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Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards

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Abstract

Brown spot, caused by *Stemphylium vesicarium*, is the main fungal disease of pear in northern Italy where it may cause severe crop losses and it requires numerous fungicide applications. Monitoring work was performed by collecting fungal populations in Po valley between 1995 and 2003 in order to study the dicarboximides resistance already detected in the 1990s for procymidone as a result of control failures in field. Sensitivity tests showed that the resistant strains occur all over the monitored areas. Where present the efficacy of procymidone in field is completely lost in spite of what is observed often in other fungi. In most of the isolates (phenotype R_1), *S. vesicarium* resistance level to procymidone (Sialex) was shown to be very high (RF≅3000) whereas it was lower towards the other dicarboximides iprodione (Rovral), vinclozolin (Ronilan) and chlozolinate (Serinal) (RF≅10). Therefore the resistance is partially crossed even if a high level of resistance was rarely observed for all dicarboximides (phenotype R_2). At least two different mechanisms of resistance seem to be involved: one that may provide a moderate resistance and the other that may give a high resistance level. Monospore isolate sensitivity tests confirmed the qualitative response suggested by such high resistance factors.

Introduction

Stemphylium vesicarium is the causal agent of brown spot, the main fungal disease of pear in Italy since the late 1970s (Ponti et al., 1982). It appeared in Spanish (Vilardell, 1988) and French (Blancard et al., 1989) orchards during the late 1980s and subsequently spread to other European countries as testified by its occurrence in The Netherlands in 1997 (Mr J. van Mourik, Rijnvallei, The Netherlands, pers. comm.) and Belgium in 2001 (Mr P. Creemers, PCF Royal Research Station Gorsem, St. Truiden, Belgium, pers. comm.). *Stemphylium vesicarium* is a mitosporic fungus and its teleomorphic form is the Ascomycetes *Pleospora allii* (Simmons, 1969; Farr et al., 1989).

Many pear orchards in Po valley, the main pear growing area in Italy, are affected by this fungal disease that may cause heavy loss of production, up to 100%. Plants may be affected during the whole season and both leaves and fruits may be damaged. Symptoms consist of necrotic areas (brown spots) on leaves and fruits that can rot before, during or after harvest.

Besides some cultural practices, scheduled preventative applications of dithiocarbamates, dicarboximides, strobilurins and tebuconazole (DMI) from petal fall to fruit ripening are the only way to control the disease (Ponti et al., 1996). Among dicarboximides, procymidone is the most widely used, iprodione is less applied because of its phytotoxicity towards some pear varieties, while

vinclozolin and chlozolinate are not used in field to control pear brown spot.

In the early 1990s problems in disease control were reported in some areas of northern Italy with schedules based on procymidone, giving rise to doubts about its efficacy. Monitoring work on procymidone started in 1995 to characterise this phenomenon. Laboratory assays were carried out on isolates collected from different places in the north of Italy to test *S. vesicarium* sensitivity to this fungicide. A high resistance level was found *in vitro* which corresponded to the failure of the disease control in field (Brunelli et al., 1997; Collina et al., 2002). Before the 1990s some brown spot control failures were reported that could either be explained by resistance problems or be related to more general difficulties in the chemical control of *S. vesicarium* on pear.

The present work was undertaken to improve the knowledge of *S. vesicarium* resistance to dicarboximides and to prevent the occurrence of a similar severe phenomenon in other European countries. The probable cross-resistance between procymidone and the other dicarboximides (iprodione, vinclozolin and chlozolinate) was studied while an analysis of monospore isolates sensitivity was performed in order to determine the nature (i.e. qualitative or quantitative) of the resistance response.

Materials and methods

Origin and isolation of Stemphylium vesicarium *strains*

Five hundred and sixteen *Stemphylium vesicarium* strains analysed in this work were collected between 1995 and 2003 from different sites in Po Valley, northern Italy, both randomly and from orchards where disease control had failed. They were isolated from symptomatic pear fruits of different cultivars, mainly Abbé Fétel, Conference but also doyenne, Kaiser and Passe Crassane. Fruit fragments, after disinfection for 60 s in sodium hypochlorite solution (2% available chlorine) and rinsing in sterile distilled water, were incubated for three days at 23 °C, 12 h of photoperiod (fluorescent light) on V8 agar containing 20% V8 (vegetable juice, Campbell's Grocery Ltd), 1.5% technical agar (agar Grade A, Becton Dickinson), 0.4% calcium carbonate (Fluka) in distilled water. The V8 agar was amended with 50 mg l^{-1} streptomycin sulphate after autoclaving. *S. vesicarium* colonies were identified using a stereomicroscope to observe the conidia obtained after further 2 days of 12 h photoperiod with near-ultraviolet light (TL 40 W/05 Philips) (Leach, 1967). Isolates identified as *S. vesicarium* were then transferred on to new V8 agar plates to obtain pure cultures.

Production of monospore isolates

Nine hundred monospore isolates were made from nine strains (100 for each one), chosen based on their different sensitivity towards dicarboximid fungicides from isolates collected in 1998, 1999, 2000 and 2002. A spore suspension was prepared from 7-day-old V8 agar plates by adding a few millilitres of sterile water and gently scraping the colony surface with a spatula. This suspension was then filtered through a 100 μm filter. The spore concentration was lower than 10^4 conidia ml^{-1} to allow easy separation of conidia. One millilitre of this spore suspension was spread onto a water agar plate and left to dry in a laminar flow hood. Using a stereomicroscope, conidia were transferred with a sterilised needle to new V8 agar 5 cm plate. Eventually the monospore isolates were incubated under the same growth conditions previously mentioned.

Fungicides

Dicarboximide fungicides were tested as formulated compounds: Sialex (procymidone 50% WG, Siapa), Rovral (iprodione 50% WP, Aventis), Ronilan (vinclozolin 50% WP, Basf), Serinal (chlozolinate 50% WP, Isagro). These products were suspended in distilled water and then added to V8 medium after autoclaving. Five to six concentrations were tested for each fungicide. All the concentrations are given as amounts of active ingredient (a.i.) per volume (mg l^{-1}).

Sensitivity tests

At first an initial qualitative assessment (preliminary test) was carried out for all the isolates. Afterwards another series of tests was made to find the EC_{50} concentration for each fungicide (i.e. the

concentration causing a 50% reduction in the growth rate compared to an unamended control). The EC_{50} values allowed the calculation of the Resistance Factor (RF), which shows the sensitivity level of isolates. Sensitivity tests to find the minimum inhibitory concentration (MIC) were carried out on the sensitive strains collected in 2000. Among the monospore isolates analysed in the preliminary test, eighteen of each population were further tested for EC_{50} determination.

For the preliminary test V8 medium was amended with 10 mg l^{-1} of a.i. and poured into 9 cm Petri dishes. Each Petri dish was inoculated with an inverted mycelium plug, cut from the edge of a 7 day-old colony. After 3 days of incubation (23 °C and 12 h light/dark) the mycelial growth was compared to the unamended control. Isolates that showed a growth at this concentration were considered resistant, whereas the inhibited ones were considered sensitive (Lorenz, 1988).

In EC_{50} assays V8 medium was amended with a different range of 5–6 concentrations of each fungicide (Table 1). The range was chosen according to the phenotype determined in the preliminary test and previous results of the same EC_{50} assays. The amended medium was poured into 9 cm plates. Each Petri dish was inoculated with an inverted mycelium plug (5 mm diam), cut from the edge of a 7 day-old colony. Three replicates were used and all the trials were repeated twice. The mycelial growth was evaluated after 3 days of incubation (Pappas and Fisher, 1979; Davis and Dennis, 1981a) at 23 °C and 12 h light/dark, measuring and averaging two perpendicular diameters of each fungal colony. EC_{50} values were determined by probits analysis.

The RF was estimated as ratios between the average EC_{50} of the resistant isolates and the average EC_{50} of sensitive ones. It was calculated both on the strains sampled each year and on the whole 516 strains collected in the 9 years of the monitoring work.

The procedure followed for the MIC test was the same described above for the EC_{50} test but different concentrations of formulated compounds were used: procymidone 0-1-2.5-5-6-7-8 mg l^{-1} a.i., iprodione 0-1-2-3-4-5-6-7.5 mg l^{-1} a.i., vinclozolin 0-1-2.5-5-8-9-10 mg l^{-1} a.i. and chlozolinate 0-2.5-5-10-15-17.5-20 mg l^{-1} a.i. The evaluation was made after 3 days incubation at 23 °C and 12 h light/dark. Fungal development was observed

Table 1. Dicarboximide concentrations (mg l^{-1}) used in the EC_{50} assays for the different sensitivity phenotypes of *S. vesicarium*

S. vesicarium phenotype as determined in preliminary test	Procymidone	Iprodione	Vinclozolin	Chlozolinate
Sensitive (S)	0-1-2.5-5-7.5	0-1-2.5-5-7.5	0-1-2.5-5-7.5-10	0-1-2.5-5-7.5-10
Resistant (R)	0-1000-2000-3000-4000-5000	0-5-10-15-20-50-100-500-1000-2000	0-5-10-20-50-100-500-1000-2000-3000	0-10-25-50-100-500-1000-2000-4000-5000

macroscopically and the concentration that was able to inhibit completely mycelium growth was recorded.

Results

Of the 516 strains isolated from 1995 to 2003 and analysed in the present study, 227 were found resistant (R) and 289 sensitive (S) in the preliminary sensitivity test. Table 2a shows the total number of strains examined each year and the respective number of the different phenotypes (sensitive and resistant).

The EC_{50} levels were quite different between sensitive and resistant isolates (Table 3 and Figure 1). EC_{50} values of sensitive isolates were around 1 mg l^{-1} for procymidone and iprodione whereas they ranged from 0.64 to 3.87 mg l^{-1} for vinclozolin and from 0.75 to 10.1 mg l^{-1} for chlozolinate. The resistant isolates showed two sensitivity levels and so they could be classified in two groups: R_1 (207 isolates) and R_2 (20 isolates) (Table 2b). R_1 showed an EC_{50} range of the order of thousands of mg l^{-1} for procymidone, from 3 to 29.8 mg l^{-1} for iprodione, from 6.2 to 28.7 mg l^{-1}

Table 2. Number and phenotype of *S. vesicarium* isolates tested from 1995–2003 (2a S_{tot} = sensitive isolate, R_{tot} = resistant isolate; 2b S = sensitive isolate, S+ = sensitive isolate with higher values of EC_{50} towards procymidone and iprodione, R_1 = isolate moderately resistant towards iprodione, vinclozolin and chlozolinate but highly resistant towards procymidone, R_2 = highly resistant isolate towards all dicarboximides)

2a				2b			
Years	Total isolates	S_{tot}	R_{tot}	Sensitive isolates S_{tot}		Resistant isolates R_{tot}	
				S	S+	R_1	R_2
1995	37	28	9	27	1	9	–
1996	13	8	5	8	–	5	–
1997	42	38	4	38	–	4	–
1998	62	43	19	43	–	17	2
1999	20	7	13	7	–	11	2
2000	70	29	41	28	1	37	4
2001	63	29	34	29	–	33	1
2002	123	66	57	65	1	50	7
2003	86	41	45	41	–	41	4
Total	516	289	227	286	3	207	20

Table 3. Range of EC_{50} values for mycelial growth in mg l^{-1} for sensitive or resistant *Stemphylium vesicarium* isolates in different years and in the whole 9 year monitoring period (total value). (P = procymidone I = iprodione V = vinclozolin C = chlozolinate)

Years	Sensitive isolates (S)				Resistant isolates (R_1)				Resistant isolates (R_2)			
	P	I	V	C	P	I	V	C	P	I	V	C
1995	0.50–1.75				1770–5000							
1996	0.58–0.93	0.35–1.04			5000	8.63–29.8						
1997	0.32–1.35	0.38–1.23	1.23–2.56	2.49–8.10	5000	5.48–13.4	8.40–23.5	36.3–43.2				
1998	0.54–1.41	0.41–1.43	1.20–2.36	3.15–10.1	3810–9690	5.67–9.50	7.40–20.0	10.7–58.3	5200–7790	456–1060	1440–1910	1030–1550
1999	0.44–1.11	0.56–1.19	1.34–3.04	2.96–6.21	1140–5550	4.50–18.8	6.63–22.3	19.4–69.6	1870–11500	124–689	729–1160	423–776
2000	0.66–1.30	0.69–1.17	0.64–3.87	1.54–6.46	1300–5360	4.50–13.4	6.40–28.7	19.8–51.4	1980–6490	721–2120	1720–2530	760–1020
2001	0.68–1.56	0.37–1.11	0.90–2.38	0.75–6.48	857–2880	3.47–11.4	6.20–16.7	21.1–56.2	5140	64.8	367	2050
2002	0.48–1.55	0.31–1.58			674–3730	4.32–10.4			1500–4750	434–983	863–2170	341–639
2003	0.74–1.57	0.44–1.69			251–5570	3.00–16.4			1370–1712	134–800	783–1981	321–473
Total value	0.32–1.75	0.31–1.69	0.64–3.87	0.75–10.1	251–9690	3.00–29.84	6.20–28.70	10.7–69.6	1370–11500	64.8–2120	367–2530	321–2050

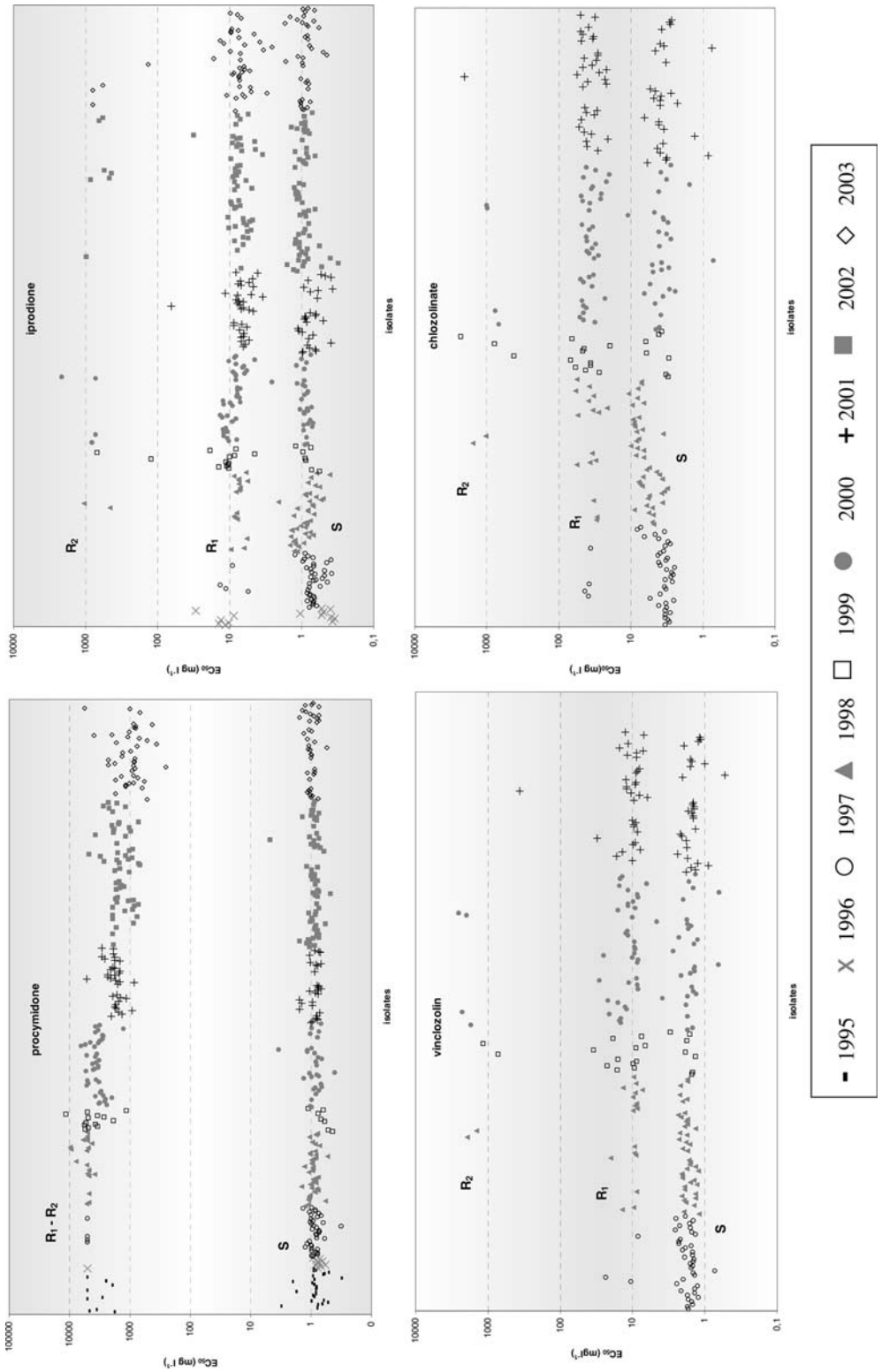

Figure 1. Sensitivity of *Stemphylium vesicarium* isolates towards dicarboximide fungicides in different years.

for vinclozolin and from 10.7 to 69.6 mg l^{-1} for chlozolinate. R_2 isolates showed the same level of resistance towards procymidone as R_1, and a higher level of resistance towards iprodione, vinclozolin and chlozolinate. Three isolates, among the 289 sensitive ones, were slightly less sensitive towards some dicarboximides, in fact the range of their EC_{50} values was 3.05–4.74 mg l^{-1} for procymidone and 2.1–3.44 mg l^{-1} for iprodione. They were called S+ (Table 2b and Figure 1).

The RF values towards procymidone were always over 1000 during the 9 years of monitoring while they ranged between 7 and 13 (Table 4) for the other dicarboximides for most of the resistant isolates (R_1). R_2 RFs were calculated separately because of their small number (20/227 isolates) and their EC_{50} values were very different for iprodione, vinclozolin and chlozolinate compared with R_1 isolates. In fact, their RF values are 780 for iprodione, 918 for vinclozolin and 159 for chlozolinate while still over 1000 for procymidone (Table 4). Differences that can be observed in some cases among the RF values in different years could be due to the reduction of dicarboximides treatments resulting in a lower fungicide selection pressure.

The MIC was determined only for sensitive isolates of *S. vesicarium*, since EC_{50} values of the resistant isolates were undoubtedly too high. *In vitro* tests carried out on sensitive isolates collected in 2000 showed the MIC values were different for each fungicide: for procymidone they ranged from 5 to 8 mg l^{-1}, for iprodione from 5 to 7.5 mg l^{-1}, for vinclozolin from 8 to 10 mg l^{-1} and for chlozolinate from 17.5 to 20 mg l^{-1}.

In order to reinforce the resistance picture described through the sensitivity parameters previously considered, a further study on monospore isolates was carried out. All monospore isolates (100 per field strain), after the preliminary test, were found to have the same sensitivity/resistance phenotype as their original strain. Moreover the 18 isolates, chosen randomly for each field strain, showed EC_{50} values very similar to their respective original population (S+, R_1, R_2).

Table 4. *Stemphylium vesicarium* resistance factors towards dicarboximide fungicides calculated on the isolates sampled every year and on all 516 isolates collected in the 9 years period

Years	Resistance Factor (for R_1)				Resistance Factor (for R_2)			
	P	I	V	C	P	I	V	C
1995	3842	–	–	–	–	–	–	–
1996	6686	28	–	–	–	–	–	–
1997	5682	14	6	11	–	–	–	–
1998	5814	8	6	6	6933	807	939	194
1999	6126	13	8	59	9879	480	524	143
2000	3706	10	7	10	4286	1161	1195	220
2001	1967	9	7	11	5670	88	235	571
2002	1510	8	–	–	2851	642	966	109
2003	1160	8	–	–	1409	627	870	88
Total	2695	9	7	13	3908	780	918	159

Discussion

The sensitivity tests, carried out during nine years, showed that all the considered pear orchard areas of northern Italy (Bologna, Ferrara, Modena, Reggio Emilia, Ravenna, Forlì, Verona, Rovigo, Padova, Mantova) were randomly affected by both sensitive and resistant strains of *S. vesicarium*. The aim of the present study was not to quantify this phenomenon but rather to evaluate qualitatively the pathogen resistance in order to confirm its previous occurrence. Therefore the ratio between sensitive and resistant strains, found during this monitoring work, is not statistically valid. In orchards where resistant *S. vesicarium* populations were detected, a complete failure in procymidone disease control was observed. Moreover, in some cases, resistant strains have been found in the field after several years during which dicarboximides were not applied, suggesting they did not have low fitness. Such field inefficacy has never been recorded as so widespread phenomenon in other plant pathogenic fungi. The most studied fungus for dicarboximides resistance is *Botrytis cinerea*. For this pathogen, problems in disease control were noticed only in some French vineyards (e.g. Champagne region) (Leroux and Clerjeau, 1985) whereas in other areas dicarboximide performance was not reduced even if resistant strains were frequently found (Pommer and Lorenz, 1982; Beever and Brien, 1983; Faretra et al., 1986). This aspect could be justified by a demonstrated lower fitness of resistant strains, and therefore they tend to disappear in the absence of the fungicide's selection pressure, giving rise to the resistance regression (Hisada et al., 1979 Davis

and Dennis, 1981b; Pommer and Lorenz, 1982; Beever and Brien, 1983; Romano et al., 1983). Also in other fungi, such as *Monilinia fructicola* and *Penicillium expansum*, resistant strains showed low fitness and often a moderate resistance level that did not cause severe difficulties in field disease control (Rosenberger and Mayer, 1981; Ritchie, 1983; Gullino and De Waard, 1984; Elmer and Gaunt, 1993, 1994). The level of resistance, which was very high towards procymidone, was another difference shown by *S. vesicarium* compared to *Penicillium* and *Botrytis* where the high resistance level was only found in laboratory mutants or in some greenhouse crops (Pommer and Lorenz, 1982; Beever and Brien, 1983; Faretra et al., 1986; Leroux et al., 1999). Comparable high resistance has been observed in field strains of different species of *Alternaria* (Iacomi-Vasilescu et al., 2004; Dry et al., 2004).

In the present study a cross-resistance among dicarboximides could be observed in *S. vesicarium* but it differs from the other fungi like *B. cinerea* (Katan, 1982; Leroux and Fritz, 1984) because it was not of the same intensity for all dicarboximides. The resistance level for iprodione, vinclozolin and chlozolinate was much lower than for procymidone, in fact *S. vesicarium* R_1-phenotype is highly resistant towards procymidone ($RF > 100$) whereas moderately resistant ($3 < RF < 100$) towards other dicarboximides. This classification is quite similar to that observed in other fungi (Gouot, 1988). Only a few isolates (R_2) could be considered highly resistant towards all these fungicides.

The presence of two resistant phenotypes of *S. vesicarium* towards different dicarboximides would suggest that at least two different mechanisms of resistance may be involved: one that leads to a moderate resistance (as shown for R_1 towards iprodione, vinclozolin and chlozolinate) and the other that gives a high resistance level (as shown for R_1 towards procymidone). The former could be given by an enhanced level of antioxidant enzymes which are able to protect the cell and remove peroxy radicals (Sisler, 1988). This ability allows the pathogen to alleviate the net fungitoxic effect of dicarboximides as showed in *B. cinerea* (Steel and Nair, 1993). The other mechanism of resistance might be due to a specific fungal mutation which would reduce the affinity of the target site only towards procymidone as the single base mutations in a two-component histidine kinase gene found on *B. cinerea* towards dicarboximides (Leroux et al., 2002; Oshima et al., 2002). This hypothesis could be considered because dicarboximide fungicides are chemically different even if they have an analogous mode of antifungal action. The rare occurrence of R_2 phenotype seems to confirm the previous theories since additional mutations have to pervade a population to make it highly resistant towards all dicarboximides.

The monospore isolate study showed that all progeny had the same sensitivity as their corresponding wild type both in the discriminatory test and in EC_{50} assays. Isolates with an intermediate sensitivity were not found. This result combined with the observed high difference in sensitivity levels between sensitive and resistant isolates suggests that *S. vesicarium* has a qualitative response in the selection of dicarboximide-resistant subpopulations. Such a qualitative response is usually acquired in a one-step pattern and it is believed to be controlled by genes of major effect (Georgopoulos, 1985; Skylakakis, 1985). As mutations in these genes might be involved in high resistance to dicarboximides, it will be useful to characterise the molecular basis of dicarboximides resistance in *S. vesicarium* by DNA analysis.

In conclusion of this nine years of monitoring work some practical recommendations can be suggested: in the case of demonstrated field resistance, the treatments with dicarboximides must be interrupted while in the orchards where sensitivity is still detected all the anti-resistance strategies suggested by FRAC (Fungicide Resistance Action Committee) must be applied, in particular the reduction of selection pressure by minimising the number of applications (not more than two to three per season) and the maintenance of regular prolonged times without exposure to dicarboximides.

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