Use of Selenate-Resistant Strains as Markers for the Spread and Survival of *Botrytis cinerea* Under Greenhouse Conditions

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ABSTRACT

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Botrytis cinerea marked strains combining traits of fungicide resistance or sensitivity (carbendazim, iprodione) with resistance to selenate were created and assessed for use in studying the dispersal of *B. cinerea* and its survival inside plant tissue under greenhouse conditions. Marked strains differed in their ability to cause lesions and to disperse in the greenhouse. A strain that was the most aggressive in infecting plants was also the most successful in spreading across the greenhouse. Following 7 to 14 days of exposure to marked inoculum, about 90% of plants showed quiescent

B. cinerea infection with no significant difference between hosts or seasons. However, in a warm season, most of the plants were infected with wild-type *B. cinerea*, whereas most of the winter-recovered *B. cinerea* strains were of the marked phenotype, showing the importance of local inoculum from within the glasshouse in winter. The air of the greenhouse contained the same population of marked *B. cinerea* in warm and in cold periods, whereas the total population was significantly higher in summer. In the warm season, mycelium of *B. cinerea* inside plant debris lost viability within 3 to 4 months, whereas it stayed viable for 4 months in the winter (December to March) and started to lose viability in April.

Additional keywords: beans, gray mold, pepper, strawberry.

Botrytis cinerea, the anamorph of *Botryotinia fuckeliana*, attacks a wide range of plant species, causing gray mold on many economically important crops such as vegetables, ornamentals, herbs, bulbs, and fruits (11). Most genetic and population studies of *B. cinerea* have used mating type and fungicide resistance as markers (3,14), because auxotrophic markers are difficult to obtain and morphological markers are frequently unstable (17). Resistance to fungicides is widely spread in *B. cinerea* populations in Israel (12), which renders resistance markers useless for many population and epidemiological studies. Mating type also has a limited application in population monitoring because sexual structures are rare in natural conditions and rather difficult to obtain in the laboratory (13). To cope with these limitations, the toxic analogs of common nutrients, such as nitrates or sulfates, were proposed for obtaining nutritional markers suitable for population, epidemiological, and ecological studies of *B. cinerea* (37). Chlorate, a nitrate analog, is reduced by nitrate reductase to hypochlorite, which is toxic to the fungal cell. Chlorate-resistant, nitrate-nonutilizing (*nit*) mutants of numerous fungi, including *Botrytis* (4), *Colletotrichum* (22), *Fusarium* (7), and *Verticillium* (24), have been reported and have been usually used for studying vegetative compatibility. Several previous studies have exploited *nit* mutants as markers in greenhouse and field experiments, mostly concerning the survival and pathogenicity of the *nit* mutants which were similar to or somewhat less than those of the wild-type parents (18,32,38). *nit* mutants were successfully used for testing survival of nonpathogenic strains of *F. fujikuroi* and *F. oxysporum* in rakkyo (*Allium chinense*) plants and their colonization of roots (21); for studying population dynamics of *F. oxysporum* f. sp. *spinaciae* after different soil treatments (33); for

quantification of root and stem colonization of watermelon by *F. oxysporum* f. sp. *niveum* (41); and for characterization of spatial and temporal dispersal of *F. oxysporum* f. sp. *radicis-lycopersici* through plants and soil (29). *nit* mutants of *B. cinerea* were used for studying the spread of internal latent infection through primula plants (2) and for testing the influence of flower-feeding thrips on *Botrytis* bunch rot in grapes (26).

Selenate, a toxic analog of inorganic sulfate, is inhibitory in many biological systems, and resistant mutants with altered sulfate transport and assimilation have been characterized in several fungal genera, including *Acremonium* (34), *Aspergillus* (1,16), *Botrytis* (37), *Fusarium* (8), *Magnaporthe* (19), and *Neurospora* (27). Information about the use of selenate-resistant mutants in ecological studies is limited. In greenhouse tests, selenate-resistant mutants of *M. grisea* were pathogenic on rice and indistinguishable from the corresponding wild type in their virulence (19), and the pathogenicity of selenate-resistant mutants of *B. cinerea* on bean was similar to that of the parent strains (37). To our knowledge, marked strains that combine the trait of resistance to selenate (or chlorate) with any other trait have not been constructed previously. Such double marked strains could widen the scope for exploiting marked strains in ecological/epidemiological studies by using, for example, two or more different selenate resistance strains in the same experiment.

The objectives of the present study were (i) to develop *B. cinerea* marked strains combining traits of resistance to selenate with resistance or sensitivity to fungicides and (ii) to test the use of the marked strains in epidemiological and ecological greenhouse studies. To achieve that aim, (i) a selection of field strains of *B. cinerea* was characterized with respect to resistance/sensitivity to benzimidazole and dicarboximide fungicides; (ii) selenate-resistant, sulfate-nonutilizing mutants were recovered from *B. cinerea* isolates with different resistance/sensitivity to fungicides and characterized; and (iii) the spread and survival of marked *B. cinerea* inoculum in a greenhouse under different climatic conditions was evaluated.

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MATERIALS AND METHODS

Media. Minimal medium (MM) comprised of modified Vogel's medium N (35), which contained (g/liter) $\text{Na}_3\text{ citrate} \times 2\text{H}_2\text{O}$, 2.5; KH_2PO_4 , 3.0; NH_4NO_3 , 0.1; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.2; $\text{KCl} \times 2\text{H}_2\text{O}$, 0.1; sucrose, 15; agar, 20; biotin (at 0.1 mg/ml), 50 μl ; and trace element solution, 5 ml. The medium was prepared according to Vogel's protocol. MM and MM amended with sodium selenate (1 g/liter) and taurine (100 mg/liter) were used to recover and subculture selenate-resistant (*sel*) mutants; MM amended with L-methionine (0.5 g/liter) was used for partial phenotyping of *sel* mutants (19); and MM supplemented with sorbose (100 mg/liter) or Triton 100 (0.5 ml/liter) was used to grow slow compact colonies of *B. cinerea* (17).

Potato dextrose agar (PDA; Difco Laboratory, Detroit) was used for routine culturing, for comparison between the growth of parental strains and *sul* mutants, and for preparing inoculum for pathogenicity tests.

A defined MM derived from the Difco Czapek-Dox agar (CDA) recipe by Yourman and Jeffers (39) and CDA amended with fungicides and/or sodium selenate (1 g/liter) were used to test parental strains and *sul* mutants for resistance to fungicides. This medium contained (g/liter) NaNO_3 , 3; KH_2PO_4 , 1; K_2HPO_4 , 1; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.5; KCl , 0.5; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 0.01; agar, 15; and glucose, 10. The final pH was 6.6. CDA supplemented with Triton 100 (0.5 ml/liter) was used to grow slow compact colonies of *B. cinerea*.

Botrytis selective media (BSM) (10) and BSM amended with selenate (1 g/liter) were used for re-isolating wild-type and *sul* mutants from infected plants. BSM contained (g/liter) NaNO_3 , 0.1; K_2HPO_4 , 0.1; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.2; KCl , 0.1; chloramphenicol, 0.2; pentachloronitrobenzene, 0.02; Maneb 80, 0.02; rose bengal, 0.05; tannic acid, 5; glucose, 2; and agar, 20. The pH was adjusted to 4.5 with 1 M NaOH prior to addition of the agar.

Botrytis spore trap medium (BSTM) and BSTM amended with selenate (1 g/liter) were used for trapping wild-type and *sul* mutants from the air. BSTM differed from BSM in that Rubigan (12% fenarimol at 0.1 ml/liter) replaced rose bengal (10).

Isolates. Twenty-two *B. cinerea* isolates were used in this study. Strain B4 originated from France; all other strains originated from 10 locations in Israel (fields and greenhouses) and were isolated during 1997 and 1998. The collection was stored in glass tubes with PDA at -20°C . One monoconidial culture was prepared from each isolate and stored on PDA at 4°C during this study.

Fungicide resistance. Carbendazim (MBC; Delsene 50DF, E.I. DuPont de Nemours & Co., Wilmington, DE) and iprodione (Rovral 50 WP, Rhone-Poulenc, Lyon, France) were used to test fungicide resistance in *Botrytis* isolates. Appropriate volumes of stock fungicide suspensions were added to molten CDA to give final active ingredient concentrations of 0.5, 1.0, 5.0, 50, and 500 $\mu\text{g/ml}$. Mycelial plugs, 5 mm in diameter, taken from the leading edge of 5-day-old colonies on PDA were placed in the center of 9-cm-diameter plates containing amended medium, or unamended medium as a control. Cultures were incubated at 20°C in the dark. The diameters of colonies were measured on the fourth day and the effective concentrations that inhibited mycelium growth by 50% (EC_{50} growth) were calculated. The growth assay was conducted twice.

Selenate-resistant sulfate-nonutilizing (*sul*) mutants. *sul* mutants (19) were generated essentially according to Weeds et al. (37). Plugs were taken from the growing edge of 3- to 5-day-old colonies on PDA and placed on MM amended with sodium selenate (1 g/liter) and taurine (100 mg/liter) at 6 to 10 points in 9-cm-diameter plates and incubated at 20°C . Selenate-resistant sectors that emerged from the restricted colonies were evident after 2 to 4 weeks; they were transferred to fresh selenate medium for purification and then to MM. The ability of selenate-resistant

mutants to utilize sulfur was determined on MM and MM amended with L-methionine (0.5 g/liter). Sectors that grew as thin expansive colonies with little or no aerial mycelium on MM, but similarly to the wild type on L-methionine-amended MM were considered to be sulfate-nonutilizing (*sul*) mutants. *B. cinerea* strains that failed to produce selenate-resistant mutants spontaneously in the course of several attempts were subjected to UV irradiation (UV-C 254nm, Vilber Lourmat, France) to enhance the yield of mutants. Slow compact colonies of *Botrytis* that developed on MM supplemented with sorbose or Triton 100 were counted and the UV dosage was adjusted to kill 95% of the conidia. The irradiated conidia were then spread on MM amended with sodium selenate at 1 g/liter. After 10 to 14 days, growing colonies were transferred to fresh selenate medium to confirm their resistance, and then to MM.

Sensitivity of *sul* mutants to selenate and agricultural fungicides. The in vitro selenate sensitivity of the *sul* mutants' mycelial growth was estimated on MM amended with taurine (100 mg/liter), to which aqueous solutions of sodium selenate were added. The response to selenate was determined with respect to retardation of radial growth rate at a series of concentrations (0.01, 0.1, 1.0, and 10.0 mg of selenate per ml of MM). Mycelial plugs taken from the edge of actively growing cultures were centrally inoculated on 9-cm-diameter selenate-amended plates and incubated in triplicates at 20°C . Colony diameters were recorded 7, 14, and 21 days after inoculation, and the area under the growth curve (AUGC) was calculated with a formula for the area under disease progress curve (5). The responses of *sul* mutants to fungicides were tested by assessing conidial germination on CDA and on CDA amended with sodium selenate and fungicides (carbendazim at 50 μg a.i./liter and/or iprodione at 5 μg a.i./liter). Suspensions of conidia were dispersed onto fungicide-amended media and onto unamended media, with all media containing Triton 100 at 5 ml/liter (100 conidia per plate, three 9-cm-diameter plates for each strain $\times$ medium combination). The plates were incubated at 20°C in the dark for 1 week. The germination rates were determined by counting the number of colonies.

Growth of parental strains and *sul* mutants. The yield of conidia (conidiation) and the colony diameters of parental isolates and *sul* mutants were determined on PDA. Conidiation was defined as the number of conidia washed from a 14-day-old colony and counted with a hemacytometer. Colony diameter was measured on the fourth day at 20°C . All measurements were carried out on two replicates of three plates. Germination rates were determined on modified CDA amended with Triton 100.

Plants. Bean (*Phaseolus vulgaris* cv. Hilda), pepper (*Capsicum annuum* cv. Yoel), and strawberry (*Fragaria ananassa* cv. 328) plants, 5 to 7 weeks old, were used throughout the study. The plants were grown in 1-liter pots containing a 1:1 (vol/vol) mixture of peat and volcanic gravel. They were irrigated every 1 to 2 days and proportionally fertilized with 0.0125:0.0075:0.02% NPK.

Pathogenicity tests. Parental strains and *sul* mutants were compared for their pathogenicity by inoculating mature intact pepper plants with mycelial disks. Mycelial plugs of 5 mm diameter, cut from the actively growing edges of 7-day-old colonies on PDA, were placed on the upper surface of 10 to 15 intact leaves per plant (one plug per leaf and three plants per strain). The pots with inoculated plants were placed in two transparent polypropylene bags and incubated in a glasshouse with mean day/night temperatures during the experiment of $22.8/20.3^\circ\text{C}$, respectively, and the relative humidity inside the bags close to 100%. The diameters of the developing disease lesions were measured 7 days after inoculation.

Spreading of marked strains in the glasshouse. The potential of marked strains of *B. cinerea* as indicators of the spread of the pathogen from an inoculum source was assessed in a glasshouse

(7 × 20 m) with uncontrolled temperature and humidity (no air conditioning), and with free access of the outdoor air via open vents on two sides. The glasshouse was painted with white-wash to protect plants from direct sunlight. Three experiments were conducted during a warm (March to June) and a cold (January to February) season, with bean, pepper, and strawberry as the target plants. The warm experiment with pepper plants lasted from 10 March to 19 April 2004 (experiment I) and that with bean plants from 28 April to 6 June 2004 (experiment II); and the cold experiment with bean and strawberry plants lasted from 11 January to 20 February 2005 (experiment III) (Table 1). A data logger (Onset Computer, Bourne, MA) was placed at plant level to record the temperature and humidity inside the glasshouse. Data were recorded hourly.

In order to provide a source of marked inoculum, mature pepper (one plant) or bean plants (three to five plants) in 1-liter pots were inoculated as described above with *B. cinerea* strains of various fungicide and selenate resistance phenotypes. The inoculated plants were incubated in the greenhouse under conditions favorable for developing *B. cinerea*, for 14 days as described above, to allow the formation of sporulating lesions. At the end of this period, *B. cinerea* lesions covered the entire surface of most leaves of source plants. The plants, still enclosed within polypropylene bags, were transferred to the experimental glasshouse and placed as a group of 20 pots in the center of the bench. The polypropylene bags were then removed and the plants were left in place until the end of the experiment (40 days). These source plants acted as a focus of inoculum for six groups of 30 to 33 pots with healthy target plants that were positioned at increasing distance from the inoculum source. The nearest targets were placed adjacent to the source plants on the same bench (=0 m). The remaining target groups within the greenhouse were placed on a series of five benches that were 3 m apart, providing distances from the inoculum source(s) of 3, 6, 9, and 12 m. In all three experiments, source plants inoculated with strain B21Sul4 were used as the source of inoculum of phenotype BenS DicR SelR. In experiment III, additional marked strains B13Sul2 (BenS DicS SelR), B15Sul15 (BenR DicR SelR), and B33Sul5 (BenR DicS SelR) were used, with the source plants of each strain being positioned on different benches. The source plants of strain B33Sul5 were positioned outside the glasshouse, 1 m from the open vent of the glasshouse, to allow the exchange of inoculum in and out of the glasshouse to be determined. No target plants were placed outside, and spread of marked inoculum out of the glasshouse was tested by trapping marked strains from the air on selective media (described below), whereas spread of marked inoculum inside the glasshouse and from outside to inside was tested by both recovery of marked strains from target plants and trapping from the air.

Spreading of inoculum was examined 7, 14, 21, and 40 days after infestation. There were no spreading lesions on target plants,

although small necrotic spots were sometimes observed. To visualize latent and quiescent infections, three or four pots were randomly selected from each group of target plants at each time point (72 to 96 pots per experiment) and exposed to high humidity at 20 to 23°C for 10 days. By that time, most plants were covered with *B. cinerea* spreading lesions. Hereafter, the term “infection” refers to quiescent and latent infection revealed by maintenance under these conditions. The phenotype of these infections was determined from three samples of mycelium from different plants or different plant parts in each pot that were transferred to plain or selenate-amended BSM using sterile loops. Representatives of apparently selenate-resistant strains were tested on fungicide-amended CDA to confirm the resistant phenotype.

Trapping of marked strains from the air. Colony forming units (CFUs) of *B. cinerea* were trapped from the air of the glasshouse and outside during “spreading” experiments II and III (Table 1). Trapping was first done before introducing the sources of infection, and repeated 1, 3, 7, 14, 21, and 40 days afterwards. After 40 days, the source of inoculum was removed from the glasshouse and a final trapping was made 20 days later, i.e., 60 days after the start of the experiment. The CFUs were trapped in 9-cm-diameter plates containing plain or selenate-amended BSTM, which were exposed near midday, when the concentration of conidia reaches a peak (15). Five plates of each medium were put on each bench inside the glasshouse and on the bench outside near the vent of the glasshouse, and were left open for 1 h, after which they were closed and incubated for colony counting as described previously. Colonies of trapped selenate-resistant *B. cinerea* were further phenotyped on CDA amended with carbendazim and/or iprodione.

Survival in plant tissues. Survival of *B. cinerea* was tested in the debris of mature pepper plants. The plants were grown and inoculated as described above, with parental isolates of various phenotypes and *sul* mutants derived from them (Table 2). Fourteen days after inoculation, pots with diseased plants covered with spreading *B. cinerea* lesions were transferred to the screen house; the polypropylene bags were perforated and the plants were allowed to dry. A hygrothermograph was placed at plant level to record the temperature and humidity inside the screen house. Each month, fragments of plant debris were taken and placed in 9-cm-diameter plates that contained plain or selenate-amended BSM. There were five to seven fragments per plate, and three plates were used for each strain × media combination. The plates with fragments were kept at 20°C in the dark until mycelium of *B. cinerea* was evident, after 7 to 10 days. The developed mycelium was then subcultured on plain and fungicide-amended CDA to confirm the strain phenotype. The viability percentages of parental *B. cinerea* and of *sul* mutants were calculated and compared. The warm season experiment (experiment I) lasted from 21 March 2004 to 21 August 2004. The cold season experiment (experiment II), which actually continued into the

TABLE 1. Microclimate conditions (temperature [T, °C] and relative humidity [RH, %]) during experiments

Experiment	Host	Month/Year	Day time	T _{min}	T _{max}	T _{mean}	RH _{min}	RH _{max}	RH _{mean}
Spreading exp.									
I	Pepper	Mar. 2004 – Apr. 2004	Day	9.4	40.6	24.3	8.4	96.6	49.0
			Night	6.2	24.8	13.7	21.2	95.0	81.1
II	Bean	May 2004 – Jun. 2004	Day	17.9	40.1	26.7	9.1	82.8	50.0
			Night	12.6	26.0	18.1	23.8	95.0	82.5
III	Bean/strawberry	Jan. 2005 – Feb. 2005	Day	7.4	37.0	19.6	16.1	96.0	55.2
			Night	5.4	19.0	11.9	41.5	97.5	77.6
Survival exp.									
I	Pepper	Mar. 2004 – Aug. 2004	Day	7.8	41.5	27.6	7.4	97.8	50.1
			Night	6.2	29.9	18.7	17.3	99.2	83.8
II	Pepper	Nov. 2004 – Mar. 2005	Day	7.4	37.0	19.6	16.1	96.0	55.3
			Night	5.4	19.0	11.9	41.5	97.5	77.6
		Apr. 2005	Day	14.8	42.7	26.9	8.8	82.7	40.6
			Night	10.6	28.7	18.4	18.7	87.1	63.3

warm season, lasted from 28 November 2004 to 1 May 2005 (Table 1).

Data analysis. Effective concentration values for mycelium growth inhibition (EC_{50} growth) were calculated by fitting a dose-response function to a logistic model by means of inverse prediction option. To compare different media, strains, and mutants, pairs of means were subjected to contrast *t* tests. All tests were performed at $P \leq 0.05$ using the JMP 4.0.0 software (SAS Institute, Cary, NC).

RESULTS

Resistance to fungicides. Out of 22 *B. cinerea* field isolates, 10 were sensitive to both benzimidazoles (carbendazim) and dicarboximides (iprodione), five were resistant to both fungicides, six were resistant to benzimidazoles only, and one isolate was sensitive to benzimidazoles and resistant to dicarboximides. The mean EC_{50} (growth) values for resistance and sensitivity to carbendazim were 267.3 and 0.25 $\mu\text{g/ml}$, respectively, and those for resistance and sensitivity to iprodione were 23.0 and 1.7 $\mu\text{g/ml}$, respectively.

Selection of selenate-resistant mutants. Field isolates did not grow on MM amended with sodium selenate at 1 g/liter, and fast-growing sectors emerging from the inoculum plugs were considered to be selenate-resistant (*sel*) mutants (Fig. 1, left). The sector production rates after 28 days ranged from 0.01 to 0.14 sectors per colony. About 80% of the *sel* mutants grew as thin expansive colonies with little or no aerial mycelium on MM with sulfate as the sole sulfur source, and these were considered to be sulfate-nonutilizing (*sul*) mutants. All *sul* mutants grew normally on L-methionine-amended MM (Fig. 1, right). Two isolates (B15 and B33) failed to produce selenate-resistant sectors spontaneously

in the course of several attempts and were subjected to UV-irradiation, which strongly enhanced the yield of *sel* mutants. More than 500 resistant colonies were recovered in strain B15, which exhibited a mutation rate of 1 in 4.7×10^4 viable conidia, compared with only 11 in strain B33, with a mutation rate of 1 in 3.7×10^6 viable conidia, in a similar experiment. Most of resistant mutants recovered from both isolates did not form thin expansive colonies on MM, which showed them to be selenate-resistant (*sel*) but not sulfate-nonutilizing (*sul*).

Growth and pathogenicity of sulfate-nonutilizing mutants. *sul* mutants showed high resistance to selenate (Fig. 2), which

Fig. 2. Radial growth of parental strains (B3, B4) and *sul* mutants of *Botrytis cinerea* on minimal medium amended with taurine at 0.1 g/liter and different concentrations of sodium selenate. AUGC = area under growth curve; 15 days at 20°C. Vertical bars represent standard errors of the means ($P \leq 0.05$).

TABLE 2. Growth, conidiation, and conidial germination on agar media, and lesion size on pepper plants of parental isolates and *sul* mutants of *Botrytis cinerea*^a

Strain	Phenotype	Conidia per colony	Germination of conidia (%)	Colony diameter (mm)	Mean lesion diameter on pepper leaves (mm), in replications		
					I	II	III
B3	BenR DicR SelS	3.2×10^7 ab	71.8 a	51.3 b	68.2 a	69.1 a	71.5 a
B3Sul3	BenR DicR SelR	1.0×10^7 c	32.4 c	55.3 ab	27.1 b	21.7 b	10.0 c
B15	BenR DicR SelS	4.2×10^7 a	58.2 b	49.4 b	66.4 a	75.8 a	41.4 ab
B15Sul15*	BenR DicR SelR	2.5×10^7 b	61.6 ab	40.6 b	43.1 ab	25.4 b	32.8 b
B21	BenS DicR SelS	2.8×10^7 ab	73.2 a	68.0 a	66.7 a	46.7 ab	45.3 ab
B21Sul4	BenS DicR SelR	2.3×10^7 b	62.6 ab	61.5 a	69.4 a	41.9 ab	65.0 a
B33	BenR DicS SelS	1.8×10^7 bc	61.6 ab	46.7 b	63.4 a	36.8 b	27.8 b
B33Sul5*	BenR DicS SelR	1.2×10^7 c	56.3 b	47.6 b	23.5 b	12.1 c	13.9 c
B13	BenS DicS SelS	1.7×10^7 bc	33.8 c	70.7 a	60.7 a	57.4 a	21.7 b
B13Sul2	BenS DicS SelR	1.0×10^7 c	34.0 c	42.9 b	22.2 b	17.8 c	13.9 c

^a Mutants marked with asterisk were UV-induced, otherwise they are spontaneous. In each column, numbers followed by the same letter are not significantly different according to the contrast *t* test ($P \leq 0.05$).

Fig. 1. Induction and identification of selenate-resistant sulfate-nonutilizing (*sul*) mutants of *Botrytis cinerea*. Left: Spontaneous fast-growing sector arising from a restricted colony on minimal medium (MM) amended with sodium selenate at 1 g/liter (isolate B18, 14 days of incubation at 20°C in 9-cm-diameter plate). Right: Thin growth of the *sul* mutant on MM (left plate) and thick growth on MM amended with L-methionine at 0.5 g/liter (mutant B18Sul1, 10 days after inoculation, 5-cm-diameter plates).

was maintained through at least 10 passages on selenate-free complete medium (PDA). Depending on the resistance or sensitivity of the parental strain to benzimidazole and dicarboximide fungicides, four different selenate-resistant phenotypes (BenR DicR SelR, BenR DicS SelR, BenS DicR SelR, and BenS DicS SelR) were recovered that could be clearly differentiated on CDA amended with selenate, carbendazim, and iprodione in various combinations (Fig. 3). Conidiation, germination, and radial growth of *sul* mutants on agar media were similar to or sometimes reduced compared with those of the parental strains. Among five mutants tested, only B21Sul4 showed the same level of pathogenicity as the parental strain (Table 2).

Trapping *sul* markers from the air. Temperature and humidity conditions in the glasshouse are given in Table 1. No marked strains were found in the glasshouse air prior to establishing the sources of inoculum. During 40 days after infestation, the mean concentration of the marked strain, B21Sul4, in the air was the same in the warm and in the cool season, at 0.5 CFU per

plate, although the proportions of marked and unmarked CFU differed significantly (Fig. 4). In the warm season, the marked CFU trapped during the first 14 days after infestation amounted to 18% of all the trapped *B. cinerea*, whereas in winter, about 90% of the trapped *B. cinerea* CFU were of the marked phenotype. These percentages changed to 10 and 63%, respectively, 40 days after infestation. No marked *B. cinerea* was found in the air of the glasshouse 2 months after infestation in the warm season, but it was still present in the air at a similar time point in winter, albeit at a relatively low level (3% of total *B. cinerea*). All the marked strains that were used as sources of inoculum in experiment III were trapped from the air inside the glasshouse albeit in differing proportions. Strain B21Sul4 was the most frequent phenotype, accounting for 57% of total *sel*, B13Sul2 was the next at 33%, whereas B15Sul15 and B33Sul5 each contributed only about 5%. Exchange of inoculum in and out of the glasshouse was evident because (i) colonies of marked strain B33Sul5, which had been established outside, were trapped inside the glasshouse, and (ii) a few colonies of other marked strains that were established inside were trapped outside at a distance of about 1 m from the open vent of the glasshouse.

Spreading of marked strains across the plants. Strain B21Sul4 (BenS DicR SelR) was the most aggressive in pathogenicity tests (Table 2) and was used as a marked strain in greenhouse experiments for testing temporal and spatial spreading across the target plants. Following exposure to inoculum of this strain, about 90% of the plants showed quiescent or latent *B. cinerea* infection, with no significant difference between seasons. However, in the warm season, most plants were infected by wild-type *B. cinerea*, with only about 20% infected by the marked strain. In contrast, in the cold period, most infected plants were infected by the marked strain (Table 3). The spreading of the marked strain was fast. Seven days after introduction of an inoculum source, the mean number of plants infected with the marked strain almost reached the maximum for the given experiment (24% in warm and 80% in cold season) and did not change further during the subsequent 3 weeks. Only on strawberry, in experiment III, was the maximum infection level reached later (2 to 3 weeks after infestation). Some decrease in the disease incidence caused by the marked strain was often observed toward the end of the 40-day period, or on the benches farthest from the source of infection (Table 4).

Additional marked strains B13Sul2 (BenS DicS SelR), B15Sul15 (BenR DicR SelR), and B33Sul5 (BenR DicS SelR)

Fig. 3. Growth of *Botrytis cinerea* parental strains and *sul* mutants on Czapek-Dox agar (CDA) and CDA amended with selenate and fungicides. From left to right, top row, parental isolates on CDA, CDA plus carbendazim (crb), CDA plus iprodione (ipr), and CDA plus selenate (sln); and bottom row, *sul* mutant from the above isolate on CDA plus selenate, CDA plus selenate plus carbendazim, and CDA plus selenate plus iprodione. A, Isolate B15 (BenR DicR). B, Isolate B21 (BenS DicR). C, Isolate B33 (BenR DicS). D, Isolate B31 (BenS DicS). Images were taken on 9-cm-diameter plates 10 days after inoculation. Triton100 was added to the medium at 0.5 ml/liter to restrict colonial growth.

Fig. 4. Relative densities of total *Botrytis cinerea* viable conidia (Bc total) and of the marked strain B21Sul4 (Bc sul) in the glasshouse air during warm and cold seasons. At each time point, bars indicated with asterisks are significantly different from others according to the contrast *t* test ($P \leq 0.05$).

were used in experiment III (cold season) for studying the contribution of different sources of inoculum in the infection of target plants and the penetration of *Botrytis* from outside into the glasshouse. In total, 348 among 538 (65%) *B. cinerea* isolates recovered from the target plants were selenate-resistant, and about 80% of these had the DicR SelR phenotype of marked strain B21Sul4. The remaining three marked phenotypes were less frequent, accounting for 1.5 to 17% on the average (Table 5). Marked strains B21Sul4 and B13Sul2 were more frequently recovered from plants on the bench with the source of inoculum or from plants on the neighboring benches, although the strains were dispersed across the whole glasshouse area. Dispersal of strain

TABLE 3. Proportions of plants infected with wild type or marked^y *Botrytis cinerea* in glasshouse experiments

Experiment	Season	Host	Infected plants ^z	
			Total (%)	Marked (% of total)
I	Warm	Pepper	88.4 a	24.1 b
II	Warm	Bean	88.3 a	23.9 b
III	Cold	Bean	92.5 a	80.5 a
III	Cold	Strawberry	71.2 b	71.1 a

^y Strain B21Sul4 of the phenotype BenS DicR SelR was used.

^z Plants infected with the marked strain were placed as a source of infection among healthy plants, and the spread of the marked strain across the target plants was monitored. Quiescent infection on target plants was expressed by exposure of plants to high humidity at 20°C. In each column, numbers followed by the same letter are not significantly different according to the contrast *t* test ($P \leq 0.05$).

TABLE 4. Temporal and spatial spreading of *Botrytis cinerea* marked strain B21Sul4 (BenS DicR SelR) through plants in the glasshouse

Host and season	Days after inoculation	Proportions of plants infected with marked strain at various distances from the source of inoculum (m) ^z				
		0	3	6	9	12
Pepper, warm	7	+	+	+	+	–
	14	+	+	+	+	+
	21	+	+	+	+	–
	40	+	+	+	–	–
Bean, warm	7	+	+	+	+	+
	14	+	+	+	+	+
	21	+	+	+	+	+
	40	+	+	–	+	–
Bean, cold	7	+++	+++	+++	+++	++
	14	+++	+++	++	+++	+++
	21	+++	+++	+++	+	+++
	40	++	++	+	+	++
Strawberry, cold	7	+	+	+	+	+
	14	+++	++	++	+	+
	21	+++	+++	+++	+++	++
	40	+++	+++	++	+	+

^z – = no infected plants; + = less than 45% of plants infected; ++ = 46 to 75% infected; and +++ = more than 75% infected.

B15Sul15 was random. The number of plants infected by any given phenotype generally matched the mean frequencies of the respective phenotypes in the air. Marked strain B33Sul5, positioned outside the glasshouse, was recovered at a low proportion from plants on the benches near the vent (Table 5).

Survival of wild type and marked strains in plant debris. The temperature and humidity inside the screen house are given in Table 1. In the warm season experiment, the temperature inside the screen house was above 30°C during 38.9% of daylight hours. The daily maximum temperature was above 40°C during May to July (during 0.3% of daylight hours). The relative humidity exceeded 80% every night, and the daily minimum relative humidity was between 7 and 40% when air temperatures reached their maximum. Under such conditions, *sul* survived in plant debris for 90 to 120 days. Strain B21Sul4 was recovered from plant debris after 150 days, although the frequency of recovery decreased from about 90% in May to about 30% in August. In the cold season experiment (December to March), the mean daily temperature was 19.6°C and the maximum temperature did not exceed 37°C; temperatures above 30°C were recorded during only 4.2% of daylight hours. Under these conditions, the viability of *sul* mutants in plant debris did not decrease during 120 days, and significant reduction was evident only from April, when temperatures above 30°C were recorded during 30% of daylight hours and maximal temperatures exceeded 40°C (Fig. 5). Selenate-sensitive *B. cinerea* isolates were recovered from plant debris at all times, with no decrease during the experiments.

DISCUSSION

Resistance to benzimidazoles and dicarboximides was common among *B. cinerea* field or greenhouse isolates in Israel, confirming previous findings (12). Benzimidazole-resistant isolates exhibited resistance levels (RL = EC₅₀ resistant/EC₅₀ sensitive) greater than 1,000, whereas dicarboximide-resistant strains showed a moderate resistance level of about 10. Such moderate resistance to dicarboximides, which is associated with either slight or no osmotic sensitivity, is common under field conditions, whereas highly resistant laboratory strains are osmotically sensitive (25). This finding demonstrates one of the advantages of using natural rather than laboratory-induced fungicide-resistant isolates as marked strains (natural mutants that have already been exposed to selection pressure should be more viable in field conditions). Four different phenotypes were found: resistant to both fungicides, resistant to one or the other, and sensitive to both. Representatives of each phenotype were used to create selenate-resistant mutants that combined the traits of fungicide resistance and selenate resistance. Traits of fungicide resistance or sensitivity enable distinction between different selenate-resistant mutants and therefore facilitate the use of more than one selenate-resistant strain in the same experiment, for example, for studying the spread of *B. cinerea* from inside to outside the greenhouse and vice versa.

TABLE 5. Comparative dispersal of different marked strains of *Botrytis cinerea* across the plants in the greenhouse

Strain	Phenotype	Number of plants (%) infected with different marked phenotypes on benches ^y						
		N1	N2	N3	N4	N5	N6	Mean
B21Sul4	BenS DicR SelR	88.9	81.6	69.7	78.3	77.3	76.2	78.7 a ^z
B13Sul2	BenS DicS SelR	11.1	7.9	27.3	17.4	18.2	19.0	16.8 b
B15Sul15	BenR DicR SelR	0	10.5	3.0	0	0	4.8	3.0 c
B33Sul5	BenR DicS SelR	0	0	0	4.3	4.5	0	1.5 c
N7								

^y Benches N1 to N6 were arranged in a row and consecutively numbered N1 to N6. Benches N4 and N5 were closest to the vent, while bench N7 was located outside the glasshouse close to this vent. Sources of inoculum (shaded area) of a given phenotype were established on benches N2, N3, and N6 inside the glasshouse; a source of inoculum B33Sul5 was established on bench N7 outside.

^z Numbers identified by different letters are significantly different according to the contrast *t* test ($P \leq 0.05$).

sul mutants of wild type and fungicide-resistant *B. cinerea* were highly resistant to selenate, with an EC₅₀ (growth) value often exceeding 10 g/liter, and were stable in passages on PDA. They combined resistance to selenate with their parental resistance or sensitivity to fungicides, and thus provided four different marked phenotypes. On MM amended with selenate and with taurine as a reduced-sulfur source, the spontaneous recovery rate of selenate-resistant mutants was between 0.01 and 0.14 sectors per colony. In *M. grisea*, the mean sectoring frequency ranged from 0 to 0.5 depending on strain and media. Significantly more *sul* mutants were recovered on media with sulfur-containing compounds, and no significant differences in mycelial dry weight were observed between the *sul* mutants and their corresponding wild types when grown in liquid media containing either L-methionine or taurine (19). Similarly, no difference in proliferation in liquid medium was observed between *nit* mutants of *F. oxysporum* and their wild-type parents, although some *nit* mutants showed slower growth on potato sucrose medium (32). In the present study, some *B. cinerea sul* mutants grew slower than the parent strains and produced fewer conidia on undefined full medium (PDA); most of them also formed slower spreading lesions on pepper leaves. In previous studies, the pathogenicity or survival of nutritional mutants was often observed to be less than that of their wild-type parents. Similarly, only one of six chlorate-resistant strains of *F. oxysporum* f. sp. *niveum* resembled the wild-type progenitor isolate in its pathogenicity and growth in soil and on PDA, and was therefore used in subsequent greenhouse and field experiments (41). *nit* mutants of *C. gloeosporioides* showed decreased competitiveness compared with their wild-type parent, which was associated with a decrease in infectivity, longer latent period, reduced lesion size, and reduced sporulation (38). On the other hand, selenate-resistant mutants of *B. cinerea* were highly pathogenic on leaves of uprooted bean plants (37). Virulence of *M. grisea* also was found to be unaffected by the *sul* mutation when tested by spraying plants with a conidial suspension (19). The difference in results is as likely to be related to the method of pathogenicity estimation as to variations in the tested mutants. In previous studies, more than one class of *sul* mutants was found in *B. cinerea*, differing, among other characteristics, in resistance to chromate (23,37). It should be noticed also, that two of the five *sul* mutants tested for cultural and pathogenic properties in the present study were induced by UV irradiation, and their virulence could be affected by undefined mutations. A spontaneous *sul* mutant (B21Sul4) that carried an additional marker of resistance to dicarboximides was the most pathogenic and was therefore chosen as the principal marked strain in glasshouse experiments to study the spread of marked inoculum in the air and through plants.

B. cinerea conidia, which are generally considered to be the most important dispersal propagules of *B. cinerea* (20), were trapped from the air of the glasshouse on selective media. The dispersal of marked conidia was very fast. Three days after establishment of inoculum, the concentration of marked CFU in the air reached its maximum; it then fell to zero in summer or near to zero in winter during the following 60 days. Similarly, the rapid evolution of the *B. cinerea* population structure following introduction of marked isolates was shown in a tomato greenhouse in France: within 14 days, simple sequence repeat profiles identical to those of the introduced strains accounted for 63% of the characterized conidia found in the air of the greenhouse (9). The mean densities of marked conidia in the air were similar in the warm and cold seasons, but the amount of wild-type *B. cinerea* was significantly higher in summer (up to tenfold). The proportion of marked *B. cinerea* in the air of the greenhouse matched that observed in plant infections. Only 24% of plants with quiescent infection showed the marked infection in summer, whereas in winter, this proportion reached 80%. The contributions of the marked phenotype to the airborne population

in those periods were about 20 and 90%, respectively. This indicates that local sources of infection from within the glasshouse could be especially harmful in the cold season, when the spore density in the air is relatively low and thus limits the incidence of the disease. These results highlight the particular importance of sanitation in cold period, even though sanitation measures should always be applied (40).

Of the four marked strains used as the sources of infection in experiment III, B21Sul4 was the most widespread in the glasshouse (it was re-isolated from 79% of plants) and, similar to its parental strain, formed the largest lesions on leaves when tested for pathogenicity. Both the parental isolate and the marked strain B21Sul4 formed fast-growing colonies on agar media, and these colonies produced large amounts of well germinating conidia. The other strains were recovered from plants in the glasshouse less frequently (3 to 17%), and they were less productive on artificial media and less pathogenic in greenhouse test.

Conidia are generally regarded as short-lived propagules whose survival on plant surfaces is largely determined by temperature extremes, moisture availability, and sunlight exposure (20). *B. cinerea* conidia were able to survive on the surface of kiwifruit, remaining viable and infectious throughout the growing season (36). On the surface of pears, the viability of *B. cinerea* conidia after 7 weeks had declined to support only a 10% germination rate (31). When *B. cinerea* conidia were exposed to direct sunlight at midday in an Israeli summer, their survival time was only minutes (30). In our present study in a glasshouse with uncontrolled climatic conditions and no direct sunlight, marked *B. cinerea* propagules, which are mostly conidia, remained viable and infective for 40 days after infestation in both warm and cold seasons. They disappeared or almost disappeared from the air of the glasshouse in both the summer and the winter at 60 days after infestation. Lesions were not formed on target plants and only latent or quiescent infection was established; therefore, no secondary inoculum was formed in the glasshouse. All marked *B. cinerea* CFU trapped from the air originated from the primary inoculum

Fig. 5. Recovery of *Botrytis cinerea* marked strains and wild-type isolates from infected plant debris during warm (upper) and cold (lower) seasons. Vertical bars represent standard errors of the means ($P \leq 0.05$).

source. This result shows the longevity of marked inoculum source and, as well, the longevity of the marked CFU (mostly conidia). The disappearance of marked CFU was as likely to be caused by the limit of the resource of marked infection, which could be dissipated in the environment, as by the death of the conidia.

Sclerotia, mycelium, and, to some extent, chlamydospores are regarded as the long-term survival structures of *B. cinerea* (6,20, 28). In Israel and in southeastern Spain, *B. cinerea* is said to overwinter mostly as mycelium in plant stems where it is protected from desiccation, whereas little importance is given to survival as sclerotia (28,40). In our present study, when heavily diseased plants were left drying in the screen house for about 5 months, we found no sclerotia in plant debris, and supposed that both the wild type and the marked strains survived in the plant debris as mycelium. Survival of mycelium depended on the weather conditions: 80 to 100% of the inoculum in pieces of infected plant debris survived during 4 months in winter (December to March), whereas a period of the same length was lethal for most of strains in summer (March to June). Long periods of high temperatures, as well as high humidity combined with relatively high temperatures seem to be responsible for the reduced viability of *B. cinerea* mycelium in the summer.

These studies confirm the value of selenate-resistant mutants for investigating both the survival of *B. cinerea* mycelium and the spread of *B. cinerea* via plants or in the air in the glasshouse. Although the parent strains used here were also marked with fungicide resistance or sensitivity, their use as markers would have been fruitless in the ubiquitous presence in the environment of *B. cinerea* that could also be resistant to fungicides. In contrast, selenate-resistant mutants were readily distinguished from naturally occurring wild-type *B. cinerea*, and from each other in the mixed population by growth on selective media amended with selenate and fungicides.

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