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Field strains of Stemphylium vesicarium with a resistance to dicarboximide fungicides correlated with changes in a two-component histidine kinase

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Abstract In *Stemphylium vesicarium*, four phenotypes were recognized according to their in vitro responses to dicarboximide fungicides: S (sensitive), S_+ (low resistant to iprodione and procymidone but moderately resistant to vinclozolin), R_1 (moderately resistant to iprodione and vinclozolin but highly resistant to procymidone), R_2 (highly resistant to all dicarboximides). Cross-resistance was observed between dicarboximides and aromatic hydrocarbon fungicides in all cases while cross-resistance to phenylpyrroles was only detected in R_2 phenotype. Moreover, no changes were noted in sensitivity to oxidative and osmotic stress inducers. An osmosensing histidine kinase gene, homologous to *OS1* from *Neurospora crassa,* was sequenced from several field isolates of *Stemphylium vesicarium*. This gene is predicted to encode a 1,329 amino acid protein, comprising a conserved histidine-kinase domain in the C-terminal region and six tandem repeats of about 90 amino acids at the N-terminal end. In S_+ and R_1 phenotype isolates, a single amino acid substitution was observed in the first amino acid repeat; F267L and L290S respectively. For the R_2 isolates, the exchanges T765R or Q777R were located within the histidine-kinase domain.

Keywords Brown spot · Fludioxonil · Iprodione · Osmotic sensitivity · Pear · Procymidone

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Introduction

The most important fungal pathogen on pear in Italy is *Stemphylium vesicarium* (Wallr.) Simm., the causal agent of brown spot. It also occurs in other European countries (Spain, France, The Netherlands, Portugal and Belgium) where, in some cases, it has begun to be a real concern in disease control. Many fungicide applications are required from petal fall to fruit ripening to protect pears from brown spot and the key products in Italy are dicarboximides (procymidone until 2007 and iprodione) as well as dithiocarbamates, and more recently fludioxonil, strobilurins and boscalid. Unfortunately dicarboximide resistance was detected in *S. vesicarium* for the first time in the early 1990s in the Po valley (Italy) associated with severe failures in field control (Brunelli et al. 1997).

Resistance to dicarboximide fungicides has been a known phenomenon since the 1970s (Leroux and Fritz 1984). Laboratory mutants highly resistant to

dicarboximides were soon obtained in several fungi including *Botrytis cinerea* (Leroux and Fritz 1984; Cui et al. 2002; Leroux et al. 2002; Oshima et al. 2002), *Neurospora crassa* (Fujimura et al. 2000; Ochiai et al. 2001) and *Cochliobolus heterostrophus* (Yoshimi et al. 2003). Dicarboximide-resistant field isolates of *B. cinerea* were also found in many countries (Beever and Brien 1983; Faretra and Pollastro 1991; Cui et al. 2004; Leroux 2004; Oshima et al. 2006) and more recently this phenomenon was recorded in several *Alternaria* species (Dry et al. 2004; Ma and Michailides 2004; Avenot et al. 2005).

Recent studies highlighted that both dicarboximides and phenylpyrroles induce an accumulation of glycerol in susceptible fungal species suggesting that these fungicides could interfere with the osmotic signal transduction pathway (Pillonel and Meyer 1997). Their target site could be a two-component histidine kinase (HK) which belongs to the III group of HKs because it contains a coiled-coil region at the N-terminus, formed by 6 repetitions of about 90 amino acids as well as the histidine kinase and the regulatory domain fused on the same protein and the presence of an ATP-binding domain (Catlett et al. 2003). The activity of this protein is strictly linked to the autophosphorylation of a histidine residue in the histidine kinase (HK) domain, that subsequently transfers its phosphoryl group to an aspartate residue in the response regulator domain. Two-component systems can consequently regulate a mytogen activated protein (MAP) kinase cascade that modulates gene expression in several metabolic pathways, such as osmotic or oxidative stress response (West and Stock 2001). Different point mutations in the corresponding HK gene sequences of different fungi result in a series of phenotypes characterized by their degree of dicarboximide resistance and by pleiotropic aspects, such as osmotic sensitivity and cross-resistance to phenylpyrroles and aromatic hydrocarbons (Table 1).

In *S. vesicarium* a 9-year monitoring study identified 4 phenotypes with different degrees of sensitivity to these fungicides: an S-sensitive phenotype ($0.31<EC_{50}<1.75$ mg l^{-1} for procymidone and iprodione); an S_+-resistant phenotype, low resistant (LR) to procymidone and iprodione (Resistance Factor on EC_{50} values (RF) =3); an R_1-resistant phenotype, highly resistant (HR) to procymidone (RF>100) but moderately resistant (MR) to the other dicarboximides (3<RF<100); an R_2-resistant phenotype, highly resistant to all dicarboximides (RF> 100) (Alberoni et al. 2005). The existence of different degrees of cross-resistance among the dicarboximides is quite uncommon. A similar behaviour was observed only in *B. cinerea* by Cui et al. (2004) where the same strains showed a high resistance to vinclozolin and procymidone (RF>100) and a moderate resistance to iprodione (9<RF< 15.8). These strains owed their resistance to mutations in the amino acid repeat region of histidine kinase gene (Table 1).

The aim of this study was to characterize *S. vesicarium* phenotypes with different degrees of sensitivity to dicarboximides in relation to their cross-resistance with phenylpyrroles and aromatic hydrocarbon fungicides (AHFs), their sensitivity to osmotic and oxidative stress inducers and the possible presence of specific mutations in the histidine kinase gene. These findings make it possible to understand whether in the case of *S. vesicarium* the osmotic regulation pathway is involved in dicarboximide resistance.

Materials and methods

Fungal isolates

Isolates, collected between 1997 and 2004, were tested for their cross-resistance among dicarboximides, fludioxonil and AHFs. They came from different cultivars and different areas of the Po valley as shown in Table 2. Some isolates were chosen for each phenotype with different degrees of dicarboximide sensitivity to study changes in osmotic and oxidative stress inducer sensitivity and to find genetic differences.

Growth inhibition assays to evaluate cross-resistance among fungicides

Isolate sensitivities to several fungicides were evaluated in mycelial tests through the effective concentration (EC_{50}) of each fungicide that inhibits 50% of mycelial growth compared to an unamended control.

V8 juice agar [20% V8 (vegetable juice, Campbell's Grocery Ltd), 1.5% technical agar (Agar Grade A, Becton Dickinson), 0.4% calcium carbonate (Fluka) in dH_2O] was amended with various ranges of 6–

Table 1 Main mutations in histidine kinase gene of several fungal species and sensitivity to dicarboximides, aromatic hydrocarbons, phenylpyrroles and osmotic pressure of related phenotypes

Species	Strain type	Histidine kinase modifications					Phenotypes						References[g]
		Nr of amino acids	Codon (s) affected	Effects	Protein domains	Null mutant	Dicarboximides [c]			Aromatic hydrocarbons [c]	fludioxonil [c]	Osmo-sensitivity to salts [d]	
							iprodione	procymidone	vinclozolin				
Stemphylium vesicarium	Field	1329	267	F to L	Repeat 1 (He) [b]	no	LR	LR	MR	HR	S	L^{+} [e]	1
	Field		290	L to S	Repeat 1 (Lo)	no	MR	HR	MR	HR	S	L^{+}	1
	Field		765	T to R	Near H box	no	HR	HR	HR	HR	HR	L^{+}	1
	Field		777	Q to R	Near H box	no	HR	HR	HR	HR	HR	L^{+}	1
Alternaria brassicicola	Lab	1329	343	Q→stop	Repeat 2	yes	HR	HR	nd[h]	nd	HR	L	2,3
	Lab/Field		495	Frameshift	Repeat 4	yes	HR	HR	nd	nd	HR	M	2,3
	Field		634	W→stop	Repeat 5	yes	HR	HR	nd	nd	HR	M	2,3
	Field		753	E to K	H box	no	HR	HR	nd	nd	HR	L	2,3
	Field		998	Q→stop	Near G2 box	yes	HR	HR	nd	nd	HR	M	2,3
Alternaria alternata [a]	Field	nd	–	Frameshift	Repeat 1	yes	HR	nd	nd	nd	MR	M	4
	Field		–	Frameshift	Repeat 5	yes	HR	nd	nd	nd	MR	M	4
	Lab		no	no	Repeat 1–6	–	HR	nd	nd	nd	nd	M	5
Alternaria arborescens [a]	Field	nd	no	no	Repeat 1–6	–	HR	nd	nd	nd	nd	L	5
Sclerotinia sclerotiorum [a]	Field	1310	635	M to T	Repeat 5	no	HR	HR	HR	nd	HR	H^{+}	6
	Field		no	no	Repeat 1–6	–	HR	HR	nd	nd	MR	M^{+}	6
Cochliobolus heterostrophus	Lab	1329	226–234	Frameshift	Repeat 1	yes	HR	HR	nd	nd	HR	H	7,8
	Lab		282	G to D	Repeat 1	no	HR	HR	nd	nd	HR	H	7,8
	Lab		327–511	ΔH-R	Repeats 2–4	no	HR	HR	nd	nd	HR	H	7,8
	Lab		573	W→stop	Repeat 5	yes	HR	HR	nd	nd	HR	H	7,8
	Lab		616	G to S	Repeat 5	no	HR	HR	nd	nd	HR	H	7,8
	Lab		647	G to D	Repeat 5	no	HR	HR	nd	nd	HR	H	7,8
	Lab		781	M to I	Near H box	no	LR/MR	LR/MR	nd	nd	S/LR	H	7,8
	Lab		834	Frameshift	After H box	yes	HR	HR	nd	nd	HR	H	7,8
	Lab		1210	R to C	Near D box	no	LR/MR	LR/MR	nd	nd	S/LR	M	7,8
	Δ^{i}-mutant		–	–	–	–	HR	nd	nd	nd	HR	H	8
Neurospora crassa	Lab	1298	294	Frameshift	Repeat 2	yes	HR	nd	nd	HR	HR	M	9,10
	Lab		308	Q→stop	Repeat 2	yes	HR	HR	HR	HR	HR	M	9,10,11
	Lab		368/639	T to P/	Repeats 3–6	no	HR	HR	HR	nd	nd	H	11

Table 1 (continued)

Species	Strain type	Histidine kinase modifications					Phenotypes						References[g]
		Nr of amino acids	Codon (s) affected	Effects	Protein domains	Null mutant	Dicarboximides [c]			Aromatic hydrocarbons [c]	fludioxonil [c]	Osmo-sensitivity to salts [d]	
							iprodione	procymidone	vinclozolin				
	Lab		418	M to V E to K	Repeat 3	no	MR	HR	HR	nd	nd	M/H	11
	Lab		459	L to M	Repeat 4	no	HR	HR	HR	nd	nd	M/H	11
	Lab		388/578	Q to S/ A to V	Repeats 3–5	no	LR	HR	HR	LR	LR	H	9,10,11
	Lab		580–582	G to R/ I to M	Repeat 5	no	HR	HR	HR	HR	MR	H	9,10,11
	Lab		625	L to P	Repeat 5	no	MR	nd	nd	HR	LR	H	9,10
	Lab		680	Δ K	Repeat 6	no	HR	HR	HR	nd	nd	H	11
	Lab		1091	Frameshift	Near D box	yes	HR	HR	HR	nd	nd	M	11
	Δ-mutant		–	–	–	yes	MR	MR/HR	MR	nd	nd	M	11
Botrytis cinerea	Lab	1315	323	G to D	Repeat 2(He)	no	HR	HR	HR	HR	HR	H^+	12
	Lab		345	E to G	Repeat 2(He)	no	LR/MR	nd	LR/MR	nd	nd	H	13
	Lab		346	V to A	Repeat 2(He)	no	HR	nd	HR	nd	nd	H	13
	Lab		355/691	T to P/ G to E	Repeats 2–6 (Lo/He)	no	HR	HR	HR	HR	HR	H^+	6
	Field		365	I to N	Repeat 2 (Lo)	no	LR	LR/MR	LR	LR	S	L	12,14
	Field		365	I to R	Repeat 2 (Lo)	no	LR/MR	LR/MR	MR	LR	S	L	12,14
	Field		365	I to S	Repeat 2 (Lo)	no	LR/MR	LR/MR	LR	LR	S	L	12,14,15
	Field		365/369	I to S/ Q to P	Repeat 2 (Lo)	no	MR	HR	HR	LR/MR	LR	L	6,14
	Field		368/369/447	V to F/ Q to H/ T to S	Repeats 2–3 (Lo)	no	LR/MR	nd	LR	nd	S	L	15
	Field		369/373	Q to P/ N to S	Repeats 2–3 (Lo/He)	no	LR/MR	nd	LR	nd	S	L	15
	Field		371–372	Ins. S	Repeats 2–3 (Lo)	no	LR/MR	LR/MR	MR	S	S	L	6
	Lab		386	L to F	Repeat 3 (He)	no	HR	nd	HR	nd	nd	H	13
	Lab		482–483	ΔVR	Repeat 4 (He)	no	HR	nd	HR	nd	nd	H	13
	Lab		531	S to N	Repeat 4 (Lo)	no	LR/MR	nd	LR/MR	nd	nd	H	13
	Lab		538	G to R	Repeat 4 (Lo)	no	HR	nd	HR	nd	nd	H	13
	Lab		570	L to W	Repeat 5 (He)	no	HR	nd	HR	nd	nd	H	13

Lab	585	A to H	Repeat 5 (Lo)	no	HR	nd	HR	nd	nd	H	13
Lab	684	L→stop	Repeat 6 (He)	yes	HR	HR	HR	HR	HR	H^+	12
Δ-mutant	–	–	–	yes	HR	HR	HR	HR	HR	H^+	16

[a] : partial sequences, changes occurring within the 6 repeated domains.

[b] : locations of changes in helix (He) or Loop (Lo) of repeated domains.

[c] : levels of sensitivity to fungicides: low (LR), moderate (MR) or high (HR) resistance and sensitivity (S). When some isolates showed intermediate values between two categories both were reported (e.g. LR/MR)

[d] : osmosensitivity evaluated in the presence of KCl or NaCl : L, M, H, low similar to that of wild-type strains, moderate or high levels of osmosensitivity, respectively.

[e] : $^+$ response also obtained with sugars.

[g] : references :

1 Alberoni et al. this study

2 Avenot et al. 2005

3 Iacomi-Vasilescu et al. 2004

4 Dry et al. 2004

5 Ma and Michailides 2004

6 Leroux P., Lanen C. and Fritz R. unpublished data

7 Yoshimi et al. 2003

8 Yoshimi et al. 2004

9 Fujimura et al. 2000

10 Ochiai et al. 2001

11 Miller et al. 2002

12 Leroux et al. 2002

13 Cui et al. 2002

14 Cui et al. 2004

15 Oshima et al. 2006

16 Viaud et al. 2006

h : nd=not determined

i : deletion

Table 2 Characteristics of *S. vesicarium* isolates

Isolate ID	Year of sampling	Cultivars	Geographic location (in Italy)	Dicarboximide-sensitivity phenotypes [a]
Sv129′97	1997	Conference	Ferrara	S
Sv173′97	1997	Abbé Fétel	Bologna	S
Sv129′98	1998	Conference	Ferrara	S
Sv173′98	1998	Abbé Fétel	Bologna	S
Sv173′04	2004	Abbé Fétel	Bologna	S
Sv563	2004	Conference	Mantova	S
Sv346	2000	Abbé Fétel	Rovigo	S_+
Sv448	2002	Conference	Modena	S_+
Sv188′97	1997	Conference	Ferrara	R_1
Sv190	1997	Conference	Verona	R_1
Sv193′97	1997	Abbé Fétel	Ravenna	R_1
Sv209′97	1997	Abbé Fétel	Bologna	R_1
Sv193′98	1998	Abbé Fétel	Ravenna	R_1
Sv209′98	1998	Passe Crassane	Modena	R_1
Sv245	1998	Conference	Mantova	R_1
Sv464′04	2004	Abbé Fétel	Mantova	R_1
Sv524	2003	Abbé Fétel	Ravenna	R_1
Sv222′04	2004	Abbé Fétel	Bologna	R_2
Sv257	1998	Abbé Fétel	Ravenna	R_2
Sv439	2002	Conference	Ravenna	R_2
Sv479	2003	Abbé Fétel	Ravenna	R_2

[a] S=sensitive; S_+=low resistant to procymidone and iprodione but moderately resistant to vinclozolin; R_1= highly resistant to procymidone but moderately resistant to the other dicarboximides; R_2= highly resistant to all dicarboximides

8 concentrations from 0.05 to 100 mg l^{-1} of the active ingredients of procymidone, iprodione, vinclozolin, dicloran, tolclofos-methyl and fludioxonil. The compounds were dissolved in organic solvents in concentrations never exceeding 1% (v/v) in the nutrient medium. An inverted mycelium plug (5-mm diameter) cut from the edge of a 7-day-old colony was used to inoculate the plates. Three replicates were prepared for each concentration and all the assays were repeated twice. The mycelial growth was evaluated by measuring and averaging two perpendicular diameters of each fungal colony after 3 days of incubation at 23°C and 12 h of photoperiod. For each compound EC_{50} was determined by probit analysis.

Growth inhibition assays to evaluate osmotic and oxidative stress sensitivity

Eight isolates (two per phenotype with different degrees of dicarboximide sensitivity) were evaluated for their sensitivity to stress inducers.

Mycelial disks were cut with a sterilized cork borer from the margin of 7-day-old colonies and placed upturned on plates with the following medium: 2 g of KH_2PO_4; 1.5 g of K_2HPO_4; 1 g of $(NH_4)_2SO_4$; 0.5 g of $MgSO_4 \cdot 7H_2O$; 10 g of glucose; 2 g of yeast extract; 12.5 g agar in distilled water for 1 litre of medium, containing glucose at 25-50-100-200 g l^{-1}, NaCl at 1.5-3.2-6.25-12.5-25-50 g l^{-1}, methanol at 0.5-1-2-4-8% (v/v), menadione at 6-16-40-100-250 mg l^{-1}. For each condition the colony diameter was measured after 3, 5, 7 and 10 days of incubation at 18°C in the dark. The EC_{50} values were calculated by regressing the mycelial growth rate relative to the control against the log of fungicide, sugar, salt, methanol or menadione concentration.

Molecular analysis of the phenotypes

Genomic DNAs of *S. vesicarium* phenotypes were isolated from mycelia grown for 7 days at 18°C in the dark in the following liquid medium: 2 g of KH_2PO_4;

1.5 g of K_2HPO_4; 1 g of $(NH_4)_2SO_4$; 0.5 g of $MgSO_4 \cdot 7H_2O$; 10 g of glucose; 2 g of yeast extract in distilled water for 1 litre of medium. They were then homogenised and incubated in fresh medium for 24 h at 23°C in the dark. Mycelia were harvested by filtration, then lyophilised and pestled in a mortar with liquid nitrogen. The lysis buffer was Tris-HCl 50 mM pH 8, EDTA 50 mM pH 8, 2% sarcosyl, NaCl 150 mM. The mixture had been incubated at 4°C with phenol (pH 8) for 3 h. After centrifuging, DNA was precipitated in isopropanol and dissolved in TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) with RNase A. The supernatant was extracted with phenol-chloroform; DNA was precipitated with ethanol and dissolved in TE.

The resultant nucleic acid of a sensitive isolate (ID Sv563, Table 2) was used in a polymerase chain reaction (PCR) with degenerate primers PDF (5′-AAG AT(C/T) GAA GC(A/G) AAC CG(C/T) ATG-3′) and PDR (5′-CAT (A/G)A(C/T) (G/T)GG CAT TTG AAC GTC CAT-3′) designed from conserved domains identified by NCBI published sequences of two-component HKs of *Alternaria brassicicola* (AY700092) and *B. cinerea* (AF396827). The PCR was carried out in a 50-µl volume containing 1 µl of genomic DNA, 5 µl 10X Titanium *Taq* PCR Buffer, 0.2 µM primers, 0.2 mM of each dNTP and 2.5 U Titanium Taq DNA Polymerase (Clontech) with the following thermal conditions: 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min then 10 min at 72°C. The PCR product was sequenced by Biofidal (Vaulx en Velin, France).

On the DNA fragment obtained, primers were designed to start a process of genome walking with Universal GenomeWalker™ kit (Clontech) according to the manufacturer's instructions. The PCR series were carried out in a 50-µl volume containing 1 µl DNA of the experimental library, 5 µl 10X BD Advantage™ 2 PCR Buffer, 0.2 µM primers, 0.2 mM of each dNTP and 0.33 µl 50X BD Advantage™ 2 Polymerase Mix (BD Biosciences) with the following program for the primary PCRs: 7 cycles of 95°C for 25 s and 72°C for 3 min; 32 cycles of 95°C for 25 s and 68°C for 3 min; then 4 min at 68°C and with the following program for the nested PCRs: 5 cycles of 95°C for 25 s and 72°C for 3 min; 20 cycles of 95°C for 25 s and 68°C for 3 min; then 4 min at 68°C. The nested PCR products were sequenced by Biofidal.

Two pairs of PCR primers were designed to amplify the coiled-coil region (SVSRFw: 5′-ACA TCG GCG CAC TTA AGC GAG AAC T-3′and SVSRRe: 5′-GCG ACA TGT TGG CGA GAA ACT CAG A-3′) and the histidine-kinase domain (SVHKFw: 5′-TAA GGA ATA CGG CAG CCA GGG AAG C-3′ and SVHKRe: 5′-CGA GAA CGG AAT CTC CTC CAT GAC C-3′). PCRs were carried out for 29 isolates belonging to the 4 phenotypes of *S. vesicarium* with different sensitivity to dicarboximides (10 S, 2 S_+, 9 R_1, 8 R_2) in a 50-µl volume containing 50 ng of genomic DNA, 5 µl 10X Ex Taq™ Buffer, 0.2 µM primers, 0.2 mM of each dNTP and 1.25 U TaKaRa Ex Taq™ (Takara) with the following program to amplify the coiled-coil region: 94°C for 3 min; 30 cycles of 94°C for 45 s, 68°C for 45 s and 72°C for 1 min and 30 s; then 7 min at 72°C and with the following program to amplify the histidine-kinase domain: 94°C for 3 min; 30 cycles of 94°C for 20 s, 68°C for 20 s and 72°C for 30 s; then 7 min at 72°C.

PCR products were purified using a GeneElute PCR Clean-up kit (Sigma) then sequenced by BMR Genomics—University of Padova Italy (http://www.bmr-genomics.it/).

To compare the HK coiled-coil region and HK domain of resistant and sensitive isolates, the DNA sequences from each isolate were translated into amino acid sequences using the standard code from the computer program Traduc (www.infobiogen.fr). The sequences of DNA and deduced amino acids were aligned using the computer program ClustalW (Des Higgins, EBI, Hinxton Hall, UK-http: npsa-pbil.ibcp.fr) and processed with the program Boxshade (http://bioweb.pasteur.fr).

Results

Growth inhibition assays

Mycelial growth inhibition assays with dicarboximides confirmed the known sensitivity for each phenotype: EC_{50} values of S isolates were around 1 mg l^{-1} while S_+ isolates had low resistance to procymidone and iprodione but were moderately resistant to vinclozolin. The R_1 phenotype was moderately resistant toward iprodione and vinclozolin ($5.48 \leq EC_{50} \leq 25.8$ mg a.i. l^{-1} and $8.4 \leq EC_{50} \leq 40.8$ mg a.i. l^{-1} respectively) and

highly resistant toward procymidone (EC_{50}>100 mg a.i. l^{-1}). The R_2-resistant isolates were highly resistant to all dicarboximides (Table 3).

The assays with other fungicides showed that the isolates sensitive to dicarboximides were inhibited by dicloran (3.2≤EC_{50}≤13.9 mg l^{-1}), tolclofos-methyl (0.41≤EC_{50}≤1.08 mg l^{-1}) and fludioxonil (0.33≤ EC_{50}≤1.48 mg l^{-1}). All the isolates resistant to dicarboximides appeared highly resistant to both AHFs dicloran and tolclofos-methyl (Table 3). The cross-resistance pattern to fludioxonil changed according to the different resistant phenotypes. Only R_2 isolates had EC_{50}>100 mg l^{-1} whereas S_+ and R_1-resistant phenotypes were similar to S with EC_{50} values ranging from 0.71 to 3.03 mg a.i. l^{-1} (Table 3).

As regards the other aspects related to dicarboximide resistance in other fungi, all *S. vesicarium* phenotypes showed a normal growth, similar to the control, in the presence of high concentrations of glucose and sodium chloride. In addition, effects linked to the phenotype were not observed in medium amended with menadione or methanol (Table 4).

Molecular analysis of the phenotypes

Through the genome walking procedure, a series of sequences were assembled to obtain the entire gene of *S. vesicarium* histidine kinase *SvHK1* (GenBank accession number EU711371) in an isolate (ID Sv563) sensitive to dicarboximides. A predicted open reading frame of 3,987 bp was interrupted by five introns of 47–55 bp. Introns were deduced by similarity to *B. cinerea* and *A. brassicicola* sequences. The deduced amino acid sequence of the putative histidine kinase was compared with those of other fungal species and a high level of homology was observed especially with the genus *Alternaria* and *Cochliobolus* (Table 5).

Table 3 Growth inhibition (EC_{50} in mg l^{-1}) of dicloran, tolclofos-methyl and fludioxonil on *S. vesicarium* isolates of different phenotypes with respect to sensitivity to dicarboximides (iprodione, procymidone and vinclozolin)

Isolate ID	phenotypes[a]	procymidone	iprodione	vinclozolin	dicloran	tolclofos-methyl	fludioxonil
Sv129′97	S	0.93	0.77	1.7	5.8	nd[b]	0.7
Sv173′97	S	0.98	0.62	1.7	13.9	nd	1.48
Sv129′98	S	1.26	1.15	1.2	7.8	nd	0.66
Sv173′98	S	1.13	1.27	1.9	7	nd	0.54
Sv173′04	S	1.12	0.86	1.8	8.5	1.08	0.33
Sv563	S	0.35	0.1	0.2	3.2	0.41	0.76
Sv346	S_+	3.43	2.55	4.69	>100	>50	0.79
Sv448	S_+	4.74	3.44	6.4	>100	>50	0.71
Sv188′97	R_1	>100	5.48	10.5	>100	nd	3.03
Sv190	R_1	>100	11.1	23.5	>100	nd	1.27
Sv193′97	R_1	>100	13.4	9	>100	nd	1.73
Sv209′97	R_1	>100	9.13	8.4	>100	nd	2.2
Sv193′98	R_1	>100	9.5	13.8	>100	nd	nd
Sv209′98	R_1	>100	6.38	9.7	>100	nd	1.54
Sv245	R_1	>100	8.4	9	>100	nd	1.32
Sv464′04	R_1	>100	25.8	16.3	>100	>50	2.25
Sv524	R_1	>100	7.99	40.8	>100	>50	1.4
Sv222′04	R_2	>100	>100	>100	>100	>50	>100
Sv257	R_2	>100	>100	>100	>100	nd	>100
Sv439	R_2	>100	>100	>100	nd	nd	nd
Sv479	R_2	>100	>100	>100	>100	>50	>100

[a] S=sensitive; S_+=low resistant to procymidone and iprodione but moderately resistant to vinclozolin; R_1= highly resistant to procymidone but moderately resistant to the other dicarboximides; R_2= highly resistant to all dicarboximides

[b] nd=not determined

Table 4 Characterization of some *S. vesicarium* isolates by their sensitivity (EC_{50} values) toward osmotic stress inducers (sodium chloride and glucose), oxidative stress inducers (menadione) and methanol

Isolate ID	Phenotypes[a]	Sodium chloride (EC_{50} in g l^{-1})	Glucose (EC_{50} in g l^{-1})	Menadione (EC_{50} in mg l^{-1})	Methanol (EC_{50} in % v/v)
Sv173′04	S	>50	>200	>250	2.4
Sv563	S	>50	>200	>250	13.4
Sv346	S_+	>50	>200	106	4.8
Sv448	S_+	>50	>200	>250	5.2
Sv524	R_1	>50	168	>250	4.2
Sv464	R_1	>50	>200	154	7.4
Sv222′04	R_2	>50	>200	130	4.8
Sv479	R_2	>50	>200	63	2.6

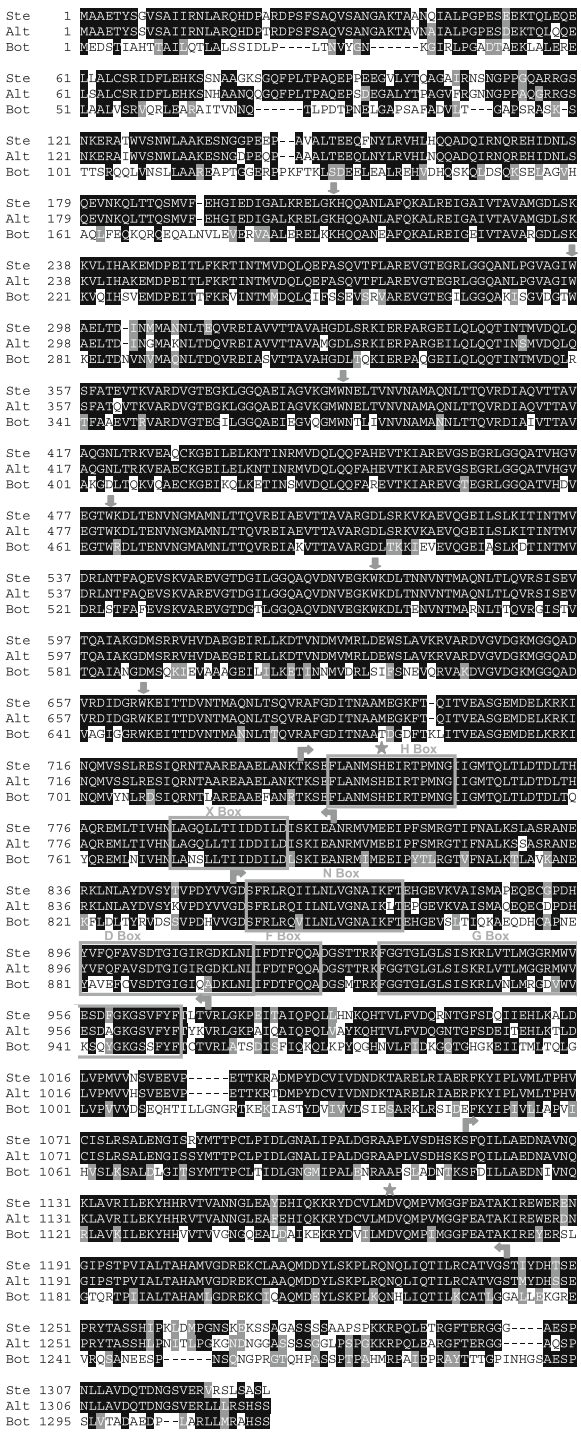
[a] S=sensitive; S_+=low resistant to procymidone and iprodione but moderately resistant to vinclozolin; R_1= highly resistant to procymidone but moderately resistant to the other dicarboximides; R_2= highly resistant to all dicarboximides

In particular, the alignment with *A. brassicicola* and *B. cinerea* sequences led to identification of the protein structure. In *S. vesicarium* the predicted 1,329 amino acid protein consisted of the histidine-kinase domain with the typical X and H boxes, containing the histidine residue with the first phosphoryl group, the ATP-binding domain, with the N, D, F and G boxes, and, at the C-terminus, the regulatory domain, with the aspartate residue with the second phosphoryl group (Fig. 1). At the N-terminus, six repeated series of about 90 amino acids (Figs. 2 and 3) were found as in other HKs of the III group.

The histidine-kinase domain and the coiled-coil region were then compared in 29 isolates belonging to the four different phenotypes. Alignments of nucleotide sequences of resistant isolates showed four base substitutions when compared to the sensitive isolates. These were different among phenotypes but identical for the isolates of the same phenotype. Observing the alignments of the deduced amino acids, each different single base caused an amino acid substitution; therefore all mutations were not silent. The S_+-resistant phenotype showed a T-to-C transition in position 898 of the nucleotide sequence that resulted in the appearance of a leucine instead of a phenylalanine at position 267 of the amino acid sequence, in the first amino acid repeat. The R_1-resistant phenotype

Table 5 Alignments of amino acid sequences of histidine kinase of *S. vesicarium* and other fungi using the computer program ClustalW (Des Higgins, EBI, Hinxton Hall, UK-http: npsa-pbil.ibcp.fr)

Fungal species	GenBank Accession number	Identity (%)	Strong similarity (%)	Weak similarity (%)	Diversity (%)
Alternaria tenuissima (partial cds)	AY559308	98.26	1.16	0	0.58
Alternaria arborescens (partial cds)	AY559302	98.26	1.16	0	0.58
Alternaria alternata (partial cds)	AF489110	97.76	1.20	0.3	0.75
Cochliobolus heterostrophus	AB095748	96.77	1.80	0.45	0.98
Alternaria brassicicola	AY700092	93.23	2.78	1.96	2.03
Botrytis cinerea	AF396827	63.37	14.07	7.37	15.19
Magnaporthe grisea	AB041647	61.67	15.06	6.41	16.85
Neurospora crassa	U53189	61.43	15.12	7.07	16.38
Aspergillus fumigatus	XM_744396	61.18	14.71	6.91	17.21



◀ **Fig. 1** Comparison of amino acid sequences of histidine kinase in *S. vesicarium* (Ste), **A**. *brassicicola* (Alt) and **B**. *cinerea* (Bot). Vertical arrows represent the beginning of amino acid repeats, curving arrows respectively the beginning and the end of histidine kinase, ATP-binding and regulatory domains, rectangles the conserved boxes and stars the phosphoryl sites (on a histidine residue in HK domain and on an aspartate residue in regulatory domain). Identical amino acids have a black background, similar amino acids have a grey background and different amino acids have a white background. Hyphens represent a lack of amino acid correspondence

showed a substitution of a leucine with a serine in the same amino acid repeat but in position 290, caused by the presence of C instead of T in position 968 of the nucleotide sequence. The R_2-resistant phenotype showed neither amino acid nor nucleotide alterations in the amino acid repeat region but differences in the histidine-kinase domain. In particular, an A-to-G substitution in position 2,484 of the nucleotide chain led to the change of a glutamine with an arginine in position 777 of the amino acid sequence or a C-to-G transition in position 2,448 caused the change of a threonine with an arginine in position 765 of the amino acid chain (Fig. 4).

Discussion

The *S. vesicarium SvHK1* gene was completely sequenced and showed a high level of homology with the histidine kinase genes of other fungi, especially of the genus *Alternaria*. Its putative protein had all the characteristic domains of class III histidine kinases, that are often involved in osmotic and oxidative stress response. In this protein, single amino acid substitutions were found in *S. vesicarium* with a specific location for each phenotype: in the first repeat of the coiled-coil region for S_+ and R_1-resistant isolates and in the HK domain for R_2 isolates. The mutations identified for each phenotype caused amino acid substitutions that do not lead to codon stop, therefore the respective proteins were different but complete. This could probably explain why *S. vesicarium* isolates were all insensitive to high osmotic pressure, as shown in the presence of both salt (sodium chloride) and sugar (glucose) and as already observed with sucrose and sodium chloride (Collina, unpublished data). This behaviour was consistent with what was observed for *B. cinerea* moderately dicarboximide resistant isolates (Leroux et al. 2002) but not for highly resistant isolates of *B. cinerea*, *C. heterostrophus* and *N. crassa* (Fujimura et al. 2000; Ochiai et al. 2001; Leroux et al. 2002; Oshima et al. 2002; Cui et al. 2002, Cui et al. 2004;

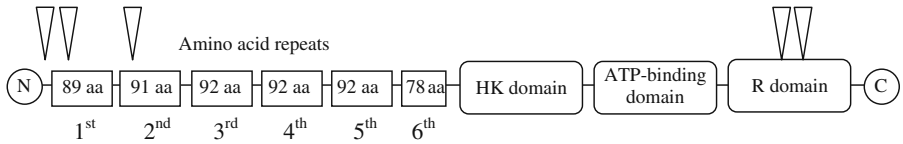


Fig. 2 Schematic representation of *S. vesicarium* putative histidine kinase. Inverted triangles represent introns

Yoshimi et al. 2003). The highly resistant field isolates of *Alternaria alternata* (Dry et al. 2004), *A. brassicicola* (Avenot et al. 2005) were more similar to *S. vesicarium* because they showed only a slightly greater osmotic sensitivity.

Mutations in the HK domain as in the R_2 phenotype isolates caused remarkable changes in fungicide sensitivity; indeed the rare dicarboximide-fludioxonil cross-resistance was identified only in this resistant phenotype (highly resistant to all dicarboximides). This observation was consistent with the findings of other authors who were able to observe cross-resistance between dicarboximides and phenylpyrroles only in *B. cinerea* and *N. crassa* laboratory mutants with high resistance to dicarboximides (Fujimura et al. 2000; Ochiai et al. 2001; Leroux et al. 2002; Oshima et al. 2002; Cui et al. 2002, Cui et al. 2004) or in *A. alternata* (Dry et al. 2004) and *A. brassicicola* (Avenot et al. 2005) highly resistant field isolates. In *B. cinerea* field isolates with moderate-low dicarboximide resistance (Leroux et al. 2002), no cross-resistance was observed between dicarboximides and phenylpyrroles, as in *S. vesicarium* S_+ and R_1 phenotypes. A response similar to *S. vesicarium* R_2 isolates is probably due to a change near the phosphorylation site that has a greater impact on protein functionality compared to a single change in amino acid repeats. In this last case the only dicarboximide resistance detected showed that the coiled-coil region is more involved in the interaction with these fungicides than with phenylpyrroles.

Although a strong impact on protein functionality was observed for the mutations in the histidine-kinase domain they do not seem to cause fitness costs as much as those in the coiled coil region, because all resistant phenotypes had similar mycelial growth rate, percentage of sporulation, germination rate and pathogenicity on detached leaves, fruits and pear seedlings to those of the sensitive phenotype (Alberoni et al. 2006 and data not shown). In *S. vesicarium* the mutations that lead to dicarboximide resistance should therefore be stable because the mutated strains seem as competitive as the non-mutated ones, also without the selection pressure of the fungicides, as confirmed by in vitro competitive assays (data not shown).

Full cross-resistance between dicarboximides and AHFs was detected for all isolates as already observed in *B. cinerea* (Leroux et al. 1992). The presence of such cross-resistance even in S_+-resistant isolates made it possible to establish that they really are resistant isolates even if their EC_{50} values toward procymidone and iprodione were closer to the values of the sensitive isolates rather than to those of the other resistant isolates. The observation of other pleiotropic effects made it possible to better characterise the four *S. vesicarium* phenotypes. Dicarboximide resistant isolates did not show a greater tolerance to methanol, confirming that they are more similar to the *B. cinerea* field isolates rather than to the laboratory mutants (Leroux et al. 2002). The oxidative stress inducer menadione did not have a particular action, unlike the case in *Aspergillus nidulans* where it blocked the generation of conidiated sectors (Appleyard et al. 2000) or in *B. cinerea* where it was able to inhibit the growth of wild type isolate and the complemented *bos1*Δ null mutants but not at all the *bos1*Δ null mutants (Viaud et al. 2006).

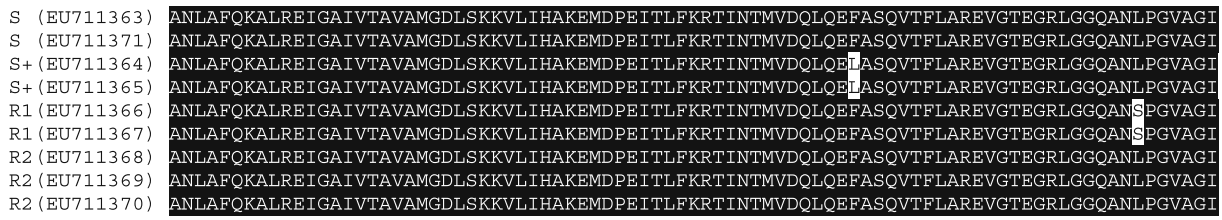
The distinct phenotypes correlated to the presence of specific single point mutations characterised in this study and may confirm the hypothesis of a qualitative response of *S. vesicarium* to dicarboximide selection pressure. This suggestion was firstly proposed by the observation that 900 monospore isolates obtained from 9 populations showed the same sensitivity as their corresponding populations (Alberoni et al. 2005). The presence of three different *S. vesicarium* resistant phenotypes is not contrary to this theory because of the greatly different frequency with which they are found in the field. The S_+ phenotype, which shows the lowest resistance to dicarboximides, is so rare (3 out of 516 isolates tested along 9 years of monitoring (Alberoni et al. 2005)) that it cannot

Fig. 3 Alignment of the six amino acid repeats of a *S. vesicarium* dicarboximide-sensitive isolate. Identical amino acids have a black background, similar amino acids have a grey background and different amino acids have a white background. Hyphens represent lack of amino acid correspondence

represent a step in a quantitative resistance process. In addition, the R_2-resistant phenotype is seldom collected in the field while the majority of resistant isolates belong to the R_1 phenotype. All resistant phenotypes are, furthermore, very rarely detected together in the same orchard (Alberoni et al. 2008); they could therefore be the result of qualitative selection processes that operate independently on pathogen populations.

In conclusion, the *S. vesicarium* phenotypes with different levels of sensitivity to dicarboximides are characterised by specific mutations in histidine kinase gene. These mutations, both when they are in the coiled-coil region and even in the HK domain, should never cause unfavourable changes in osmotic and oxidative stress response and fitness, compared to the sensitive phenotype. The resistant phenotypes may, therefore, be stable in the field and their decline within the populations may need a long period of the suspension of dicarboximide applications especially when a considerable number of resistant strains are selected in the field. Avoiding a strong selection using anti-resistance strategies should thus be an important concern for pear growers. The cross-resistance between dicarboximides and fludioxonil is not, by contrast, a real practical problem because it is

Part of the 1st Unit of amino acid repeats (212-296 aa)



Histidine-kinase domain (742-806 aa)

Fig. 4 Comparison of amino acid sequences of *SvHK1* fragments among *S. vesicarium* phenotypes with different sensitivity to dicarboximides: S=sensitive; S_+=low resistant to procymidone and iprodione but moderately resistant to vinclozolin; R_1= highly resistant to procymidone but moderately resistant to the other dicarboximides; R_2= highly resistant to all dicarboximides (only 2–3 isolates for each phenotype are reported). Identical amino acids have a black background and different amino acids have a white background

related only to the R_2 dicarboximide-resistant phenotype, which is very rarely found in the field.

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