Stability and Fitness of Anilinopyrimidine-Resistant Strains of *Botrytis cinerea*

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ABSTRACT

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The fitness of anilinopyrimidine-resistant isolates of *Botrytis cinerea* compared with that of sensitive isolates, collected from vegetable crops in Greece during 2005, was investigated. Stability of resistance to anilinopyrimidine fungicides was determined after consecutive transfers of the fungal isolates on fungicide-free potato dextrose agar for 16 culture cycles or on fungicide-untreated cucumber seedlings for eight disease cycles. Results showed that after the consecutive transfers of the isolates either in vitro or in vivo sensitivity to cyprodinil was not changed significantly compared to the initial sensitivity in all the isolates tested, suggesting a stable genetically controlled trait. Fitness parameters measured were mycelial growth, spore production in vitro, osmotic sensitivity, virulence, spore production in vivo, percentage of spore germination, and competitive ability of the resistant isolates in four pairs with sensitive isolates both on artificial nutrient medium or on cucumber seedling plants. The measurements of the fitness components in individual isolates showed high variability within both sensitivity groups in all, except viru-

lence, fitness components tested. As a group, resistant isolates showed significantly lower ($P < 0.05$) mycelial growth and virulence, while they were more osmotically sensitive than the sensitive isolates. In addition the resistant isolates showed higher ($P < 0.05$) spore production in vivo but there was no difference ($P > 0.05$) between the two sensitivity groups in spore production in vitro and in the percentage of spore germination. However, the correlation to test if there is any relationship between the values of each fitness component tested and the level of cyprodinil sensitivity of each isolate was for all, except the spore production in vivo, fitness components not significant ($P > 0.05$). This absence of significant correlation coefficient values suggests that the development of resistance to anilinopyrimidine fungicides did not affect the fitness of the resistant isolates. Competition of the resistant versus sensitive isolates was isolates-dependent, since in two of the isolate pairs the resistance frequency decreased significantly after five culture or disease cycles, while in the remaining two pairs resistance frequency increased significantly after five disease cycles or remained stable for one pair after five culture cycles on artificial nutrient media.

Additional keywords: fungicide resistance, gray mold.

Gray mold caused by *Botrytis cinerea* Pers.: Fr. (teleomorph *Botryotinia fuckeliana*) is among the most important diseases of greenhouse-grown vegetable crops in many countries worldwide, including Greece. The control of the disease is based on an integration of several cultural methods with the use of fungicides belonging to several groups. Until the middle of the 1990s, chemical control of gray mold was mainly achieved by site-specific fungicides belonging to benzimidazoles, dicarboximides, and N-phenylcarbamate, while multi-specific inhibitors such as chlorothalonil, dichlofluanid, iminactodine, and captan were used only in tank mixtures or in rotation with site-specific inhibitors (8,19). During the previous decade, a novel fungicide group, anilinopyrimidine fungicides, with a new mode of action, was registered for use and introduced into the spray programs applied for the control of gray mold on vegetable crops worldwide (32).

Three active ingredients, pyrimethanil, cyprodinil, and mepanipyrim belong in the group of the anilinopyrimidines. They have a broad activity spectrum including several important plant pathogens that belong to the Ascomycetes and to the Imperfect Fungi. They share a common mode of action consisting of inhibition of biosynthesis of methionine and other amino acids while they also inhibit the secretion of cell wall degrading enzymes required for the infection process of *B. cinerea* (6,10,23). Strains of *B. cinerea* obtained from grapes and vegetable crops with high or weak resistance to anilinopyrimidines have already been detected (9,11,

18,24). Moreover, a recent monitoring program to determine the sensitivity of *B. cinerea* isolates obtained from vegetable crops showed the development of strong resistance to anilinopyrimidine fungicides (25). Sensitivity distribution of *B. cinerea* populations to anilinopyrimidine fungicides was largely abnormal, suggesting a qualitative, disruptive selection for resistance, which is typical for one-gene mutation. Several previous studies reported that resistance to anilinopyrimidine fungicides is monogenically controlled (2,4,11).

The development and evolution of fungicide resistance in fungal populations are largely dependent on the fitness of the resistant fraction of the population and this has important implications on disease management (27,34). Fitness can be defined as the survival and reproductive success of an allele, individual, or group (29). The development and the evolution of fungicide resistance would be lessened if resistant subpopulation had lower parasitic or saprophytic fitness. In contrast, absence of fitness costs in the resistant fraction of the population would lead to a stable resistance frequency in the absence of fungicide selection force or to rapid development and evolution of resistance under the fungicide selection force.

Due to the high importance of fitness, development of resistance to fungicide classes such as the benzimidazoles and the dicarboximides by strains of *B. cinerea*, was followed by reports regarding the fitness of the resistant strains compared with that of the sensitive ones. In most of these reports, development of resistance was associated with fitness costs (12,31,37), while in another study development of resistance was not associated to fitness costs (30). In addition, studies on the fitness of pathogen fungicide-resistant strains have also been conducted prior to the

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introduction of novel fungicides such as pyraclostrobin, fenhexamid, and fludioxonil to determine the inherent risk for resistance development using isolates with laboratory-induced resistance (21,39,40).

After the emergence of anilinopyrimidine resistance, an optimal gray mold management program in the vegetable crops grown in greenhouses in Greece had to be developed. To achieve an effective disease management program, a detailed knowledge of the fitness of the resistant strains is required. Therefore objectives of this study were (i) to determine the stability of anilinopyrimidine-resistant phenotypes when the isolates are subcultured and the competitive ability of the resistant isolates, either in vitro or in vivo in the absence of anilinopyrimidine fungicides, and (ii) to measure several fitness components such as mycelial growth, spore production in vitro and in vivo, osmotic sensitivity, virulence, and spore germination for each isolate used in this study.

MATERIALS AND METHODS

Fungal isolates. Ten anilinopyrimidine-resistant and ten anilinopyrimidine-sensitive single-spore isolates of *B. cinerea* were used in the experiments. The isolates were collected during a monitoring program carried out in 2005, to determine the fungicide sensitivity of *B. cinerea* isolates obtained from vegetable crops grown in greenhouses in the island of Crete, Greece. The EC₅₀ values were determined based on the inhibition of mycelial growth according to a procedure described previously (25). Eight out of ten anilinopyrimidine-resistant strains used in the study belonged to the AniR1 type while the remaining two isolates were also moderately resistant to diacboximides and thus belonged to the AniR2/AniR3 type, according to the classification suggested by Leroux et al. (18). According to this classification, AniR1 strains are resistant only to anilinopyrimidine fungicides and characterized by high resistance factors, while strains belonging to AniR2/AniR3 types are simultaneously resistant to anilinopyrimidines and fungicides of other classes and characterized by low resistance factors to anilinopyrimidine fungicides (4,18). Details on the isolates sensitivity to cyprodinil and the host of origin are given (Table 1). Until use, the isolates were maintained on potato dextrose agar (PDA) (Oxoid, Basingstoke, UK) slants in a refrigerator at 4°C.

Stability of resistance. To measure the stability of resistance in vitro the 20 (10 each sensitive and resistant) selected isolates were cultured on PDA plates. The cultures of the pathogen were renewed every 2 weeks by transferring a 5-mm inoculum plug on fresh anilinopyrimidine-free PDA plates. Cultures were incubated at 22°C in the dark. For each isolate, four replicate plates were tested and in total the isolates were recultured 16 times.

To measure the stability of resistance in vivo, mycelial plugs (5 mm in diameter) were transferred on cucumber seedling cotyledons according to an inoculation procedure described by Ziogas et al. (40). Cucumber seedlings (cv. Cetriolo Telegrafo) were

grown in plastic pots (18 cm diameter) containing a 2:1 mixture of peat and perlite. After sowing, pots were kept in the growth chamber at 23°C and after the appearance of the cotyledons, the plants were inoculated. In total, four plants (8 cotyledons) were inoculated per isolate. The center of each cotyledon was punctured with a needle and a 3-mm mycelial plug from the edges of actively growing cultures on PDA, was placed on the wound. Then, the inoculated plants were transferred in the moist chamber at 23°C for 5 days. After the appearance of disease symptoms, the cotyledons were removed and the pathogen was reisolated from the diseased tissues and used for the inoculation of a new set of cucumber seedlings. The procedure was repeated in total eight consecutive times.

The sensitivity to cyprodinil was measured, after the 8th and the 16th culture cycle in the in vitro experiment and after the 8th culture cycle in the in vivo experiment, according to the method described previously by Myresiotis et al. (25).

The fungicide used in the study was the commercial formulation of cyprodinil (Chorus 50 WG, Syngenta Hellas, Athens, Greece). For the measurement of pathogen sensitivity to cyprodinil, a minimal nutrient medium was used, containing 10 g of glucose, 1.5 g of K₂HPO₄, 2 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 0.5 g of MgSO₄·7H₂O, and 12.5 g of agar (Oxoid, Basingstoke, UK) per liter. The fungicide was dissolved in sterilized distilled water and a stock solution was prepared. Autoclaved medium was amended with cyprodinil at 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 µg ml⁻¹. Control medium was not amended with the fungicide. Both experiments were repeated twice.

Fitness components. The following fitness components were measured for anilinopyrimidine-resistant and -sensitive isolates of *B. cinerea*: (i) mycelial growth on PDA, (ii) osmotic sensitivity, (iii) spore production in vitro, (iv) virulence, (v) spore production in vivo, and (vi) germination ability (%) of conidia. All the experiments were repeated in triplicate.

Mycelial growth. Four 5-mm mycelial plugs of the isolates grown on water agar were transferred to the center of PDA plates for radial growth measurements. The plates were incubated at 22°C in the dark for 3 days. The colony diameter of each isolate was measured after 72 h of incubation. For each isolate tested, four replicate plates were prepared.

Osmotic sensitivity. Osmotic sensitivity was determined by comparing the radial growth of isolates on PDA with that on PDA-amended with 25 µg ml⁻¹ of KCl. The colony diameter was measured after 3 days of incubation at 22°C, in the dark. The percentage of mycelial growth inhibition was calculated using the formula RGI% = (C-N)/(C-5) × 100, where C is the colony diameter of the non KCl treatments and N is that of KCl treatment (20). For each isolate four replicate plates were prepared.

Spore production in vitro. To determine the spore production of the isolates, PDA plates were inoculated with a 5-mm mycelial plug and incubated for 10 days at 22°C with a 14-h photoperiod. Then, sporulating colonies were rinsed with 20 ml of distilled

TABLE 1. Sensitivity to cyprodinil and host of origin of *Botrytis cinerea* isolates used in the study

Sensitive isolates ^z			Resistant isolates		
Isolate	EC ₅₀ (µg ml ⁻¹)	Host of origin	Isolate	EC ₅₀ (µg ml ⁻¹)	Host of origin
BCS 2	0.005	eggplant	BCR 1d	5.0	eggplant
BCS 3	0.008	tomato	BCR 7a	20.4	eggplant
BCS 16e	0.004	tomato	BCR 7e	10.0	eggplant
BCS 6	0.004	tomato	BCR 4a	10.2	tomato
BCS 1c	0.010	eggplant	BCR 5a	7.6	tomato
BCS 8e	0.007	tomato	BCR 11c	4.5	cucumber
BCS 9e	0.010	tomato	BCR 12b	2.8	cucumber
BCS 13e	0.009	tomato	BCR 13b	17.1	tomato
BCS 14a	0.007	eggplant	BCR 16c	22.2	tomato
BCS 14d	0.010	eggplant	BCR 18a	25.2	tomato

^z The isolates were collected during 2005 in a monitoring program carried out to determine fungal sensitivity to several fungicides (25).

water and the conidial suspension was filtered through double-layered sterile cheesecloth. The spore concentration in the suspension was measured with the aid of hemacytometer and expressed as number of conidia per cm² of the PDA culture. Four replicate droplets were counted for each plate and four plates were used per isolate.

Virulence. The virulence of the isolates was determined by inoculating cucumber seedling cotyledons, according to the method of Ziogas et al. (40). In total, 12 plants (24 cotyledons) were inoculated per isolate. Then the inoculated plants were transferred in the moist chamber at 23°C for 5 days and the infection was recorded by evaluating the lesion on each cotyledon. The evaluation was carried out by using a disease-index scale suggested by Ziogas et al. (40) as follows: 0 = no infection; 0.5 = rot only under the inoculum; 1 = less than 20% rot; 2 = 21 to 50% rot; and 4 = rot on more than 50% of the cotyledon surface.

Spore production in vivo. After measurement of virulence, the inoculated plants were transferred again in the moist chamber (100% relative humidity (RH) and 23°C) for an additional incubation of 24 h to induce sporulation on the diseased cotyledons. After 24 h, the seedlings were removed from the moist chamber and the cotyledons with sporulating lesions were cut off from the plants. Leaf segments (about 20 mm²) with sporulating lesions were removed with the aid of a 5-mm diameter cork borer. One segment was removed from each cotyledon. Twelve plants (24 cotyledons) were tested per isolate. The cotyledon segments were placed in 3 ml of distilled water in an Eppendorf tube, vortexed to detach the conidia from the lesions, and subsequently the cotyledon segments were removed. Droplets of the conidial suspension were placed on the hemacytometer and the number of conidia was counted. Four replicate droplets were counted for each tube. The number was expressed as the number of conidia per square millimeter of diseased cotyledon surface.

Germination of conidia. After the measurement of spore production, aliquots (0.2 ml) from the spore suspensions were transferred onto water agar plates for the measurement of conidial germination. The inoculated plates were incubated at 20°C for 12 h and the percentage of spore germination was determined by scoring 100 conidia per plate, in three replicate plates per isolate. A conidium was considered as germinated if the germ tube length was at least twice the length of the conidium.

Competitive ability in vitro and in vivo. For the competition experiment, four resistant (R1 to R4) and four sensitive (S1 to S4) isolates were arbitrarily selected and used. The isolates were cultured on PDA at 22°C for 10 days, and the produced conidia were harvested by adding 15 ml of distilled sterilized water in each plate. Mixed-isolate inocula were prepared by mixing appropriate volumes of conidial suspensions of R1 with S1, R2 with S2, R3 with S3, and R4 with S4 isolates, so as to produce suspensions containing 80%R:20%S, 50%R:50%S, and 20%R:80%S conidia. All suspensions contained a final concentration of 8×10^3 conidia per ml. One milliliter from each of the prepared conidial suspensions was transferred to new PDA plates. For each pair of isolates and conidial ratio, three replicate PDA plates were used. Then, the plates were incubated for 10 days at 22°C with 14-h photoperiod and after this period the produced conidia were harvested by adding in each plate 10 ml of distilled sterilized water. One milliliter of the resulting conidial suspension was used to inoculate fresh PDA plates to start a new culture cycle. The experiment was terminated after five culture cycles and repeated twice.

To measure competitive ability in vivo, the same four pairs of resistant and sensitive isolates were used, at the same three R:S ratios. Cucumber seedling plants at the stage of cotyledons that had been grown under the conditions described previously were inoculated by spore suspensions of each isolate pair at each of the three conidial ratios. For each pair and conidial ratio combination,

four replicate pots with four plants per pot were inoculated. Before inoculation, the cotyledons were punctured at several points with a needle. Inoculation was carried out by a hand atomizer by spraying plants on both cotyledon surfaces. Pots were covered with plastic bags to ensure conditions favorable for infection and incubated in the moist chamber at 23°C for 10 days. Subsequently, the plants were removed and the cotyledons were cut off from the plants to prepare the inoculum for the next disease cycle. For this purpose, all the infected cotyledons from each treatment were flooded into tap water and conidia were collected by gently shaking the leaves. The resulting suspension was used to inoculate a new set of plants. In this way a new disease cycle was started. The experiment was terminated after five disease cycles and was repeated twice.

The determination of the percentage of cyprodinil-resistant conidia in the mixed populations was carried out after the end of the fifth culture cycle or the fifth disease cycle, using single-spore isolations of the pathogen. Fifty single-spore isolates were obtained from each replicate plate in the in vitro experiment or 10 isolates per plant for the in vivo experiment. To determine resistance frequency to cyprodinil, one 5-mm mycelial plug from each isolate was transferred onto PDA plates amended with cyprodinil at a concentration of 0.1 µg ml⁻¹. The concentration of cyprodinil at 0.1 µg ml⁻¹ was completely inhibitory for mycelial growth of the sensitive isolates while allowing the growth of resistant isolates. The discrimination between sensitive and resistant isolates was carried out after 3 days of incubation at 23°C in the dark. In total, 150 isolates (fifty isolates per plate) were examined per treatment in the in vitro experiment and 160 isolates (10 isolates per plant) in the in vivo experiment.

Data analysis. In the stability experiments, to measure the extent of the changes in sensitivity of the isolates tested, a factor of sensitivity change (FC) was calculated by dividing the initial EC₅₀ value by the final EC₅₀ value of each isolate tested. All data for each fitness component were subjected in an analysis of variance (ANOVA). Mean values of each isolate within sensitivity groups and means among sensitivity groups were compared using the Fisher's protected least significant difference (LSD) test. Analysis of percentage data were based on arcsine-square root-transformed percentage values. In the competition experiments, the observed (%) frequency of resistant isolates was compared with the initial frequency of resistant isolates by the pairwise Student's *t* test. All the statistical analysis tests were supported by SPSS version 11.0.

RESULTS

Stability of resistance. In the in vitro experiment, the first EC₅₀ value determination was carried out after the 8th culture cycle but no change in the sensitivity was observed, compared to the initial sensitivity (data not shown). However, isolate sensitivity to cyprodinil appeared to be stable even after either 16 successive subcultures on artificial nutrient media or after eight disease cycles on host tissue. In most of the isolates tested, either anilino-pyrimidine-resistant or -sensitive the final cyprodinil sensitivity was similar to the initial one (Table 2). None of the resistant isolates used in this study turned to sensitive after 16 subcultures on PDA or after eight subcultures on cucumber plants. Moreover, calculation of the FC showed that this had a value of around one for most of the isolates and only in a few resistant or sensitive isolates the FC value was lower or higher than one (Table 2).

Fitness components. In total, six fitness components were measured for the 20 *B. cinerea* isolates tested. For all the six, except virulence, fitness components measured showed a great variability even among isolates within the same sensitivity group.

The anilino-pyrimidine-sensitive isolates of *B. cinerea*, as a group, had a mean mycelial growth of 84.6 mm which was significantly higher ($P < 0.05$) than that of the group of the resistant

isolates, which was 74.8 mm (Table 3). Within the group of the resistant isolates, two isolates, BCR7a and BCR11b, showed significantly lower mycelial growth compared with the remaining resistant or sensitive isolates. However, when the mean values of mycelial growth and the level of sensitivity in terms of EC₅₀ values were correlated, the correlation coefficient of 0.17 was not significant ($P > 0.05$) (Fig. 1A).

Mean spore production in vitro for the group of the sensitive isolates had a value of 7.69×10^4 spores per cm² of medium, which was not significantly different ($P > 0.05$) from the mean spore production in vitro of the resistant isolates (Table 3). In addition, the correlation coefficient value of 0.38 between the level of sensitivity to cyprodinil and the number of spores produced in vitro was not significant ($P > 0.05$) (Fig. 1B).

The group of anilinopyrimidine-resistant strains was osmotically more sensitive than the group of anilinopyrimidine-sensitive

isolates. The mean mycelial growth of the anilinopyrimidine-resistant strains on KCl-amended PDA was 83.7% of that on KCl-free PDA, while the anilinopyrimidine-sensitive strains had a mycelial growth of 90.6% of that on KCl unamended medium (Table 3). Within the group of anilinopyrimidine-resistant isolates, there were three isolates with relative growth on KCl less than 80%, while within the group of anilinopyrimidine-sensitive strains there was only one isolate with relative growth lower than 80%. However, when the mean values of the relative mycelial growth and the level of sensitivity in terms of EC₅₀ values were correlated, the correlation coefficient of 0.38 was not significant ($P > 0.05$) (Fig. 1C).

All the isolates, either anilinopyrimidine-sensitive or -resistant, with the exception of the isolate BCR11c, were virulent causing severe symptoms on the cucumber seedling cotyledons. Virulence was the only fitness component tested, for which the variability

TABLE 2. Changes of cyprodinil sensitivities of *Botrytis cinerea* strains after 16 consecutive culture cycles on fungicide-free artificial culture medium (in vitro) and after eight consecutive disease cycles on cucumber seedlings (in vivo)

Resistant isolates						Sensitive isolates					
Isolate	Initial EC ₅₀ ^y	In vitro		In vivo		Isolate	Initial EC ₅₀	In vitro		In vivo	
		EC ₅₀	FC ^z	EC ₅₀	FC			EC ₅₀	FC	EC ₅₀	FC
BCR 1d	5.0	4.6	1.08	5.2	0.96	BCS 2	0.005 a	0.006	0.83	0.005	1.00
BCR 7a	20.4	18.5	1.10	19.2	1.06	BCS 3	0.008 a	0.009	0.88	0.008	1.00
BCR 7e	10.0	9.2	1.08	10.8	0.92	BCS 16e	0.004 a	0.005	0.80	0.005	0.80
BCR 4a	10.2	10.0	1.02	8.2	1.24	BCS 6	0.004 a	0.008	0.50	0.004	1.00
BCR 5a	7.6	7.0	1.08	7.8	0.97	BCS 1c	0.020	0.020	1.00	0.011	1.10
BCR 11c	4.5	4.3	1.04	4.7	0.95	BCS 8e	0.007	0.008	0.87	0.006	1.16
BCR 12b	2.8	3.0	0.93	4.0	0.70	BCS 9e	0.010	0.010	1.00	0.008	1.25
BCR 13b	17.1	17.5	0.97	17.7	0.96	BCS 13e	0.009 a	0.009	1.00	0.009	1.00
BCR 16c	22.2	21.5	1.03	20.2	1.09	BCS 14a	0.007 a	0.006	1.16	0.007	1.00
BCR 18a	25.2	25.0	1.00	24.6	1.02	BCS 14d	0.010 a	0.012	0.83	0.008	1.20

^y Effective concentration at 50% (EC₅₀) values in µg ml⁻¹.

^z FC, factor of change, initial EC₅₀ value (µg ml⁻¹) divided by the final EC₅₀ value.

TABLE 3. Fitness components of anilinopyrimidine-resistant and -sensitive field isolates of *Botrytis cinerea*

Fitness components						
Isolate	In vitro experiments			In vivo experiments		
	Sporulation ^u	Mycelial growth (mm)	Osmotic sensitivity ^v	Virulence ^w	Sporulation ^x	Spore germination (%)
BCR 1d	9.9 cde ^y	81.1 de	90.9 bc	4.0 a	5.1 efgh	88.8 abcdef
BCR 7a	7.6 ef	14.6 i	83.2 cdef	4.0 a	1.4 h	88.4 bcdef
BCR 7e	2.9 ijk	81.1 de	73.9 f	4.0 a	1.8 h	84.1 fgh
BCR 4a	3.9 hijk	79.3 ef	85.1 cdef	4.0 a	1.4 h	87.9 bcdefg
BCR 5a	8.4 def	78.3 ef	81.6 cdef	4.0 a	6.6 defg	88.6 abcdef
BCR 11c	2.3 ijk	50.7 h	98.1 b	1.2 b	nd	nd
BCR 12b	1.0 k	72.5 g	80.6 cdef	4.0 a	1.2 h	74.3 j
BCR 13b	7.0 efg	92.1 a	78.1 def	4.0 a	20.7 b	92.6 ab
BCR 16c	12.7 bc	94.1 a	77.0 ef	4.0 a	24.2 ab	91.2 abc
BCR 18a	20.5 a	93.8 a	87.9 bcde	4.0 a	26.7 a	93.2 a
Mean	7.65 A ^z	73.8 B	83.7 B	3.7 B	9.86 A	87.7 A
BCS 2	4.3 ghij	89.0 ab	90.2 bcd	4.0 a	1.8 h	87.1 cdefgh
BCS 3	5.0 ghi	75.1 fg	113.2 a	4.0 a	4.5 efgh	88.9 abcde
BCS 16e	13.8 b	88.6 abc	90.5 bc	4.0 a	3.6 fgh	86.3 defgh
BCS 6	9.6 de	89.8 ab	90.6 bc	4.0 a	2.7 gh	86.5 cdefgh
BCS 1c	1.3 jk	92.6 a	85.7 cdef	4.0 a	14.1 c	84.6efgh
BCS 8e	9.4 de	93.3 a	88.9 bcde	4.0 a	9.1 d	89.9 abcd
BCS 9e	11.3 bcd	85.2 bcd	89.0 bcde	4.0 a	7.8 de	83.2 hi
BCS 13e	8.2 ef	78.5 ef	82.8 cdef	4.0 a	1.6 h	90.9 abcd
BCS 14a	8.2 ef	72.5 g	98.2 b	4.0 a	7.1 def	83.6 ghi
BCS 14d	6.2 fgh	83.1 cde	77.4 ef	4.0 a	5.1 efgh	79.3 i
Mean	7.69 A	84.6 A	90.6 A	4.0 A	5.7 B	86.0 A

^u Spore number $\times 10^4$ /cm² of medium.

^v Growth on potato dextrose agar (PDA) amended with KCl as percentage of growth on PDA nonamended with KCl.

^w Measurements based on the 5-scale disease index suggested by Ziogas et al. (40).

^x Spore number $\times 10^4$ per mm² of lesions.

^y Statistical analysis within groups; means of individual isolates followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

^z Statistical analysis between groups; mean values of groups followed by the same capital letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

within each sensitivity group was almost absent since all the isolates, except BCR11c, had a disease index value of four (Table 3). Additionally, the correlation coefficient value of 0.1 between level of sensitivity to cyprodinil and virulence of the isolates was not significant ($P > 0.05$) (Fig. 1D).

Resistant isolates, as a group, produced 9.86×10^4 conidia per mm^2 of lesions on diseased cucumber tissues, a number which was significantly higher ($P < 0.05$) than the respective number of 7×10^4 conidia per mm^2 of lesions produced by the group of the sensitive isolates (Table 3). The isolate BCR11c did not sporulate and was not included in the calculation of the mean spore number. A significant ($P < 0.05$) correlation coefficient of 0.61 existed between the level of isolate sensitivity and spore production on host tissues (Fig. 1E).

Germination of conidia produced on the host tissues was the last fitness component tested. The results showed that both the group of resistant and the sensitive isolates had similar ($P > 0.05$) germination percentage of 87.7 and 86.0%, respectively (Table 3). Moreover, when the level of sensitivity to cyprodinil was correlated with the percentage of spore germination of each isolate, it was shown that the correlation coefficient of 0.32 was not significant ($P > 0.05$) (Fig. 1F).

Competitive ability in vitro and in vivo. The changes in the resistance frequency were largely dependent on the competing isolates. In two among the four isolate pairs, the sensitive isolates became dominant over the resistant isolates after either five culture cycles in vitro or after five disease cycles on cucumber seedlings. In these two isolate pairs (R1/S1 and R4/S4) in all three

ratios tested, the frequency of the resistant isolates decreased significantly ($P < 0.05$) both in the in vitro and the in vivo experiments (Table 4).

In the second pair (R2/S2) the resistant isolate R2 was a strong competitor and consequently resistance frequency significantly ($P < 0.05$) increased compared to the initial resistance frequency in all three ratios tested either in vitro or in vivo (Table 4).

In the third pair (R3/S3) the two competing isolates showed equal competitive ability when tested in vitro (Table 4). In all three ratios tested, the final resistance frequency after five cultures on artificial nutrient medium remained similar to the initial ($P > 0.05$). In contrast, the resistant isolate R3 was a better competitor than the sensitive isolate S3 in the in vivo experiments, since resistance frequency was significantly increased ($P < 0.05$) in all three ratios tested (Table 4).

DISCUSSION

B. cinerea represents a classical high risk pathogen for fungicide resistance development due to its high genetic variability, the abundance of sporulation, the short generation time, and the wide host range (19,28,38). During the last decade, chemical control of gray mold on vegetable crops and on grape vines has been based upon the use of anilinopyrimidine fungicides in many other countries worldwide, including Greece. However, development of resistance to this promising fungicide class in pathogen isolates from both grapes and vegetable fruits (11,17,18,24,25) necessitates predictions regarding the risk for resistance evolution. In

Fig. 1. A, Correlation between level of sensitivity to cyprodinil and mycelial growth, B, spore production in vitro, C, osmotic sensitivity, D, virulence, E, spore production in vivo, and F, percentage of spore germination of anilinopyrimidine-resistant and -sensitive strains of *Botrytis cinerea*.

the current study the relationship between resistance of *B. cinerea* to anilinopyrimidine fungicides and fitness of the resistant strains was investigated. Fitness was evaluated both in terms of “predicted fitness” (measurement of several components in individual isolates) and in terms of “realized fitness” (competition between sensitive and resistant isolates) (1).

Results on the measurement of cyprodinil sensitivity after consecutive transfers in vitro or in vivo in the absence of cyprodinil, verified the phenotypic stability of anilinopyrimidine resistance in *B. cinerea*. The observed stability indicates that the decreased sensitivity of the pathogen populations on the Crete Island is not a temporary adaptation of the pathogen to fungicides. In most of the resistant isolates the level of sensitivity to cyprodinil in terms of EC₅₀ values after 16 culture cycles, remained similar to the initial, while in some isolates EC₅₀ values after 16 culture cycles were slightly decreased compared to the initial values. However, no isolate that initially was resistant to cyprodinil became sensitive to this fungicide during repeated subculturing. A similar cyprodinil-resistance stability in the absence of cyprodinil selection pressure has already been reported by Babij et al. (2) in *Tapesia acuformis*, while in the same report anilinopyrimidine-resistant isolates of *T. yallundae* lost their resistance after one or two subcultures on cyprodinil-free growth medium (2). Moreover, a similar phenotypic stability has also been observed in benzimidazole-resistant strains of *B. cinerea*, while dicarboximide-resistant strains of the pathogen showed to be unstable (38). Stability of dicarboximide resistance has only been reported for strains that were simultaneously resistant to both dicarboximide and benzimidazole fungicides (16,38). In contrast, the levels of resistance have been shown to decrease after consecutive transfers in the absence of fungicide selection pressure in other fungicide-pathogen systems, such as *Monilinia fructicola* and *Cercospora beticola*, resistant to sterol demethylation inhibiting fungicides (5,13).

When the fitness components of 10 cyprodinil-resistant and 10 -sensitive isolates were determined, the resistant isolates appeared to have similar fitness characteristics to those of the sensitive isolates. The results presented in this study showed that, as a group, resistant isolates had similar spore production capacity in vitro, virulence, and percentage of spore germination to those of the sensitive isolates group. In contrast, resistant isolates as a group, produced more spores on host tissues than the sensitive isolates did, while sensitive isolates as a group showed higher mycelial growth and lower osmotic sensitivity compared with the group of

the resistant isolates. Moreover, for no fitness component tested except spore production in vivo, the correlation coefficient values between resistance level in terms of EC₅₀ values and fitness component values showed a significant positive correlation. Within each sensitivity group of isolates a great variability was observed to the several fitness components studied. This variability suggests that there is the possibility for selection of highly resistant isolates with high fitness in the field as has already been reported by Uyenoyama (36). This author stated that fitness-modifying genes could increase the fitness of the resistant field strains to a level similar to that of the sensitive strains. Similarly, the competition experiments carried out between pairs of a sensitive and a resistant isolate showed that the outcome of the competition was isolate-dependent. In some pairs the resistance frequencies increased while in some others resistance frequencies decreased.

In one of the first published reports regarding the use of anilinopyrimidine fungicides against gray mold, Forster and Staub (9) provided sensitivity data from wood samples suggesting that a certain proportion of the less sensitive strains of the pathogen is fit enough to survive over winter. Similarly, in a recent study Moyano et al. (24) compared the sporulation capacity and the lesion growth rate of pyrimethanil-resistant and -sensitive strains of *B. cinerea* and found no significant differences. In contrast, in a long-term monitoring program carried out to determine the sensitivity of *B. cinerea* isolates from grapes, it was found that the cyprodinil resistance frequency was significantly decreased during the overwintering period. These authors assumed that this was due to the decreased survival ability of the resistant strains during the winter period (3). To the best of our knowledge, this is the first detailed report on several fitness parameters of *B. cinerea* anilinopyrimidine-resistant strains or any other fungal species that has developed resistance to anilinopyrimidine fungicides, suggesting the absence of an obvious fitness cost in the anilinopyrimidine-resistant strains of the pathogen. The absence of fitness penalty in the anilinopyrimidine-resistant strains can be supported by the fact that in most of the sensitivity determination studies carried out prior to the introduction of the anilinopyrimidines into the spray programs or in areas never treated with anilinopyrimidine fungicides, resistance frequencies were easily detectable ranging from 0.4 to 7% (3,9,24). Moreover, the absence of correlation between fitness and resistance to fungicides has been observed in several fungicide-pathogen systems (14,22,26,30) and such an absence supports the hypothesis stating that fitness differences between resistant and sensitive isolates may be due to differences in the genetic background rather than to a real fitness cost (22).

The results of the monitoring program carried out during 2005 to determine the fungicide sensitivity of *B. cinerea* populations on vegetable crops, in the island of Crete showed that there was a wide distribution of anilinopyrimidine resistant strains (25). Most of the anilinopyrimidine resistant strains were characterized as AniR2 or AniR3 isolates since they were not only resistant to anilinopyrimidines but they were simultaneously resistant to other classes of fungicides such as the benzimidazoles or the dicarboximides. However, there was also a significant fraction of AniR1 isolates representing the 20% of the population that were resistant only to anilinopyrimidines and characterized by high resistance factors. According to a long-term trial conducted by Forster and Staub (9), emergence of AniR1 isolates could be responsible for practical resistance development in the field. Obviously, this is the case in greenhouses in the Crete Island, Greece, where control failures have been observed recently, with anilinopyrimidine fungicides. However, it is not the case with *B. cinerea* populations on grape vines. According to the Fungicide Resistance Action Group (FRAC) reports, throughout Europe, there are pathogen strains with high resistance levels to anilinopyrimidine fungicides but their frequency remained stable while the performance of

TABLE 4. Competition between four pairs of anilinopyrimidine-resistant and -sensitive *Botrytis cinerea* isolates coinoculated on anilinopyrimidine-free artificial growth medium (in vitro) and on untreated cucumber seedlings (in vivo) with three different initial conidial ratios for five consecutive culture or disease cycles

Isolate pairs	Initial resistance frequency (%)	Final resistance frequency (%)	
		In vitro	In vivo
R1:S1 ^y	80	21.6 ^z	17.8*
	50	10.0*	0.0*
	20	0.0*	0.0*
R2:S2	80	97.5*	100.0*
	50	72.0*	77.6*
	20	48.7*	52.3*
R3:S3	80	76.2	95.7*
	50	52.5	73.3*
	20	18.0	52.8*
R4:S4	80	35.0*	42.5*
	50	23.3*	18.3*
	20	8.3*	10.7*

^y Isolates designated as R1, R2, R3, R4, S1, S2, S3, and S4 were the isolates BCR 12b, BCR 18a, BCR 13b, BCR 7e, BCS 9e, BCS 3, BCS 14d, and BCS 8e, respectively, listed in Table 1.

^z * Indicates resistance frequency significantly different from the initial one according to Student's *t* test at *P* = 0.05.

anilinopyrimidine fungicides in the field was not affected after more than 10 years of commercial use (FRAC webpage, online information). The results of the current study showed that the selected resistant isolates both in terms of predicted fitness components and in terms of realized fitness components were similar to the sensitive strains. The absence of a fitness cost in the anilinopyrimidine resistant strains could explain their emergence and evolution in the field. Fitness costs are more frequently associated with quantitative polygenic-controlled resistance than with qualitative monogenic-controlled resistance. It is well documented that resistance to anilinopyrimidine fungicides has a qualitative, disruptive pattern, and is monogenically controlled (2,4,11). In the past, development of resistance to benzimidazole fungicides in *Cercospora beticola*, representing a classical example of monogenic resistance, was followed by reports related to the fitness and the competitive ability of the resistant strains, suggesting that benzimidazole resistance would be stable (7,33). However, although with very low rate, the resistance frequency was significantly declined as has been reported after a sensitivity monitoring program carried out many years after the cessation of benomyl use (15). A similar decrease in the benomyl-resistance frequency was also observed during the past in *Mycosphaerella fijiensis* in banana, despite the predictions for a stable resistance frequency (35). Consequently, past experience has shown that despite the predictions for a stable and long-term duration of resistance, continuous monitoring of the population sensitivity is required to detect any change in the resistance frequency.

It is well known that fitness of the pathogen resistant strains has important implications to the disease management. The existence of fitness cost for the resistant strains could lead to a suggestion for alternations with chemicals of different mode of action, as a strategy to delay evolution of resistance. If development of resistance is not associated with fitness cost, other strategies such as use of mixtures, reduction in the application dose or in the number of spray applications, should be implemented. The results of this study showed that anilinopyrimidine-resistant strains have similar fitness characteristics to the anilinopyrimidine-sensitive strains and consequently the use of anilinopyrimidines in mixtures with chemically unrelated compounds rather than in alternation schemes should be suggested. However, it should be noted that the experiments presented in the current study were carried out under optimal infection and growth conditions, while remain unknown the fitness of the resistant strains under nonoptimal infection and growth conditions and the survival ability of the resistant strains, during the periods without crop in greenhouses. In addition, the population dynamics of the resistant and sensitive subpopulations with sensitivity measurements carried out early in the spray season and after its end should be investigated.

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