.4 WEATHER DATA

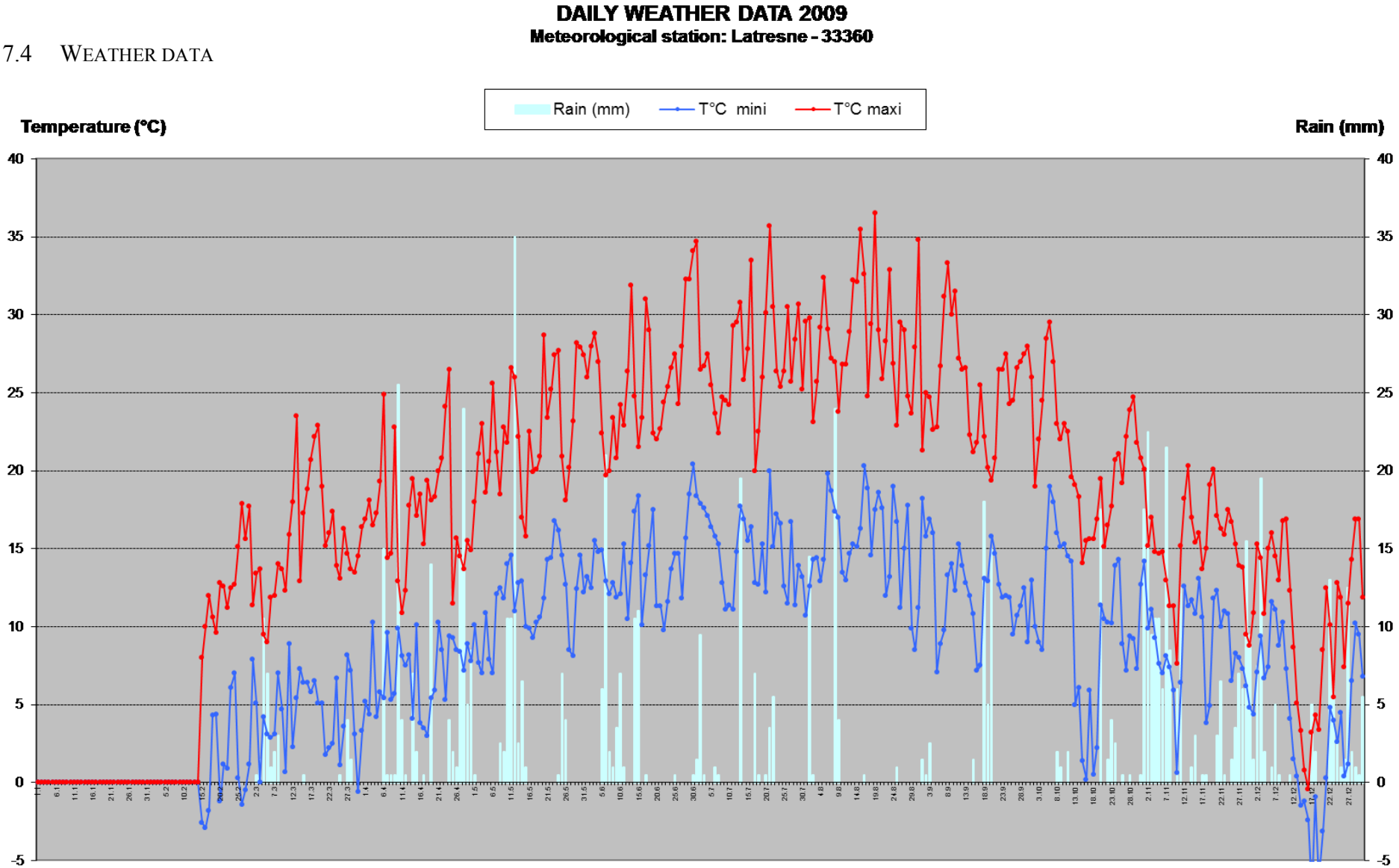

Figure 7-2 Daily weather data measured by the meteorological station Latresne (station number: 33360) in 2009. Data was kindly provided by Staphyt SARL.

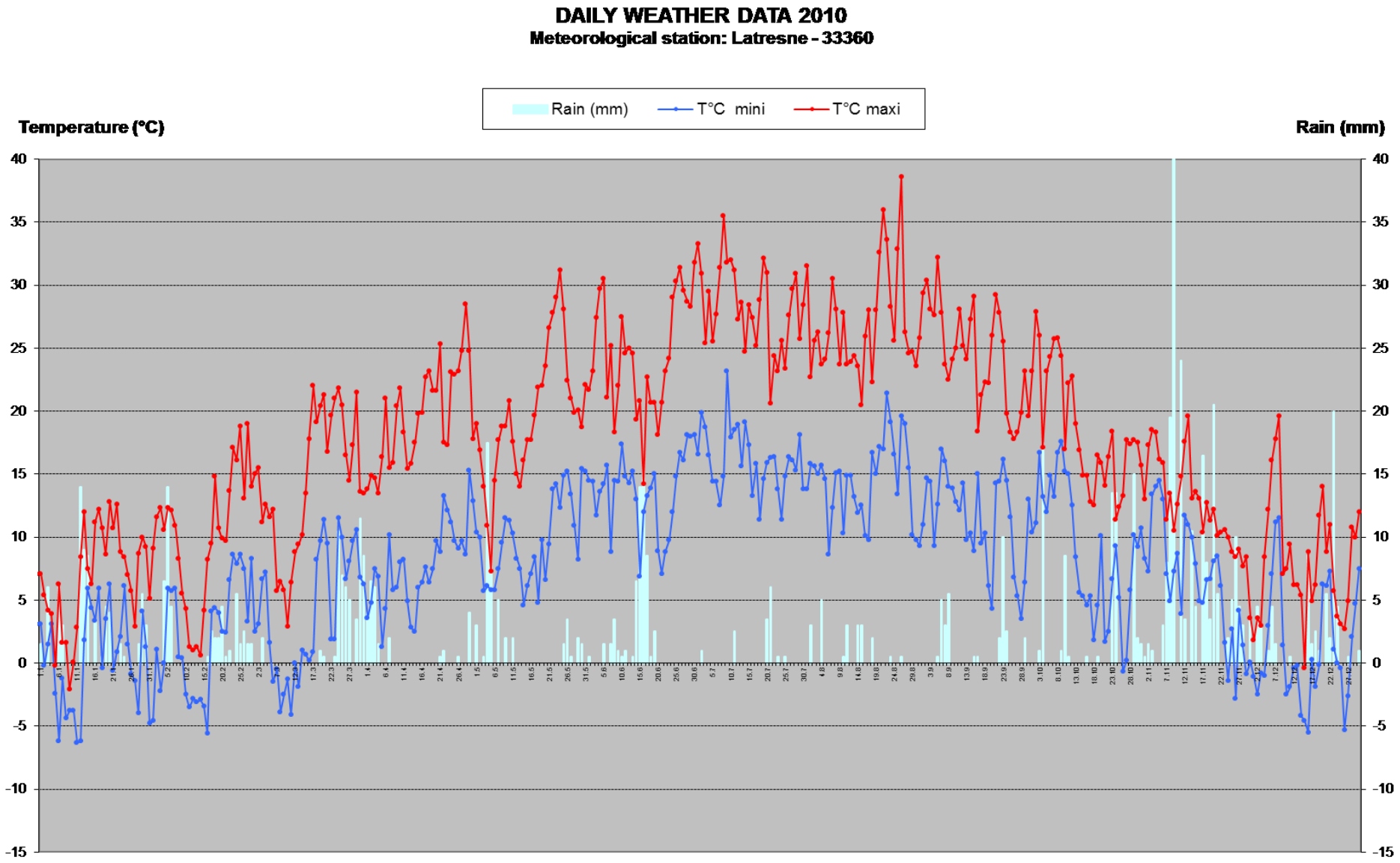

Figure 7-3 Daily weather data measured by the meteorological station Latresne (station number: 33360) in 2010. Data was kindly provided by Staphyt SARL.

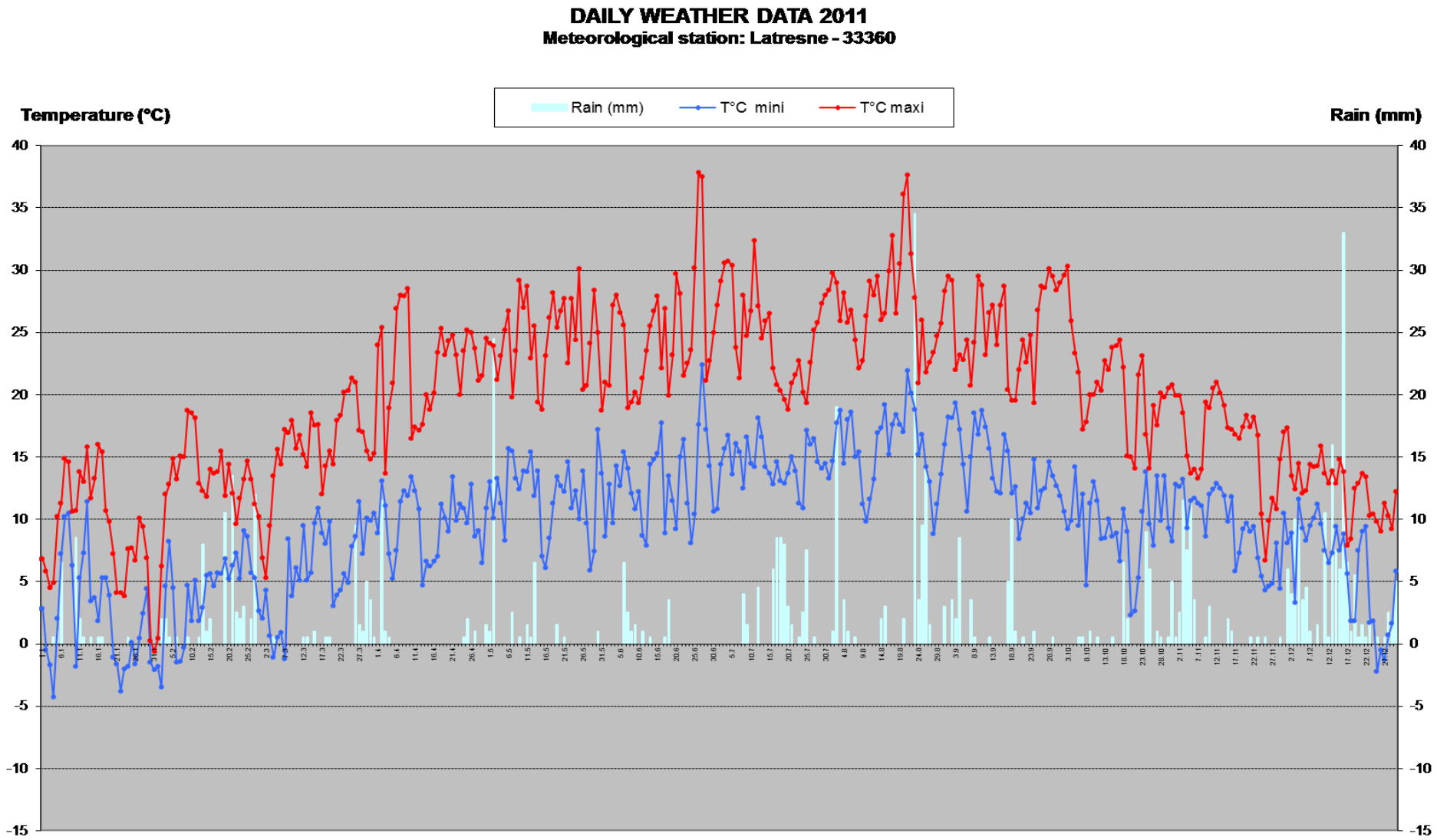

Figure 7-4 Daily weather data measured by the meteorological station Latresne (station number: 33360) in 2011. Data was kindly provided by Staphyt SARL.

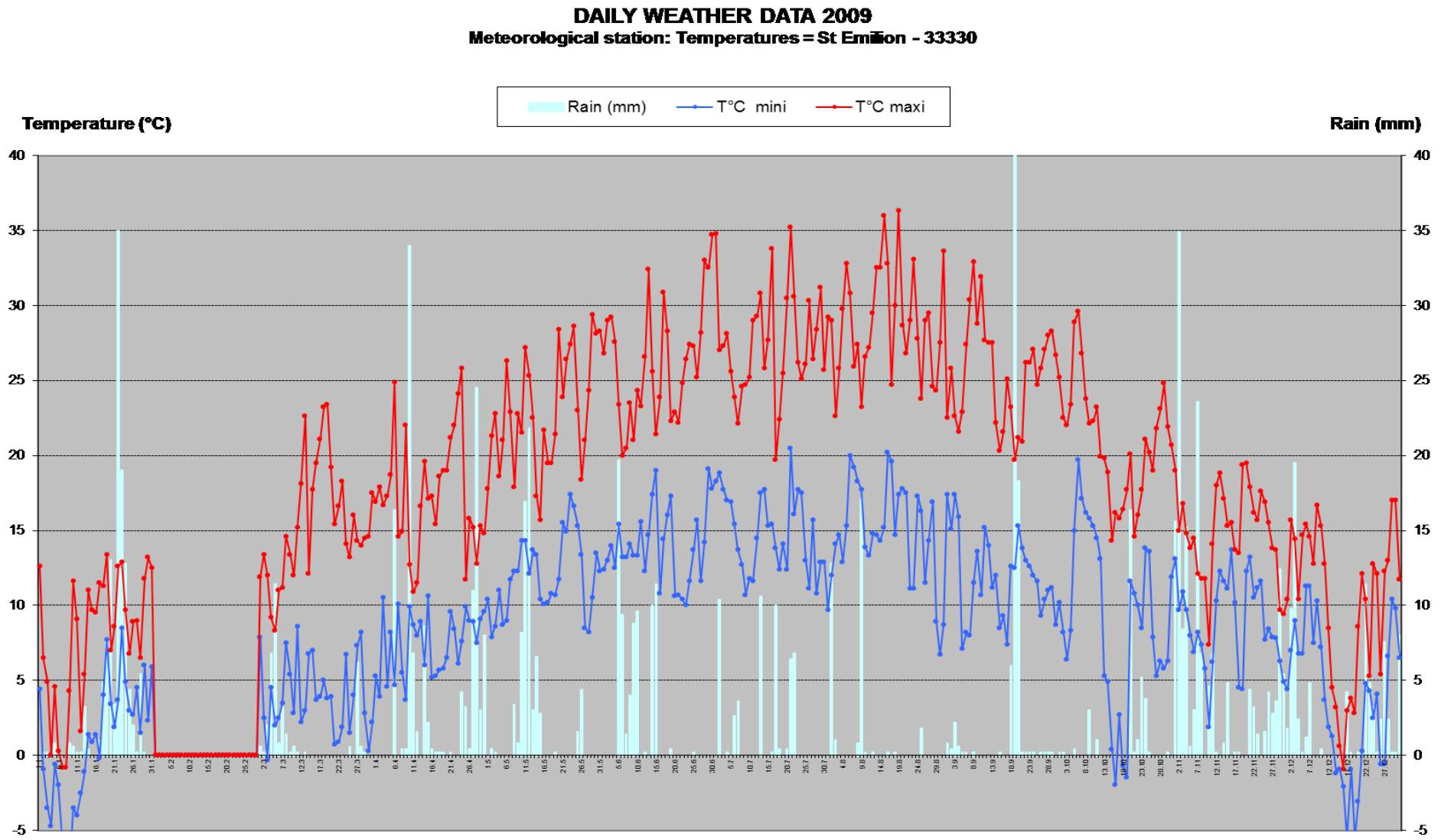

Figure 7-5 Daily weather data measured by meteorological station St. Emilion (station number: 33330) in 2009. Data was kindly provided by Staphyt SARL.

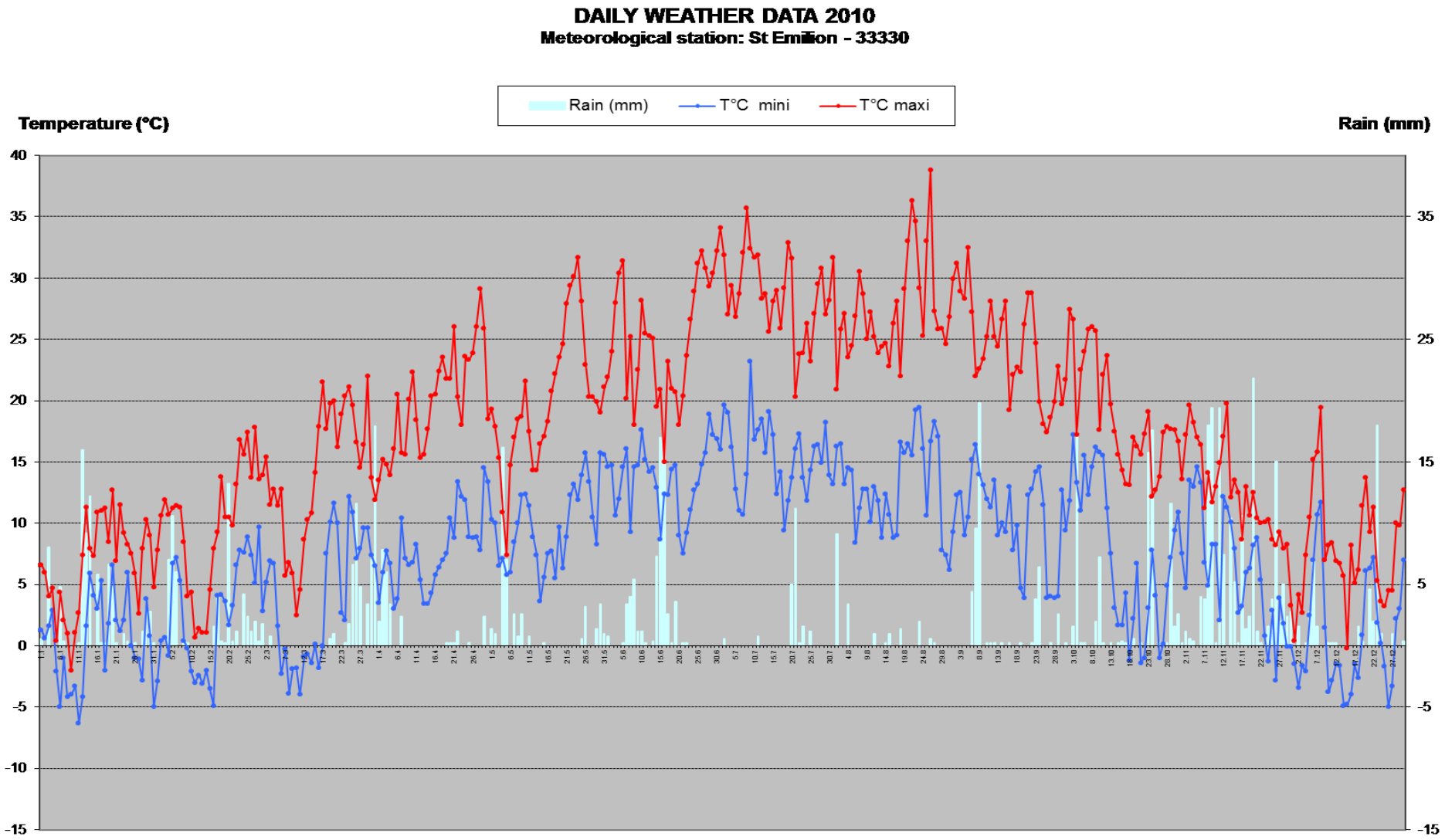

Figure 7-6 Daily weather data measured by meteorological station St. Emilion (station number: 33330) in 2010. Data was kindly provided by Staphyt SARL.

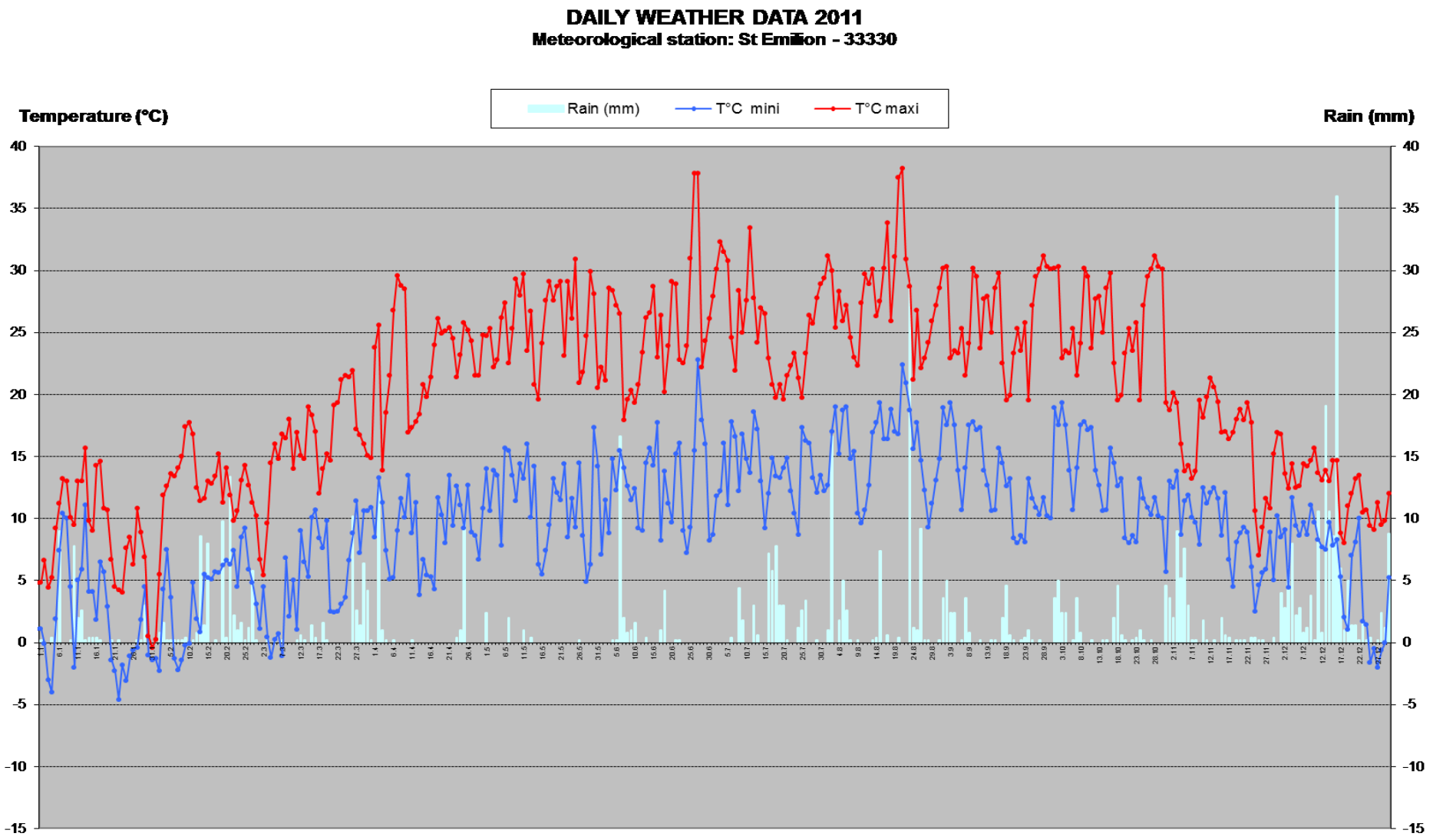

Figure 7-7 Daily weather data measured by meteorological station St. Emilion (station number: 33330) in 2011. Data was kindly provided by Staphyt SARL.

Table 7-10 Thirty year average rainfall, minimum temperature (T min) and maximum temperature (T max) measured by the meteorological station Latresne (station number: 33360) from 1961 – 1990. The data was kindly provided by Staphyt SARL.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rain [mm]	100,4	85,5	76,4	72,2	77,3	56,2	46,5	54,2	73,9	87,6	94,1	98,7
T min [°C]	2,3	3,1	3,9	6,3	9,5	12,4	14,4	14,2	12,2	9,1	5,1	2,9
T max [°C]	9,4	11,2	13,7	16,3	19,7	23,2	26,1	25,6	23,7	18,9	13,1	9,9

Table 7-11 Thirty year average rainfall, minimum temperature (T min) and maximum temperature (T max) measured by the meteorological station St. Emilion (station number: 33330) from 1961 – 1990. The data was kindly provided by Staphyt SARL.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rain [mm]	72,5	56,7	59,5	74,3	72,5	43,8	50,7	70,5	62,0	67,3	105,3	78,2
T min [°C]	2,8	3,1	5,0	7,3	10,9	14,0	15,4	15,4	12,2	14,7	5,5	3,0
T max [°C]	10,0	11,8	15,5	18,1	22,2	25,9	27,3	27,4	23,9	20,0	13,2	9,7

7.5 SPATIAL AND TEMPORAL DISTRIBUTION OF ISOLATES OF *B. CINEREA*

Figure 7-8 Spatial distribution of benzimidazole-resistant (BenR) and –sensitive (BenS) isolates of *Botrytis cinerea* for six dates of monitoring (a – f) at Grezillac. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: annual alternation: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

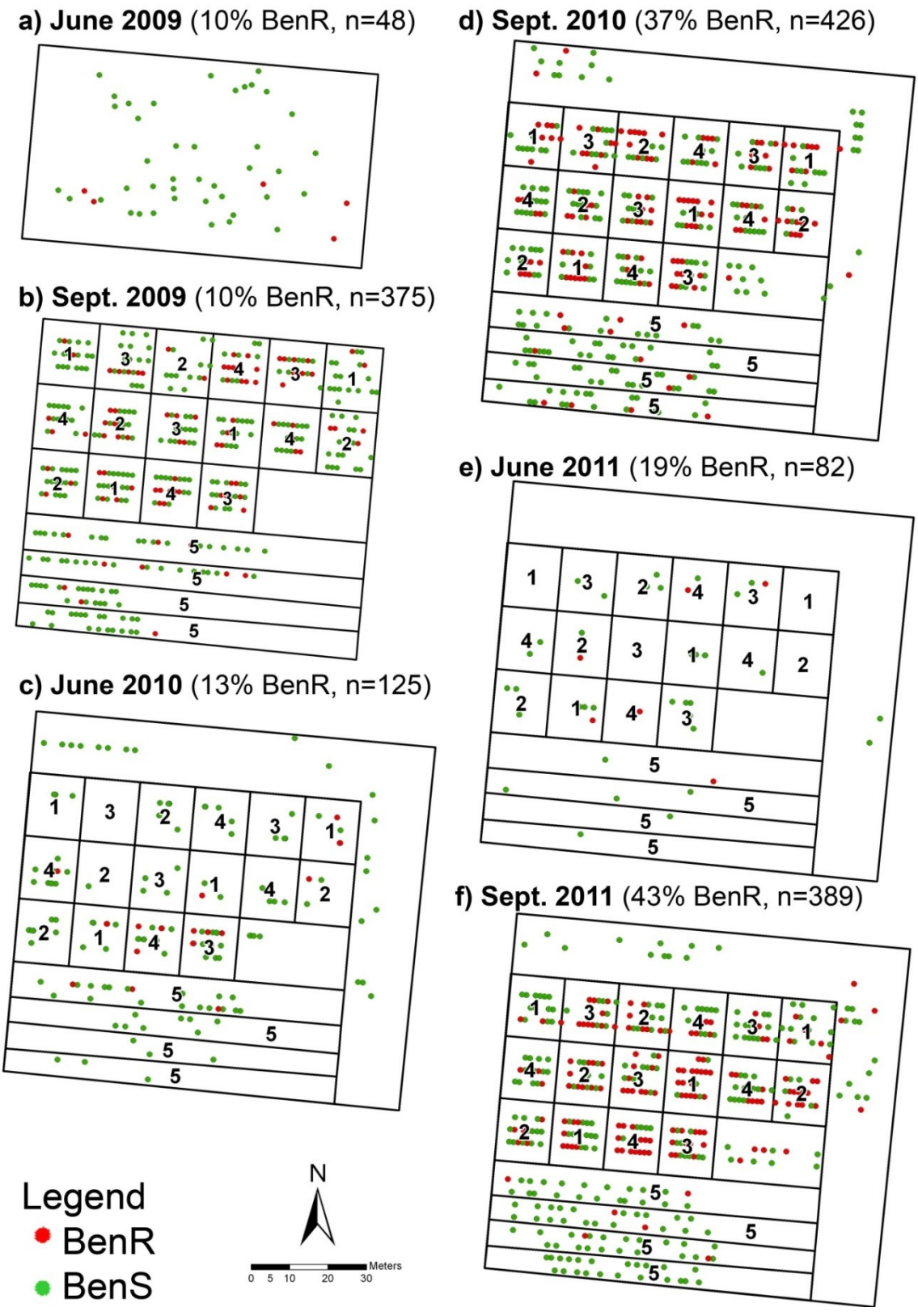

Figure 7-9 Spatial distribution of benzimidazole-resistant (BenR) and –sensitive (BenS) isolates of *Botrytis cinerea* for six dates of monitoring (a – f) at Saint Brice. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: annual alternation: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

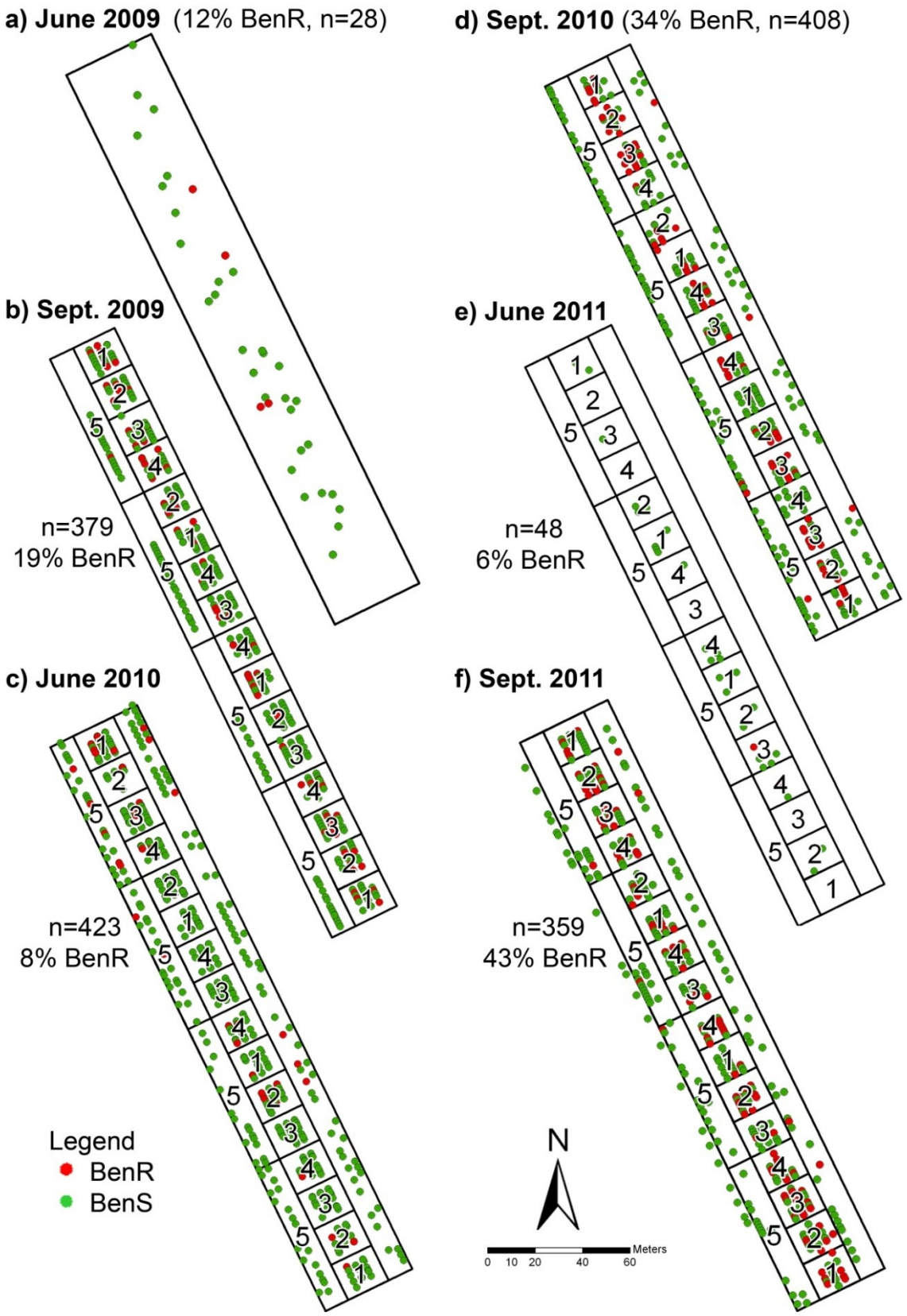

Figure 7-10 Spatial distribution of benzimidazole-resistant (BenR) and –sensitive (BenS) isolates of *Botrytis cinerea* for six dates of monitoring (a – f) at Loupes. Numbers in plot indicate fungicide treatments: 1: solo application of thiophanate methyl (TM); 2: mixture of TM and mepanipyrim (MP); 3: alternation of TM and MP; 4: annual alternation: 2009: TM; 2010: MP; 2011: TM; 5: Conventional fungicide treatment, where no TM was applied.

Table 7-12 Moran`s I indexes for six phenotypes of *Botrytis cinerea* resistant to fungicides at three locations near Bordeaux for six dates of monitoring. Statistical analysis: p-values derived by hypothesis tests under H_0 of randomness. Indices in boldface indicate significant autocorrelation at $p \leq 0.05$.

Location	Resistance Phenotype	Date of Monitoring					
		2009-06	2009-09	2010-06	2010-09	2011-05	2011-08
Grezillac	BenR	-0,037	**0,111****	-0,016	**0,106****	0,005	**0,045***
	AniR	-0,030	**0,066****	**0,081****	**0,037***	**0,137****	-0,006
	AniHR	-	-0,003	0,004	-0,002	-0,004	-0,003
	BenRDietR	-	0,021	-0,014	-0,003	-0,028	-0,028
	BenRAniR	0,031	0,004	0,021	-0,031	0,004	-0,003
	BenRAniHR	-	-	-	-	-	-
Saint Brice	BenR	0,033	**0,015****	**0,215***	**0,028****	0,001	**0,057****
	AniR	-0,030	**0,036****	0,026	**0,010***	0,039	**0,009***
	AniHR	-	0,001	-0,016	-0,001	-	-0,003
	BenRDietR	0,052	-0,001	-	0,003	-	-0,003
	BenRAniR	0,041	**0,011***	-0,004	0,004	-0,016	0,007
	BenRAniHR	-	-	-	-0,003	-	-
Loupes	BenR	0,035	**0,039***	**0,077****	**0,191****	-0,011	**0,016****
	AniR	-0,006	0,009	**0,034***	**0,069****	-0,009	**0,013****
	AniHR	-	-0,003	-0,001	-	-0,025	0,001
	BenRDietR	-	-0,011	-0,010	-0,005	-	**0,025****
	BenRAniR	-	0,001	-0,002	**0,120****	-0,025	**0,021****
	BenRAniHR	-	-	0,004	-	-	-

7.6 FREQUENCY OF ALLELES CONFERRING BENZIMIDAZOLE RESISTANCE IN *B. CINEREA*

Table 7-13 Efficacy of as-PCR using pairs of allele-specific primers at four annealing temperatures. The first column indicates the forward primer and the second row indicates the reverse primer used. Each combination of primers resulted in an amplification visualized by gel electrophoresis. Fluorescence was scored as follows: green: strong fluorescence, pale green: medium fluorescence; pale red: weak fluorescence; red: no fluorescence. S: benzimidazole-sensitive isolate (BC 11.3); R: benzimidazole-resistant isolate (BC 266.6); Y: diethofencarb-resistant isolate of *Botrytis cinerea* (B-70-3).

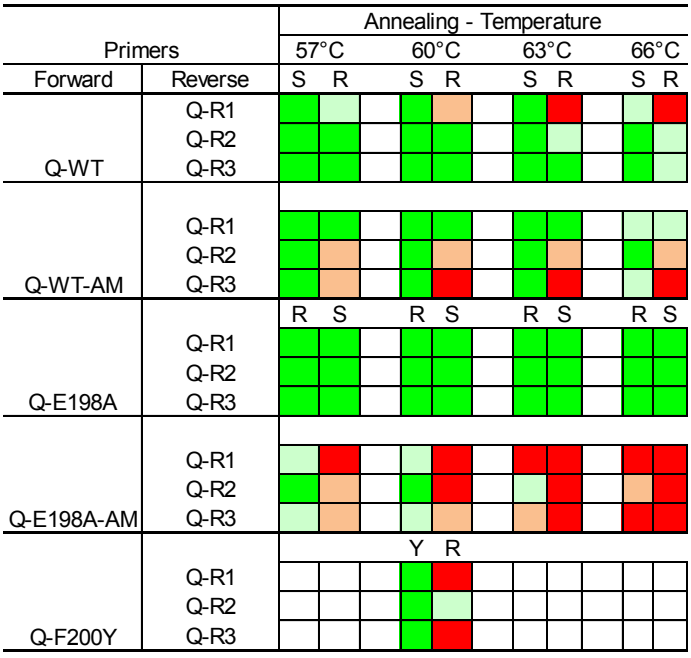

Table 7-14 Threshold cycle number (Ct) and fluorescence at threshold cycle of primer pair Q-E198A-AM / Q-R2 amplifying DNA of a benzimidazole-resistant *Botrytis cinerea* isolate (BC 266.6)using seven mastermixes in a EvaGreen® as-qPCR. The chosen mastermix is in boldface.

Company	Mastermix	Ct match	Ct mismatch	deltaCt	Fluorescence at threshold-cycle
AB	TaqMan PCR MaterMix	32,87	34,94	2,07	ca. 5.000
Takara	Premix Ex Taq	15,28	21,91	6,63	ca. 30.000
Qiagen	QuantiFast Probe PCR	15,97	22,78	6,81	ca. 20.000
Eurogentec	FAST qPCR MM (4mM MgCl)	17,85	25,29	7,44	ca. 10.000
Bioline	SensiFast Probe	10,44	11,25	0,81	ca. 15.000
Biometra	FAST Probe qPCR MM	20,2	24	3,8	ca 15.000
Biometra	**FAST PLUS EvaGreen MM**	**20,01**	**29,29**	**9,28**	**ca. 70.000**

Table 7-15 Validation of EvaGreen® as-qPCR protocol using allele-specific primer pairs Q-E198A-AM / Q-R2 and Q-F200Y / Q-R3 as well as the species specific primer pair Bc1-F / Bc1-R using DNA pools of *Botrytis cinerea* with known allele frequencies. The measured allele frequency was calculated by interpolation of gene copy numbers using standard curves. Ct: threshold cycle numbers. SD: Standard deviation.

E198A allele		allele-specific primer pair		species specific primer pair		difference	measured allele frequency	
frequency [%]	n	mean Ct	SD Ct	mean Ct	SD Ct	in mean Ct	mean [%]	RSD [%]
0	3	29,2	0,1	15,6	0,1	13,6	0,15	6,5
1	3	26,5	0,0	15,6	0,1	10,9	0,97	0,8
5	3	24,6	0,2	15,6	0,2	9,0	3,6	8,3
10	3	23,6	0,1	15,7	0,1	7,9	7,8	8,2
20	3	22,4	0,1	15,7	0,1	6,7	18,5	6,6
30	3	21,8	0,1	15,7	0,0	6,1	28,5	4,1
40	3	21,4	0,0	15,7	0,1	5,8	35,1	2,4
50	3	21,1	0,0	15,6	0,1	5,5	47,7	1,6
60	3	20,8	0,0	15,8	0,1	5,0	58,1	1,6
70	3	20,6	0,0	15,6	0,1	5,0	65,9	3,0
80	3	20,4	0,1	15,7	0,1	4,7	77,8	6,2
90	3	20,3	0,1	15,7	0,1	4,6	88,0	3,5
100	3	20,1	0,1	15,8	0,0	4,3	97,5	4,4

F200Y allele		allele-specific primer pair		species specific primer pair		difference	estimated allele frequency	
frequency [%]	n	mean Ct	SD Ct	mean Ct	SD Ct	in mean dCt	mean	SD
0	3	36,7	0,4	15,6	0,1	21,1	0,00	0,00
1	3	29,1	0,4	15,7	0,1	13,4	0,74	0,21
5	3	26,7	0,2	15,8	0,1	11,0	4,1	0,7
10	3	25,4	0,2	15,6	0,1	9,8	11,0	1,5
20	3	24,7	0,3	15,7	0,1	9,0	18,7	3,9
30	3	24,1	0,1	15,6	0,1	8,5	28,7	3,0
40	3	23,5	0,1	15,5	0,1	8,0	45,1	3,3
50	3	23,4	0,1	15,6	0,2	7,9	48,4	2,9
60	3	23,1	0,1	15,8	0,1	7,3	62,7	6,7
70	3	22,9	0,1	15,6	0,1	7,3	71,4	3,2
80	3	22,6	0,0	15,7	0,1	7,0	84,5	2,6
90	3	22,5	0,0	15,7	0,1	6,8	91,4	2,5
100	3	22,2	0,1	15,8	0,0	6,5	102,2	4,5

7.7 FITNESS OF BENZIMIDAZOLE-RESISTANT ISOLATES OF *B. CINEREA*

Table 7-16 Isolates of *Botrytis cinerea* used in the frost tolerance experiment. 20 – 30 isolates were used per fungicide-resistant phenotype: BenR1: benzimidazole-resistant isolates; AniR: isolates with a reduced sensitivity to anilinopyrimidines; BenRAniR: isolates with a reduced sensitivity to anilinopyrimidines and a resistance to benzimidazoles; AniHR: anilinopyrimidine-resistant isolates; BenR2 isolates resistant to benzimidazoles as well as diethofencarb; Sens: isolates sensitive to the fungicides mentioned above.

	Resistant phenotype					
Number	Sens	BenR1	AniR	BenRAniR	AniHR	BenR2
1	C-T1-R2-11	C-T1-R1-19	C-T1-R2-6	C-T1-R2-9	B-T1-R1-22	C-T1-R1-17
2	C-T1-R2-17	C-T1-R1-21	C-T1-R4-17	C-T1-R2-16	B-T2-R1-14	C-T1-R4-22
3	C-T1-R2-18	C-T1-R2-3	C-T1-R4-18	C-T2-R2-3	B-T3-R1-17	C-T2-R3-15
4	C-T1-R2-19	C-T1-R2-4	C-T2-R3-6	C-T2-R2-6	B-T3-R2-13	C-T2-R3-21
5	C-T2-R2-1	C-T1-R2-5	C-T2-R3-19	C-T2-R2-14	B-T4-R2-17	C-T3-R2-12
6	C-T2-R2-2	C-T2-R3-16	C-T2-R4-19	C-T2-R4-2	B-T3-R3-7	C-T4-R3-1
7	C-T2-R2-7	C-T2-R3-18	C-T3-R1-17	C-T2-R4-12	B-T3-R3-12	C-T4-R2-14
8	C-T2-R4-9	C-T2-R4-5	C-T3-R2-16	C-T2-R4-17	B-T3-R3-19	C-T3-R3-5
9	C-T2-R4-10	C-T2-R4-14	C-T4-R2-5	C-T3-R1-6	B-T3-R3-22	C-T3-R1-7
10	C-T2-R4-13	C-T2-R4-16	C-T4-R2-11	C-T3-R1-8	B-T4-R3-18	C-T2-R4-3
11	C-T3-R1-1	C-T2-R4-20	C-T4-R2-13	C-T3-R1-11	B-T1-R4-13	C-T2-R3-17
12	C-T3-R1-2	C-T3-R1-3	C-T4-R2-16	C-T3-R1-12	B-T1-R4-20	C-T1-R1-18
13	C-T3-R1-22	C-T3-R1-4	C-T4-R2-18	C-T3-R1-20	B-T1-R4-22	B-T1-R1-9
14	C-T3-R2-6	C-T3-R1-5	C-T4-R2-21	C-T3-R1-21	B-T2-R4-5	B-T2-R1-6
15	C-T3-R2-10	C-T3-R1-9	C-T4-R3-3	C-T3-R2-1	B-T2-R4-12	B-T3-R1-1
16	C-T3-R2-13	C-T3-R1-10	C-T4-R4-13	C-T3-R2-2	B-T2-R4-19	B-T3-R2-7
17	C-T3-R2-14	C-T3-R1-13	C-T5-R1-19	C-T3-R3-4	B-T4-R4-8	B-T3-R3-3
18	C-T3-R2-15	C-T3-R1-14	C-T5-R1-20	C-T3-R3-6	B-T5-R3-21	B-T2-R4-18
19	C-T3-R4-1	C-T3-R1-18	C-T5-R4-3	C-T3-R4-8	B-T5-R4-3	B-T2-R4-9
20	C-T3-R4-2	C-T3-R1-19	C-T5-R4-6	C-T3-R4-9	B-T5-R4-4	B-T4-R4-7
21	C-T3-R4-3	C-T3-R2-3	C-T5-R4-12	C-T3-R4-12		
22	C-T3-R4-5	C-T3-R2-4	C-T5-R4-19	C-T3-R4-14		
23	C-T4-R1-10	C-T3-R2-7	B-T3-R1-6	C-T3-R4-15		
24	C-T4-R1-11	C-T3-R2-8	B-T3-R2-2	C-T3-R4-17		
25	C-T4-R1-13	C-T3-R2-9	B-T4-R2-13	C-T3-R4-19		
26	C-T4-R1-15	C-T3-R2-18	B-T4-R2-15	C-T3-R4-20		
27	C-T4-R1-17	C-T4-R1-9	B-T2-R4-20	C-T4-R1-6		
28	C-T4-R1-18	C-T4-R3-14	B-T3-R3-2	C-T4-R1-7		
29	C-T4-R4-6	C-T4-R3-21	B-T4-R4-15	C-T4-R1-8		
30	C-T4-R4-7	C-T4-R4-8	B-T4-R4-9	C-T4-R4-22		

Table 7-17 Comparison of mycelium growth of ten benzimidazole-sensitive and ten -resistant isolates of *Botrytis cinerea* at four combinations of temperature and nutrition medium. Statistical analysis: p-values calculated by Student`s *t*-test ($p \leq 0.05$) comparing two sensitivity groups.

			Mycelium growth [mm²]				
Enviromental conditions		n	Sensitive group		Resistant group		p-value
Temperature	Nutrition		Mean	SE	Mean	SE	
6°C	CDA 10%	10	41,6	1,3	37,1	1,1	0,016
6°C	PDA	10	54,9	3,0	51,2	2,0	0,329
21°C	CDA 10%	10	51,2	5,2	53,9	3,2	0,669
21°C	PDA	10	59,5	5,3	65,3	2,5	0,402

Table 7-18 Comparison of fitness parameters of ten benzimidazole-sensitive to ten -resistant isolates of *Botrytis cinerea* under favourable and unfavourable development conditions for the fungus. Fitness parameters tested: mycelial growth, spore production, spore germination and lesion size on leaves of grapevine. Statistical analysis: identical small letters show no significant difference between isolates according to Tukey's HSD test at p = 0.05. Identical capital letters show no significant difference between sensitivity groups according to Student`s *t*-test at p = 0.05.

Isolate code	Favourable Development conditions (21°C)											
	Mycelium growth (on PDA)			Spore production (on PDA)			Spore germination (on PDA)			Lesion size		
	[mm]		SE (n=5)	[conidia*mm^{-1}]		SE (n=5)	[%]		SE (n=3)	[mm²]		SE (n=6)
May6	30,3	cd	0,8	3.220	ab	917	95,2	ab	1,1	146,5	abc	16,1
Rech4	18,7	b	0,9	960	ab	501	94,4	ab	1,4	69,7	a	12,9
V5-3-1	44,1	ef	1,3	1.140	ab	648	93,0	ab	0,9	120,2	abc	15,1
V5-3-5	36,8	de	0,5	2.360	ab	1.033	95,3	ab	1,2	334,7	c	23,5
V5-5-2	50,3	f	0,5	680	ab	277	95,7	ab	0,9	158,2	abc	16,8
V6-1-4	35,3	d	0,8	2.620	ab	631	91,3	ab	1,0	288,7	c	24,7
V6-3-3	39,8	e	1,2	140	a	76	94,2	ab	1,1	386,5	c	8,0
V6-3-4	45,7	f	0,7	60	a	54	94,2	ab	1,1	53,5	a	6,1
V6-3-5	48,4	f	0,8	2.170	ab	1.163	93,5	ab	1,3	350,0	c	21,9
V6-3-6	44,1	ef	1,8	2.860	ab	854	96,9	b	0,8	217,7	bc	13,1
Resistant group	39,4	A	4,3	1.620	A	519	94,4	A	1,1	212,6	A	49,3
May3	44,8	ef	2,3	1.000	ab	344	86,7	ab	6,6	255,2	bc	13,8
Rech1	46,7	f	0,9	770	ab	523	92,7	ab	1,4	323,5	c	15,5
V1-2-1	51,6	f	2,4	560	ab	304	93,5	ab	2,9	71,3	ab	9,6
V1-5-2	41,5	e	2,0	1.180	ab	456	93,3	ab	1,8	171,0	abc	6,3
V3-2-2	40,4	e	1,4	3.050	ab	988	97,6	b	0,9	325,0	c	11,2
V3-3-2	8,8	a	0,9	1.710	ab	1.167	98,6	b	0,6	147,5	abc	11,3
V5-1-4	48,1	f	6,6	1.180	ab	751	87,7	a	0,3	306,9	c	22,3
V5-2-4	45,1	ef	6,0	1.810	ab	1.029	98,3	b	1,1	164,3	abc	14,1
V5-2-6	35,1	d	0,7	3.850	b	568	97,5	b	1,6	139,8	abc	12,7
V6-4-3	24,9	bc	1,9	400	ab	197	95,6	ab	0,7	201,3	abc	14,5
Sensitive group	38,7	A	5,8	1.550	A	497	94,1	A	1,8	210,6	A	35,8
p-value	0,772			0,837			0,89			0,775		

Isolate code	Unfavourable Development conditions (6°C)											
	Mycelium growth (on CDA$_{10\%}$)			Spore production (on leaf discs)			Spore germination (on CDA$_{10\%}$)			Lesion size		
	[mm]		SE (n=5)	[conidia*mm^{-1}]		SE (n=10)	[%]		SE (n=3)	[mm²]		SE (n=6)
May6	40,4	abcd	1,9	25	a	5,1	96,7	bc	0,1	31,9	ab	10,9
Rech4	33,0	a	2,3	118	ab	20,9	96,5	bc	0,2	4,6	a	0,8
V5-3-1	34,4	ab	1,7	15	a	3,9	95,4	ab	0,4	3,1	a	0,8
V5-3-5	40,6	abcd	1,5	233	bc	47,4	92,0	a	0,4	5,0	a	0,8
V5-5-2	36,2	ab	1,6	104	a	22,8	96,5	ab	0,4	25,3	ab	4,8
V6-1-4	39,9	abc	0,8	142	ab	27,2	95,1	ab	0,9	144,9	b	37,2
V6-3-3	37,0	ab	0,9	21	a	3,8	91,0	a	0,8	9,1	a	1,8
V6-3-4	41,2	abcd	0,7	145	ab	30,8	94,0	ab	0,7	2,9	a	0,5
V6-3-5	37,8	abc	1,7	37	a	14,6	94,2	ab	0,1	39,9	ab	9,6
V6-3-6	40,5	abcd	1,7	268	bc	54,7	94,6	ab	0,7	297,8	bc	38,5
Resistant group	38,1	A	1,3	111	A	28,3	94,6	A	1,1	56,5		38,8
May3	39,9	bc	0,9	386	bc	96,3	96,9	bc	0,7	51,1	b	8,9
Rech1	42,3	bcd	0,9	772	d	117,7	95,5	ab	0,2	65,4	b	12,0
V1-2-1	39,1	abc	2,2	250	bc	62,0	96,8	c	0,0	41,0	ab	0,6
V1-5-2	39,1	abc	2,6	476	c	76,6	94,6	ab	0,7	96,4	bc	17,9
V3-2-2	48,9	d	0,4	139	ab	35,0	96,1	b	0,8	87,4	bc	7,5
V3-3-2	42,8	bcd	2,1	134	ab	21,1	96,0	bc	1,5	360,4	c	20,4
V5-1-4	37,9	abc	0,5	103	a	15,0	95,8	b	0,5	295,6	bc	49,0
V5-2-4	46,0	cd	0,4	217	b	51,9	96,3	bc	0,5	22,4	ab	0,8
V5-2-6	36,3	ab	0,5	203	b	33,9	95,1	ab	0,2	589,9	c	31,7
V6-4-3	45,0	bcd	1,2	83	a	26,7	95,9	ab	0,7	42,3	ab	6,6
Sensitive group	41,7	B	1,8	276	B	67,7	95,9	A	0,4	165,2		77,0
p-value	**0,031**			**0,037**			0,79			**0,012**		

Table 7-19 Effect of incubating temperature and fungicide application on population dynamics of benzimidazole-resistant conidia of *Botrytis cinerea* (Mean and standard deviation (SD)). Four ratios of benzimidazole-sensitive (BenS) and -resistant (BenR) isolates of *B. cinerea* were inoculated onto two month-old plants of grapevine for two subsequent generations. Inoculum was produced by mixing ten isolates per sensitivity group.

Fungicide treatment	Temperature	Generation	Ratio of benzimidazole sensitive and resistant conidia							
			90% BenR, 10% BenS		50% BenR, 50% BenS		10% BenR, 90% BenS		1% BenR, 99% BenS	
			Mean [%]	SD [%]	Mean [%]	SD [%]	Mean [%]	SD [%]	Mean [%]	SD [%]
Water	21 °C	0	93,4	0,6	94,4	1,5	95,9	1,9	-	-
		1	52,5	3,2	50,2	3,3	63,4	3,4	-	-
		2	14,1	1,2	11,5	1,4	12,9	1,7	-	-
	6 °C	0	93,4	0,6	91,3	0,8	86,9	6,5	-	-
		1	52,5	3,2	50,4	1,5	32	3,6	-	-
		2	14,1	1,2	8,5	1,8	3,9	1,8	-	-
Thiophanate-Methyl	21 °C	0	-	-	52,5	3,2	100	0	100	0
		1	-	-	14,1	1,2	100	0	100	0
		2	-	-	1,6	0	99,9	0,1	100	0
	6 °C	0	-	-	52,5	3,2	100	0	100	0
		1	-	-	14,1	1,2	100	0	100	0
		2	-	-	1,6	0	98	0,3	100	0

Table 7-20 Effect of incubating temperature on population dynamics of benzimidazole-resistant conidia of *Botrytis cinerea* (Mean and standard deviation (SD)). Three ratios of benzimidazole-sensitive (BenS) and -resistant (BenR) isolates of *Botrytis cinerea* were inoculated onto autoclaved leaf discs of grapevine for two subsequent generations. Inoculum was produced by mixing ten isolates per sensitivity group or mixing the BenS isolate Rech1 and the BenR isolate V5-3-5.

Isolates tested	Temperature	Generation	Ratio of benzimidazole sensitive and resistant conidia					
			90% BenR, 10% BenS		50% BenR, 50% BenS		10% BenR, 90% BenS	
			Mean [%]	SD [%]	Mean [%]	SD [%]	Mean [%]	SD [%]
Mixture of ten BenS and ten BenR isolates	21 °C	0	90,1	1,2	80,5	5	79,4	1,8
		1	48,1	0,3	47,7	1,4	46,4	1,5
		2	16,4	0,2	22,2	6,3	13,6	2
	6 °C	0	90,1	1,2	83	1,2	64,8	1,6
		1	48,1	0,3	45	1,9	34,8	4,4
		2	16,4	0,2	8,4	0,7	4,5	0,8
Mixture of BenS (Rech1) and BenR isolate (V5-3-5)	21 °C	0	9,2	1,9	19,2	8,2	7,6	2,6
		1	50	0,7	36	3,1	19,7	7,2
		2	92,1	0,7	78,4	3,9	73,6	9,7
	6 °C	0	92,1	0,7	0,3	0,1	0	0
		1	50	0,7	0,2	0,2	0,1	0,1
		2	9,2	1,8	0,1	0,1	0	0

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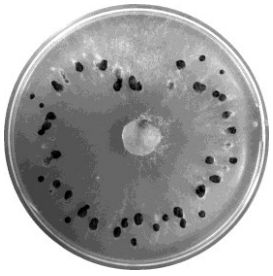

-lichen Dank für Eure Unterstützung!!