Fitness on Grape Berries of *Botrytis cinerea* Isolates Belonging to Different Dicarboximide Sensitivity Classes

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Seasonal fluctuations in the frequency of dicarboximide-resistant *Botrytis cinerea* isolates in Western Cape vineyards suggest a reduced fitness of these isolates. In this study fitness of sensitive, ultra-low-level and low-level dicarboximide-resistant isolates of *B. cinerea* were compared on grape berries. Conidia were dispersed as single cells on berry surfaces from airborne inoculum in a settling tower or deposited as clusters in water droplets. Investigations were conducted at two potential infection courts on dicarboximide treated and untreated berries, namely the intact cuticle and wounds. Surface sterilisation, isolation and freezing techniques were used to determine surface colonisation and penetration by airborne inoculum. Decay incidence, severity and sporulation incidence were determined following inoculation with conidial clusters. The different tests indicated that germlings of dicarboximide-sensitive and -resistant isolates had similar surface-colonising abilities of dicarboximide-free berries. However, sensitive strains penetrated significantly more often. Fitness decreased with an increase in the level of dicarboximide resistance. Iprodione caused a drastic disturbance in the ratio of different dicarboximide sensitivity classes that occupied the berry surface and allowed the development of germlings of predominantly resistant isolates, but with few successful infections. Significantly higher levels of infection and proliferation of dicarboximide-resistant isolates on sprayed or unsprayed berries were facilitated by wounding or the termination of host resistance (freezing). According to these findings, these modes of infection should not contribute to a gradual build-up of inoculum of either dicarboximide-sensitive or -resistant isolates. Trends by airborne conidia described here suggest that another primary infection event in the vineyard, most likely floral infection and subsequent debris colonisation, should largely regulate the dynamics between dicarboximide-sensitive and -resistant isolates in *B. cinerea* populations on grapevine.

Botrytis cinerea Pers.: Fr. infects leaves, buds, canes and bunches of grapevine and causes grey mould. This disease is controlled in vineyards by the application of fungicides, of which the dicarboximides are the most commonly used (Nair & Hill, 1992). Fitness of *B. cinerea* isolates resistant to fungicides is an important consideration in disease management. In the case of dicarboximides the characteristics of *B. cinerea* isolates belonging to different dicarboximide sensitivity classes are well documented. Dicarboximide-resistant strains were found to be less ecologically competent or fit than dicarboximide-sensitive strains. This is attributed to abnormal osmotic sensitivity (Beever, 1983; Beever & Brien, 1983), reduced growth rate (Katan, 1982; Northover, 1983; Fraile *et al.*, 1986), reduced virulence (Northover, 1983; Leroux & Clerjeau, 1985; Beever *et al.*, 1989), less sporulation and varying stability (Katan, 1982; Pommer & Lorenz, 1982; Leroux & Clerjeau, 1985; Beever *et al.*, 1989). Based on these characteristics, Beever *et al.* (1991) proposed a hypothesis to account for the behaviour of *B. cinerea* in vineyards subjected to different dicarboximide programmes. They postulated that under a given dicarboximide programme and disease environment the resistance frequency tends to a value, termed the balance value. Selection for fungicide-resistant strains is balanced by selection against these strains, due to their low fitness. The balance value is achieved in the first vintage year and maintained during the second year. In areas where the disease intensity is low, the resistant strains will therefore tend to remain at low levels, unless high selection pressures are applied.

In a survey (Fourie & Holz, 1998) of dicarboximide resistance in *B. cinerea* populations in table grape vineyards in the Western Cape province of South Africa, a drastic decline in the frequency of dicarboximide-resistant isolates was found during winter in the pathogen populations. Maximum levels were recorded during bunch closure. The study furthermore showed that in vineyards with a history of dicarboximide resistance, an early-season increase in resistance frequency occurred irrespective of whether dicarboximides were applied or not. The increase was ascribed to nutrients (pollen, flower remnants and aborted flowers) in early-season bunches which may enhance the proliferation of the less competent dicarboximide-resistant isolates in dicarboximide treated bunches (Fourie & Holz, 1998), or to incomplete cross-resistance between folpet and dicarboximides in the pathogen population (Fourie & Holz, 2001). However, it might also point to the existence of dicarboximide-resistant isolates in the local *B. cinerea* population that are, compared to dicarboximide-sensitive isolates, ecologically competitive. Northover (1988) and Moorman and Lease (1992) found no significant difference in vir-

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ulence between sensitive and resistant isolates when tested on Chardonnay grapes and geranium leaves, respectively. Faretra and Pollastro (1993) substantiated this finding by demonstrating that *B. cinerea* has the genetic potential required to differentiate isolates with high resistance to dicarboximide fungicides without seriously diminishing their biological fitness.

Botrytis cinerea exists in grapevines as sclerotia (Nair & Nadtotchei, 1987), conidia (Corbaz, 1972; Bulit & Verdu, 1973) and mycelia (Gessler & Jermini, 1985; Northover, 1987). Conidia-bearing sclerotia on grapevines are a source of primary inoculum for infection of berries, on which the most prominent symptom of the disease is found (Nair & Nadtotchei, 1987). Conidia are dispersed in air currents (Jarvis, 1962a), in splashing water droplets (Jarvis, 1962b) and by insects (Fermaud & Le Menn, 1989; Louis *et al.*, 1996). Floral debris bearing mycelia are dispersed in wind and rain (Jarvis, 1980). Conidia are deposited as single cells on berry surfaces primarily from airborne inoculum or in water (Holz *et al.*, 2000), or occasionally in clusters by insects (Fermaud & Le Menn, 1989, 1992; Louis *et al.*, 1996; Engelbrecht, 2002). Germlings that developed from conidia enter grape berries through different pathways, namely through stigmata (McClellan & Hewitt, 1973; Nair & Parker, 1985), pedicels (Pezet & Pont, 1986; Holz, Coertze & Basson, 1997; Holz *et al.*, 2002), natural openings (Pucheu-Planté & Mercier, 1983), wounds (Nair, Emmett & Parker, 1988), or by direct penetration of the cuticle (Nelson, 1956; Coertze & Holz, 1999; Coertze, Holz & Sadie, 2001). Each mode of infection might therefore contribute in a given way to a gradual build-up of secondary inoculum and *B. cinerea* epiphytotics in vineyards. Knowledge of the mode of infection and the relationship between isolates belonging to different dicarboximide sensitivity classes is needed in order to understand the dynamics of fungicide-resistant subpopulations of *B. cinerea* and to plan management strategies for fungicide resistance in vineyards.

The aim of this study was to determine the fitness characteristics of *B. cinerea* isolates belonging to different dicarboximide sensitivity classes on mature, cold-stored grape berries, which were shown to be susceptible to infection by the pathogen (Coertze & Holz, 1999, 2002). Two modes of infection were studied: conidia were dispersed as single cells on berry surfaces from airborne inoculum or inoculated as clusters in water droplets. Investigations were conducted at two potential infection courts on dicarboximide treated and untreated berries, namely the intact cuticle and wounds.

MATERIALS AND METHODS

Grapes

Grape bunches (cultivar Dauphine) were selected at harvest (19°Brix) from a vineyard with a history of no dicarboximide usage and low *B. cinerea* incidences. Bunches were surface sterilised (30 sec in 70% ethanol, 2 min in 0.35% sodium hypochlorite, 30 sec in 70% ethanol), air-dried, packed in polythene bags in boxes and kept at -0.5°C. This treatment completely eliminated *B. cinerea* from the berry surface (Sarig *et al.*, 1996) and prevented natural infection (Coertze & Holz, 1999). At each inoculation bunches were removed from cold storage and kept overnight at 22°C. Berries were cut from clusters with short stem segments attached, surface-sterilised as described previously and packed on sterile epoxy-coated steel mesh screens (53 x 28 x 2 cm). Previous studies have shown that grape berries are more susceptible to *B. cinerea* infection following cold storage (Coertze & Holz, 1999, 2002).

Isolates

Twelve sensitive (ED_{50} < 0.1 µg vinclozolin/mL), 12 ultra-low-level resistant (ED_{50} < 1.5 µg vinclozolin/mL) and 12 low-level resistant (ED_{50} > 2 µg vinclozolin/mL) isolates were selected at random from a *B. cinerea* culture collection kept on malt extract agar slopes at 5°C in the dark. Isolates in the collection were previously characterised for dicarboximide resistance and were obtained from sporulating lesions on grape berries collected from table grape vineyards in different geographical regions in the Western and Northern Cape provinces (Fourie & Holz, 1998). The selected isolates were grown at 22°C under diurnal light in Petri dishes on a synthetic agar medium amended with sugars, minerals and malic acid at concentrations occurring in grape berry exudate (1.85 g glucose; 1.95 g fructose; 0.25 g sucrose; 0.15 g malic acid; 5 g peptone; 5 g sodium chloride; 15 g agar; and 2 g yeast extract per litre deionised water). Preliminary studies showed that *B. cinerea* isolates sporulate profusely on this medium (G. Holz, Department of Plant Pathology, Stellenbosch University, Private Bag X1, 7602 Stellenbosch). Conidia were harvested dry with a suction-type collector from 14-day-old cultures and stored dry until use. Storage time did not affect germination; the dry conidia could therefore be used in all experiments (Spotts & Holz, 1996).

Germination of each isolate was verified before studies on fitness were conducted. Dry conidia of each isolate were suspended in water and spread-inoculated onto the synthetic agar plates, incubated for 18 h at 22°C and the percentage germinated conidia recorded (100 conidia per Petri dish, three replicates). Dicarboximide sensitivity of each isolate was also verified by doing mycelial growth tests on PDA amended with 3 and 7.5 µg vinclozolin/mL as described by Fourie and Holz (1998).

Inoculation

Dry airborne conidia (freshly deposited)

The abilities of freshly deposited airborne conidia to colonise grape berry surfaces and to penetrate the intact cuticle of berries, which were unsprayed or sprayed with iprodione, were determined. Inoculum consisting of conidia of sensitive and resistant isolates in a 1:1 (w:w) ratio was prepared by mixing conidia of six sensitive, six ultra-low-level resistant and six low-level resistant isolates in a 2:1:1 (w:w:w) ratio. Berries were placed on six screens and divided into two groups on each screen (40 berries per group). The berries of one group were left unsprayed and those of the other group were sprayed with iprodione (500 µg a.i./mL sterile water) to pre-runoff with a gravity feed mist spray gun (ITW DEVILBISS Spray Equipment Products) used at 2 bar. The berries were kept overnight at 22°C to air-dry before inoculation. Two screens at a time were inoculated with 3 mg of the mixture of dry conidia that was dispersed by air pressure into the top of an inoculation tower (Plexiglass, 3 x 1 x 1 m [height x depth x width]) according to the method of Salinas *et al.* (1989) and allowed 20 min to settle onto the berries that were positioned on the two screens. At this dosage approximately three conidia were evenly deposited as single cells on each mm^2 of berry surface (Coertze & Holz, 1999). To recognise the inoculated side of

the berry at a later stage, a 1-cm mark was made on the berry near the pedicel with a felt-tipped pen. Previous studies (Coertze & Holz, 1999) showed no phytotoxic effect on berries. Following inoculation, the screens were placed in an ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) chamber (60 x 30 x 60 cm) lined with a sheet of chromatography paper with the base resting in deionised water to establish high relative humidity (≥93% RH). The chamber was kept at 22°C for 48 h with a 12 h photoperiod. Studies (Coertze *et al.*, 2001) with airborne conidia of *B. cinerea* on grape berries under similar conditions showed that germination and surface colonisation reached a maximum during this period. After 48 h the screens were removed from the chambers and the berries were air-dried. Surface colonisation and skin penetration were determined by using a differential set of isolation and freezing techniques (one screen from each inoculation was used for each technique) on berries subjected to two sterility regimes. Berries from the sprayed and unsprayed groups on each screen were divided into groups of 20 berries each; one group from each treatment was left unsterile to determine surface colonisation, while the other group was sterilised in 70% ethanol for 5 sec to eliminate the pathogen on the berry surface (Coertze & Holz, 1999; Coertze *et al.*, 2001) and to determine penetration. In the isolation studies six adjoining epidermal tissue segments (5 x 5 mm) were cut from the inoculated cheek of each berry (20 berries from each group on each screen), placed with the cuticle upward on *B. cinerea* selective medium (Kerssies, 1990) and incubated for 14 days at 22°C. In the freezing studies berries were kept for 1 h at -12°C and incubated in a dry chamber for 14 days at 22°C. Studies (Holz, Körte & Coertze, 1995) conducted with naturally infected and artificially inoculated berries showed that a 1-h freezing period at -12°C is needed to promote the development of latent infections. Segments yielding *B. cinerea* or conidial tufts developing on frozen berries were transferred to PDA plates amended with streptomycin sulphate. The dicarboximide sensitivity of *B. cinerea* isolates recovered from the epidermal segments and frozen berries (one isolate per berry) was determined as described above. The experiment was repeated.

Dry airborne conidia (previously deposited)

The effect of iprodione on germlings that have established on grape berry surfaces prior to the fungicide treatment was determined in a separate experiment. Berries were stacked on six screens (three screens each for freezing and isolation technique) and divided into three groups each (30 berries per group). Berries were inoculated and incubated in moist chambers for the colonisation of berry surfaces by *B. cinerea* as described previously. After 48 h berries of the one group were left untreated, those of the second group were surface-sterilised for 5 sec in 70% ethanol, whereas those of the third group were sprayed with iprodione (500 µg a.i./mL sterile water) until pre-runoff. The berries were air-dried and surface colonisation and skin penetration determined by isolation and freezing studies as described previously. The dicarboximide sensitivity of the isolates recovered from epidermal segments and from frozen berries was determined as described before. The experiment was repeated.

Multiple conidia in a water droplet (unsprayed berries)

The ability of freshly deposited conidial clusters to induce decay and sporulation was investigated on unwounded and on wounded berries. Dry conidia of the 12 sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates were suspended in sterile, deionised water. The density of the conidial suspension of each isolate was determined with a haemocytometer and adjusted to 1 x 10^5 spores per milliliter. A circle 0.5 cm in diameter was drawn with a felt-tipped pen on the surface of the berries to indicate the inoculation site. Berries were placed on 12 screens: 90 berries per screen divided into three groups. In the first experiment the conidia were applied to unsprayed berries (30 berries per isolate) by placing a 20-µL drop of conidial suspension inside the marked area. The drops were air-dried to facilitate conidial attachment (Spotts & Holz, 1996). After 2 h half the number of berries was wounded 1 mm deep at the inoculation site with a sterile needle, while the other berries were left unwounded. The screens with berries were then placed in the moist chambers kept at 22°C with a 12-h photoperiod. At regular intervals (2, 5, 7 and 9 days) after inoculation each berry was indexed according to lesion development on a disease rating index ranging from 1-6 (1 = no decay; 2 = 1-3 mm lesion diameter; 3 = 3-5 mm lesion diameter: 4 = 5-8 mm lesion diameter; 5 = 8-12 mm lesion diameter; 6 = >12 mm lesion diameter). The numbers of berries with sporulation at lesion sites were also determined. The experiment was repeated twice.

Multiple conidia in a water droplet (sprayed berries)

The effect of vinclozolin, procymidone and iprodione on wound infection by freshly deposited conidial clusters was determined in separate trials. In each experiment berries were divided into two groups of 15 berries each. Berries of the one group were left unsprayed and those of the other group were sprayed with the fungicide (500 µg a.i./mL sterile water) until pre-runoff and air-dried. The berries were drop-inoculated with the 36 isolates, wounded, incubated in moist chambers and rated at regular intervals as described previously. The experiments were repeated twice.

Statistical analyses

The data obtained from the isolation and freezing experiments with dry, airborne conidia were subjected to analysis of variance, chi-squared and Student's t-tests. Analysis of variance was done for data on decay incidence, severity and sporulation incidence from the other experiments. An arcsin transformation (Snedecor & Cochran, 1967) was done on decay and sporulation incidence and least significant difference values (95% significance) were obtained from these transformed values. Data on disease severity were subjected to Proclogistic analysis, which compares the values on an underlying logistic scale (Agresti, 1984).

RESULTS

Isolates

No significant differences ($P < 0.05$) were found in the germination of sensitive and resistant isolates. The percentage germination of the different isolates ranged from 75% to 98%. Dicarboximide resistance in all the isolates was found to be stable.

Inoculation

Dry airborne conidia (freshly deposited)

Incidences of epidermal segments and frozen berries yielding *B. cinerea* and the dicarboximide sensitivity status of isolates recovered in each treatment are given in Table 1. Chi-squared tests of the frequencies of the different dicarboximide sensitivity classes of *B. cinerea* recovered from epidermal segments and frozen berries are given in Table 2. In the unsterile regime iprodione

TABLE 1

Mean percentages of epidermal segments and frozen berries yielding *Botrytis cinerea* after iprodione-sprayed and unsprayed grape berries were inoculated with dry, airborne conidia belonging to different dicarboximide sensitivity classes[a] and the proportions at which isolates of the different classes were recovered[b].

	Epidermal segments[d]				Frozen berries[e]			
		Proportion (%)				Proportion (%)		
Treatment[c]	Total	S	ULR	LR	Total	S	ULR	LR
Not sterilised								
Unsprayed	98.5	56.7	28.9	14.4	70.0	62.9	19.4	17.7
Sprayed	43.5	18.0	48.1	33.9	41.1	2.8	63.9	33.3
Sterilised								
Unsprayed	13.6	72.4	22.1	5.5	50.6	78.9	17.8	3.3
Sprayed	1.9	22.2	33.3	44.4	21.7	7.7	51.3	41.0

[a]Berries inoculated with a 2:1:1 (w:w:w) mixture of sensitive (S), ultra-low- (ULR) and low-level resistant (LR) strains.

[b]Dicarboximide sensitivity of isolates recovered from epidermal segments and frozen berries was determined by doing mycelial growth tests on PDA amended with 3 and 7.5 µg vinclozolin/mL as described by Fourie & Holz (1998).

[c]Not sterilised, unsprayed = sprayed with sterile deionised water prior to inoculation, not sterilised after incubation; not sterilised, sprayed = sprayed with 500 µg/mL iprodione prior to inoculation, not sterilised after incubation; sterilised, unsprayed = sprayed with sterile deionised water prior to inoculation, surface sterilised in 70% ethanol after incubation; sterilised, sprayed = sprayed with 500 µg/mL iprodione prior to inoculation, surface sterilised with 70% ethanol after incubation.

[d]Epidermal segments = six adjoining epidermal segments (5 x 5 mm) were cut from the inoculated cheek of the berries, placed with the cuticle upward on Kerssies's *B. cinerea* selective medium (Kerssies, 1990) and incubated for 7 days at 22°C.

[e]Frozen berries = berries were kept at -12°C for 1 h and incubated in a dry chamber for 14 days at 22°C.

TABLE 2

Chi-square tests of frequencies of different dicarboximide sensitivity classes of *Botrytis cinerea* isolates obtained from iprodione-sprayed and unsprayed grape berries that were inoculated with a mixture of dry conidia and subjected to various treatments[a].

	Sensitive *vs* Resistant[b]		Ultra-low *vs* low-level resistant[c]	
Treatment	Chi^2 value	*P* value	Chi^2 value	*P* value
Epidermal segments				
Not sterilised				
Unsprayed	1.6000	0.2059	4.3333	0.0374
Sprayed	97.9456	<0.0001	5.8979	0.0152
Sterilised				
Unsprayed	29.1379	<0.0001	14.3990	0.0001
Sprayed	5.5555	0.0184	0.2857	0.5930
Frozen berries				
Not sterilised				
Unsprayed	4.1290	0.0422	0.0435	0.8348
Sprayed	80.2778	<0.0001	3.4571	0.0630
Sterilised				
Unsprayed	30.0444	<0.0001	8.8946	0.0029
Sprayed	13.9628	<0.0001	0.4444	0.5050

[a]See Table 1.

[b]Frequency of sensitive isolates (S) compared with the combined frequency of the ultra-low- and low-level resistant isolates (R). $P < 0.05$ strong evidence against tested hypothesis (S:R = 1:1).

[c]Frequency of the ultra-low-level resistant isolates (ULR) compared with that of the low-level resistant isolates (LR). $P < 0.05$ strong evidence against tested hypothesis (ULR:LR = 1:1).

nearly halved the percentage of segments (98.5% to 43.5%) and frozen berries (70.0% to 41.1%) yielding *B. cinerea*. According to the dicarboximide sensitivity test, germlings of sensitive and resistant isolates occupied the surfaces of unsprayed berries in both the segment and frozen berry tests in approximately a 1:1 ratio. The opposite was found on sprayed berries, where the corresponding values were 1:5 and 1:35, respectively. The isolation and frozen berry tests conducted with surface-sterilised berries showed that iprodione also effected a marked reduction in skin penetration (13.6% to 1.9% and 50.6% to 21.7%, respectively). It was obvious from the unsprayed, surface-sterilised treatment that resistant germlings were significantly less able to penetrate the cuticle ($P < 0.0001$). On these berries sensitive germlings penetrated the cuticle approximately three (segment test) to five (frozen berry test) times more frequently than dicarboximide-resistant germlings did. It was furthermore found that significantly more ultra-low-level resistant strains penetrated the cuticle than low-level resistant strains ($P = 0.0001$ [segments]; $P = 0.0029$ [berries]). On iprodione-sprayed berries significantly more germlings of resistant isolates penetrated the cuticle ($P = 0.0184$ [segments]; $P < 0.0001$ [berries]): for each germling of a sensitive isolate that penetrated the cuticle, approximately three (segment test) to 12 (frozen berry test) germlings of resistant isolates had penetrated.

Dry airborne conidia (previously deposited)

Incidences of epidermal segments and frozen berries yielding *B. cinerea* and the dicarboximide sensitivity status of isolates recovered in each treatment are given in Table 3. Results of the chi-squared tests of the frequencies of the different dicarboximide sensitivity classes of *B. cinerea* recovered from epidermal segments and frozen berries are given in Table 4. Results obtained

TABLE 3

Mean percentages of epidermal segments and frozen berries yielding *Botrytis cinerea* and the proportion of sensitive, ultra-low- and low-level resistant isolates[a] from grape berries that were inoculated with a mixture of dry conidia[b] and subjected to various treatments.

	Epidermal segments[d]				Frozen berries[e]			
		Proportion (%)				Proportion (%)		
Treatment[c]	Total	S	ULR	LR	Total	S	ULR	LR
Unsprayed	98.5	50.6	31.7	17.8	41.1	58.9	27.4	13.7
Sterilised	15.6	81.8	11.5	6.8	32.2	74.6	15.3	10.2
Sprayed	6.7	28.3	51.7	20.0	33.9	12.5	60.7	26.8

[a]Dicarboximide sensitivity of isolates recovered from epidermal segments and frozen berries was determined by doing mycelial growth tests on PDA amended with 3 and 7.5 µg vinclozolin/mL as described by Fourie & Holz (1998).

[b]Berries inoculated with a 2:1:1 (w:w:w) mixture of sensitive (S), ultra-low- (ULR) and low-level resistant (LR) strains.

[c]Unsprayed = not sterilised or sprayed after incubation; sterilised = sterilised with 70% ethanol after incubation; sprayed = sprayed with 500 µg/mL iprodione after incubation.

[d]Epidermal segments = six adjoining epidermal segments (5 x 5 mm) were cut from the inoculated cheek of the berries, placed with the cuticle upward on Kerssies's *B. cinerea* selective medium (Kerssies, 1990) and incubated for 7 days at 22°C.

[e]Frozen berries = berries were kept at -12°C for 1 h and incubated in a dry chamber for 14 days at 22°C.

TABLE 4

Chi-squared tests of frequencies of different dicarboximide sensitivity classes of *Botrytis cinerea* isolates recovered from grape berries inoculated with a mixture of dry conidia and subjected to various treatments[a].

	Sensitive *vs* Resistant[b]		Ultra-low *vs* low-level resistant[c]	
Treatment	Chi^2 value	*P* value	Chi^2 value	*P* value
Epidermal segments				
Unsprayed	0.0222	0.8815	7.0224	0.0080
Sterilised	59.7027	<0.0001	1.8148	0.1779
Sprayed	11.2660	0.0008	8.3954	0.0038
Frozen berries				
Unsprayed	2.3151	0.1281	3.3333	0.0679
Sterilised	14.2530	0.0002	0.6000	0.4386
Sprayed	10.9590	0.0009	7.8473	0.0051

[a]See Table 3.

[b]Frequency of sensitive isolates (S) compared with the combined frequency of the ultra-low- and low-level resistant isolates (R). $P < 0.05$ strong evidence against tested hypothesis (S:R = 1:1).

[c]Frequency of the ultra-low-level resistant isolates (ULR) compared with that of the low-level resistant isolates (LR). $P < 0.05$ strong evidence against tested hypothesis (ULR:LR = 1:1).

from the unsprayed treatment confirmed those of the non-sterilised, unsprayed treatment reported in Table 1. The application of iprodione reduced the percentage segments and frozen berries yielding *B. cinerea* as effectively as did surface sterilisation. However, on the sprayed berries predominantly resistant isolates survived and penetrated.

Multiple conidia in a water droplet (unsprayed berries)

The unwounded berries inoculated with the different isolates remained mostly asymptomatic (Table 5). On wounded berries, dicarboximide-sensitive isolates proved to be more virulent than the resistant isolates. Significantly more berries inoculated with the sensitive than with resistant isolates developed decay after 2 and 5 days. The sensitive and ultra-low-level resistant isolates behaved similarly after 7 and 9 days and were significantly more virulent than the low-level resistant isolates. Significantly more berries infected by sensitive isolates yielded sporulating lesions at all ratings. Analysis of variance of disease severity indices indicated significant resistance class x treatment interaction (Table 6). A summary of the percentages of the disease severity index values of wounded dicarboximide-free grape berries 9 days after inoculation is given in Table 7. Decay was more severe after 9 days on berries infected by sensitive isolates than by resistant isolates. Significantly more berries were completely rotted (index 6) by the sensitive isolates than by the resistant isolates.

Multiple conidia in a water droplet (sprayed berries)

Analysis of variance of disease severity indices indicated significant resistance class x treatment interaction for each of the fungicides used (Table 8). Isolates of the three dicarboximide sensitivity classes behaved in a similar pattern on the wounded berries sprayed with the three fungicides. Data are therefore given for the vinclozolin treatment only. On unsprayed berries dicarboximide-resistant isolates were as virulent as the dicarboximide-sensitive

TABLE 5

Mean percentages[a] of dicarboximide-free table-grape berries showing decay by *Botrytis cinerea* and yielding conidia after inoculation[b] with isolates belonging to different dicarboximide resistance classes[c].

	Incubation period							
	2 days		5 days		7 days		9 days	
Resistance class	D[d]	S[d]	D	S	D	S	D	S
Unwounded								
Sensitive	0 c	0	0.4 d	0 b	0.4 c	0.3 c	0.5 c	0.3 d
ULR	0 c	0	0.2 d	0 b	0.2 c	0 c	0.2 c	0.1 d
LR	0 c	0	0.3 d	0 b	0.3 c	0.1 c	0.3 c	0.2 d
Wounded[e]								
Sensitive	3.2 a	0	27.6 a	0.4 a	38.8 a	10.1 a	45.4 a	19.5 a
ULR	1.3 b	0	19.1 b	0 b	34.4 a	4.0 b	42.1 a	13.9 b
LR	1.1 b	0	13.5 c	0 b	26.7 b	2.1 b	33.0 b	6.0 c

[a]Means in each column followed by the same letter do not differ significantly (P=0.05%) according to the LSD test derived from arc sin transformed data.

[b]Berries were inoculated by placing a 20 µL drop of conidial suspension onto the berries.

[c]Twelve sensitive, 12 ultra-low-level resistant (ULR) and 12 low-level resistant (LR) isolates.

[d]D = decay; S = sporulating.

[e]Berries were wounded 1 mm deep at the inoculation site with a sterile needle.

TABLE 6

Analysis of variance of disease severity index values obtained on wounded and unwounded grape berries 9 days after inoculation with *Botrytis cinerea* isolates belonging to different dicarboximide sensitivity classes[a].

Source	Degrees of Freedom	Sum of squares of variance	Mean squares of variance	F value	*P* value
Repititions 5	7.974	1.5974	0.5627	0.7270	
Resistance classes	2	24.124	12.0620	4.2493	0.0462
Fault (a)	10	28.386	2.8368		
Treatment	1	1228.690	1228.6900	1781.7430	<0.0001
Class x treatment	2	10.119	5.0595	7.3369	0.0060
Fault (b)	15	10.344	0.6896		
Total	35				

[a]Twelve sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates.

TABLE 7

Mean percentages of wounded, dicarboximide-free table grape berries recorded in different disease severity indices[a] 9 days after inoculation[b] with *Botrytis cinerea* isolates belonging to different dicarboximide sensitivity classes[c].

	Mean percentage decayed berries					
Resistance class	**Index 1**	**Index 2**	**Index 3**	**Index 4**	**Index 5**	**Index 6**
Sensitive	53.1	5.1	4.3	5.2	7.4	25.1
Ultra-low-level resistant	56.4	7.0	4.2	7.2	7.8	17.4
Low-level resistant	65.2	7.4	3.3	6.1	5.8	12.3

[a]Disease rating index ranged from 1-6: 1 = no decay; 2 = 1-3 mm diameter lesion; 3 = 3-5 mm diameter lesion; 4 = 5-8 mm diameter lesion; 5 = 8-12 mm diameter lesion; 6 = ≥12mm diameter lesion.

[b]See Table 5 for wounding and inoculation.

[c]Twelve sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates.

TABLE 8

Analysis of variance of disease severity index values obtained on fungicide-sprayed and unsprayed grape berries 7 days after inoculation with *Botrytis cinerea* isolates belonging to different dicarboximide sensitivity classes[a].

Source	**Degrees of Freedom**	**Sum of squares of variance**	**Mean squares of variance**	**F value**	***P* value**
Vinclozolin					
Resistance classes	2	460.485	230.2425	58.6112	<0.0001
Fault (a)	15	58.925	3.9283		
Treatment	1	566.988	566.9880	431.1368	<0.0001
Class x treatment	2	493.235	246.6175	187.5276	<0.0001
Fault (b)	15	19.727	1.3151		
Total	35				
Iprodione					
Resistance classes	2	132.984	66.4920	32.1373	0.0006
Fault (a)	6	12.414	2.0690		
Treatment	1	172.247	172.2470	73.9734	0.0001
Class x treatment	2	158.691	79.3455	34.0758	0.0005
Fault (b)	6	13.971	2.3285		
Total	17				
Procymidone					
Resistance classes	2	27.077	13.5385	13.6230	0.0059
Fault (a)	6	5.963	0.9938		
Treatment	1	28.288	28.2880	13.3560	0.0106
Class x treatment	2	46.334	23.1670	10.9381	0.0100
Fault (b)	6	12.708	2.1180		
Total	17				

[a]Twelve sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates.

isolates (Table 9). The fungicide did not prevent infection and decay by sensitive isolates on wounded berries, but reduced decay significantly. Resistant isolates, however, caused substantial decay on a high proportion of the vinclozolin-treated berries. Vinclozolin furthermore completely inhibited sporulation of sensitive isolates on symptomatic berries, whereas a significant inhibition of sporulation of the resistant isolates was first observed after 7 days' incubation. Disease severities for ultra-low-level and low-level resistant isolates were comparable (Table 10) and nearly half the number of sprayed berries were completely decayed by the resistant isolates after 7 days, whereas decay development by sensitive strains was markedly inhibited.

TABLE 9

Mean percentages of wounded, vinclozolin-sprayed table grape berries showing decay and sporulation by *Botrytis cinerea* after inoculation[a] with isolates belonging to different resistance classes[b].

	Decayed berries (means %)[c]					
	2 days		5 days		7 days	
Resistance class	**D[d]**	**S[d]**	**D**	**S**	**D**	**S**
Unsprayed[e]						
Sensitive	72.1 a	0	83.1 b	7.3 b	85.9 b	41.3 b
Ultra-low-level resistant	70.9 a	0	89.2 a	24.3 a	92.8 a	63.0 a
Low-level resistant	53.8 b	0	78.2 bc	6.6 b	84.1 b	45.4 b
Sprayed[f]						
Sensitive	15.7 c	0	25.1 e	0 c	28.7 d	0 d
Ultra-low-level resistant	60.0 b	0	77.5 b	4.5 b	81.6 bc	30.4 c
Low-level resistant	51.9 b	0	72.6 c	5.9 b	78.5 c	31.0 c

[a]Each berry was inoculated with a 20 µL drop of 1 x 10^5 conidia/mL suspension. A wound was made 1 mm deep at the dry inoculation site 2 h after inoculation.

[b]Twelve sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates.

[c]Means in each column followed by the same letter do not differ significantly ($P = 0.05\%$) according to the LSD test derived from arc sin transformed data.

[d]D = decay; S = sporulating

[e]Unsprayed = sprayed with sterile, deionised water.

[f]Sprayed = sprayed with 500 µg/mL vinclozolin.

TABLE 10

Percentages of wounded, dicarboximide-treated table grape berries recorded in different disease severity indices[a] 7 days after inoculation[b] with *Botrytis cinerea* isolates belonging to different dicarboximide sensitivity classes[c].

	Decayed berries (means %)					
Resistance class	**Index 1**	**Index 2**	**Index 3**	**Index 4**	**Index 5**	**Index 6**
Unsprayed[d]						
Sensitive	17.8	3.5	3.3	3.4	8.2	63.9
Ultra-low-level resistant	9.7	3.6	4.7	6.9	10.7	64.4
Low-level resistant	18.7	9.4	8.2	8.1	14.0	41.6
Sprayed[d]						
Sensitive	68.5	7.7	10.0	5.7	7.1	1.0
Ultra-low-level resistant	21.6	5.5	3.8	5.2	5.7	58.2
Low-level resistant	25.2	7.4	5.8	6.3	8.6	46.8

[a]Disease rating index ranged from 1-6: 1 = no decay; 2 = 1-3 mm diameter lesion; 3 = 3-5 mm diameter lesion; 4 = 5-8 mm diameter lesion; 5 = 8-12 mm diameter lesion; 6 = ≥12mm diameter lesion.

[b]See Table 1 for wounding and inoculation.

[c]Twelve sensitive, 12 ultra-low-level resistant and 12 low-level resistant isolates.

[d]Unsprayed = sprayed with sterile, deionised water.
Sprayed = sprayed with 500 µg/mL vinclozolin.

DISCUSSION

The findings in this study confirm the superior fitness of dicarboximide-sensitive strains over resistant strains in the absence of the fungicide as was suggested in associated and related studies (Pommer & Lorenz, 1982; Leroux & Clerjeau, 1985; Fourie & Holz, 1998). Ecological competence furthermore decreased with an increase in the level of dicarboximide resistance. However, the mode of infection should be considered when studying fungicide resistance and when planning strategies for the management of *B. cinerea* fungicide resistance in vineyards. The different tests conducted on cold-stored berries, which were shown to be susceptible to infection by *B. cinerea* (Coertze & Holz, 1999, 2002), indicated that germlings of dicarboximide-sensitive and -resistant isolates had equal ecological competence on dicarboximide-free berry surfaces. The studies with dry, airborne conidia showed that sensitive isolates were more virulent than resistant isolates and

larger proportions of germlings of sensitive isolates penetrated the dicarboximide-free cuticle. Likewise, the ultra-low-level resistant isolates were found to be more virulent than the low-level resistant isolates. The presence of iprodione caused a drastic disturbance in the ratio of the different dicarboximide sensitivity classes that occupied the berry surface and allowed predominantly the development of germlings of resistant isolates. The fungicide had a similar action on berries with established germlings. However, the amount of infections on grape berry cheeks that established by this mode was low. Working with single, airborne conidia, Coertze and Holz (1999) found that both immature and mature berries of the table grape cultivar Dauphine remained asymptomatic after extended periods (3 to 96 h) of moist or wet incubation. Other studies (Holz *et al.*, 2000) with airborne conidia confirmed this trend on table grapes (cultivars Barlinka and Waltham Cross) and wine grapes (cultivars Merlot noir, Chenin blanc and Shiraz). According to these findings, these infections *per se* should not lead to symptom expression at either phenological stage in the vineyard. Thus, irrespective of the dicarboximide spray programme used or the dicarboximide sensitivity status of the *B. cinerea* population in the vineyard, this mode of infection should not contribute to a gradual build-up of inoculum of either dicarboximide-sensitive or -resistant isolates.

The study indicated that wound infection should facilitate the proliferation of dicarboximide-resistant isolates in vineyards subjected to frequent dicarboximide applications. Grape berries can be wounded by insects, frost, hail, alluvial sand, sun or rapid water intake leading to splitting (Jarvis, 1980; Savage & Sall, 1983). The role of this mode of infection in the proliferation of inoculum in the early season is, however, uncertain. Previous studies showed that, following adhesion and the first stages of growth, *B. cinerea* did not survive for extended periods on surfaces of immature berries (Coertze & Holz, 1999; Coertze *et al.*, 2001). Therefore, in the event of wounding, a combination of fresh wounds, freshly dispersed conidia and free water on the berry surface is necessary for successful wound infection (Coertze & Holz, 2002). A synchronisation of these events might not commonly occur in vineyards. Furthermore, in the case of berries inoculated at bunch closure and harvest stages, proportions of wounds infected by this mode were extremely low. According to these findings, wound infection should not lead to an early-season increase in resistance frequency of *B. cinerea* in vineyards (Fourie & Holz, 1998).

Trends by airborne conidia described here and elsewhere (Coertze & Holz, 1999; Coertze *et al.*, 2001) suggest that another primary infection event in the vineyard, most likely floral infection and subsequent debris colonisation, should largely regulate the dynamics between dicarboximide-sensitive and -resistant isolates in *B. cinerea* populations on grapevine. Estimations of the amount of *B. cinerea* on grapevine in the Western Cape province showed that the amount of the pathogen is high at pea-size stage, then decreases significantly as the season progresses (Holz *et al.*, 2002). Studies made with spore traps in commercial vineyards in South Africa confirmed this decline in *B. cinerea* inoculum (G. Holz, unpublished data). The early increase in dicarboximide resistance frequency in Western Cape vineyards, which reach maximum levels at bunch closure (Fourie & Holz, 1998), can therefore be ascribed to the dynamics of the *B. cinerea* populations under the local climate, which usually favours the pathogen from the pre-bloom to the pea-size stage. Pollen and flower debris provide an excellent nutrient source for the development of *B. cinerea* at flowering and colonisation of aborted flowers and floral debris within bunches has been recorded (Gessler & Jermini, 1985; Nair & Parker, 1985; Northover, 1987; De Kock & Holz, 1994). Iprodione and procymidone were ineffective in eradicating the pathogen from aborted flowers and dead flower parts during these growth stages (De Kock, 1989; De Kock & Holz, 1994). The relative importance of infection by direct mycelial growth from colonised debris might furthermore have implications for the proliferation of dicarboximide-resistant isolates. Hoksbergen and Beever (1984) showed that on bean leaves *B. cinerea* isolates with low-level dicarboximide resistance could still be controlled by dicarboximide applications when inoculum was in the form of conidia, but not when the inoculum was in the form of a mycelial plug. Aborted flowers and flower remnants in clusters removed for sampling during the pea-size stage might also have been colonised by dicarboximide-resistant strains, whereas these parts might have been absent in bunches sampled later. De Kock and Holz (1994) found that dead flowers that were colonised by *B. cinerea* occurred in bunches of the table grape Barlinka until late pea size, but not at vèraison. The phenological stages of table grape development when free nutrients are less available for the fungus (vèraison to harvest), thus act as a resistance "filter", causing the resistance frequency to decline. The decline in resistance frequency would be highest in the absence of free nutrients, as well as the absence of selection pressure exerted by dicarboximide applications, as was seen over the post-bunch closure and winter stages in Western Cape province table grape vineyards (Fourie & Holz, 1998).

To ensure the efficacy of dicarboximides for control of *B. cinerea*, early season use of dicarboximide fungicides in vineyards with a history of high grey mould incidence and a high dicarboximide schedule and/or vineyards with high dicarboximide resistance balance values should be avoided. Products with unrelated chemistry (such as cyprodinil/fludioxonil, pyrimethanil and fenhexamid) should be used during the flowering to pea-size stages. Van Rooi (2002) investigated infection and fungicide efficacy at different positions in grape bunches and showed that these fungicides were as effective as iprodione in preventing *B. cinerea* at all growth stages tested. Her studies furthermore indicated that proper applications of these fungicides in commercial vineyards during flowering and bunch closure should effectively prevent infection and symptom expression and thus the development of *B. cinerea* epiphytotics.

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