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ABSTRACT

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In tomato glasshouses, the population structure of airborne inoculum of *Botrytis cinerea* depends on the production of endogenous inoculum on diseased plants as well as on incoming exogenous inoculum. Both types of inocula may contribute differently to the development of epidemics. Two strains of *B. cinerea* were introduced in each of four separate compartments of an experimental tomato glasshouse. We monitored their impact on disease development and on the genetic diversity of *B. cinerea* populations using microsatellite markers. The naturally occurring airborne inoculum of *B. cinerea* displayed a high level of genetic diversity and was rapidly displaced in the glasshouse, as isolates with microsatellite profiles identical to the introduced strains amounted to 66% of the inoculum sampled from the air 14 days after inoculation and 91% of those collected from stem lesions 60 days after inoculation. This suggested an important role of secondary inoculum in disease development, which is compatible with the hypothesis of a polycyclic development of gray mold epidemics in tomato glasshouses. In controlled-environment tests on tomatoes, a wide range of aggressiveness levels was observed, both for isolates sampled from the air and from lesions on plants. Hypotheses are proposed to explain the negligible impact of naturally incoming isolates on the epidemics observed inside the four glasshouse compartments.

Additional keywords: clonal population, fitness, *Solanum lycopersicum*, spora flux, sustainable control.

Gray mold, caused by *Botrytis cinerea* Pers.:Fr., is one of the most damaging diseases in greenhouse tomato (*Solanum lycopersicum*) production. In heated glasshouses, symptoms generally consist of stem lesions which result from the infection of the pruning wounds left by the removal of leaves and axillary buds throughout the growing season (21,23). Moderate temperature, high relative humidity, and free moisture on plant surfaces are considered to be highly conducive to disease development (23, 24). No resistant varieties are commercially available (5,27) and conventional chemical control of gray mold is complicated by the presence of strains of the pathogen with reduced sensitivity to fungicides (15) and by the relatively small number of active ingredients available on the market. Efficient and sustainable management of this disease must be conceived as a strategy integrating a variety of control measures, including chemical, cultural, climatic, and biological methods (21,30).

In absence of efficient control, *B. cinerea* can very rapidly produce massive amounts of asexual spores (conidia) on diseased plants. Nicot et al. (22) estimated that over 15 million spores could be produced in 7 days on a 2-cm-long stem segment. The release of conidia from conidiophores is stimulated by rising and falling humidity (11), a common event in heated glasshouses. These spores are easily disseminated by air movements and also, to a lesser extent, by water droplets and insects (10). In tomato greenhouses, susceptible host tissue and environmental conditions conducive to disease development are available during most of the growing season. Therefore, many cycles of infection-sporulation-dissemination are theoretically possible and epidemics of gray mold commonly are assumed to be polycyclic. However, we were unable to find any report with formal evidence for this basic point at crop level. Some of the available data on the genetic diversity of the fungus even appear to provide contradictory indirect evidence. They reveal a high level of diversity in *B. cinerea* populations, both in greenhouse and in open-field situations (1,3,4, 13,19,25,31). However, the occurrence of multiple epidemic cycles and abundant sporulation on diseased plants would presumably result in the gradual selection of strains with the greatest fitness in the confined environment of a greenhouse. In the course of a 10-month (or longer) growing season in greenhouse production, such isolates would likely become more abundant (possibly dominant) than those with lower fitness. This poses the question of what may be the relative contribution of endogenous (secondary) and of incoming exogenous inoculum to the total inoculum present inside a greenhouse, and epidemic development of the disease. Because disease development and spore production may be influenced by complex environmental conditions, we endeavored to address this question in the controlled environment of an experimental glasshouse.

The objectives of the present study were to monitor the epidemiological impact and to assess changes in the genetic diversity of *B. cinerea* populations following the introduction of known inoculum in a tomato glasshouse. To this end, two strains of *B. cinerea*, differing in aggressiveness to tomato were introduced in each of four glasshouse compartments and the genetic diversity of *B. cinerea* populations was followed with the help of microsatellite markers previously developed by Fournier et al. (6). Specifically tested hypotheses include the following: (i) populations before and after introduction of *B. cinerea* strains are significantly differentiated, and (ii) endogenous inoculum outcompetes incoming inoculum and contributes more to the development of epidemics.

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MATERIALS AND METHODS

Experimental design. The experiment was conducted in four adjacent 60 m^2 compartments in an experimental glasshouse of Centre Technique Interprofessionnel des Fruits et Legumes (CTIFL, Balandran, France). Fifty days prior to planting, the compartments were sprayed with two anti-*Botrytis* fungicides Sumico L (Philagro France) and Scala (BASF Agro) and then with a disinfectant of glasshouse surfaces (Desogerm, Laboratories A.C.I., France). In each compartment, 42-day-old tomato plants (cv. Palmiro) were arranged in six rows of 13 plants each, with two single rows on each side and two double rows in the center. The width of the three pathways delimited by these rows was 1.1 m and the overall plant density was 1.6 plants m^{-2}. The crop was grown in a soilless, drip-irrigation system with practices similar to those of commercial glasshouse tomato production. A cooling system and two fans were located on the north side of each compartment, opposite the door. Temperature and relative humidity were regulated separately in each compartment with the help of a computerized environment control system (DGT Volmatic, Denmark) and sensors (Vaisala Humicap, Finland) placed 30 cm above the canopy in the center of each compartment.

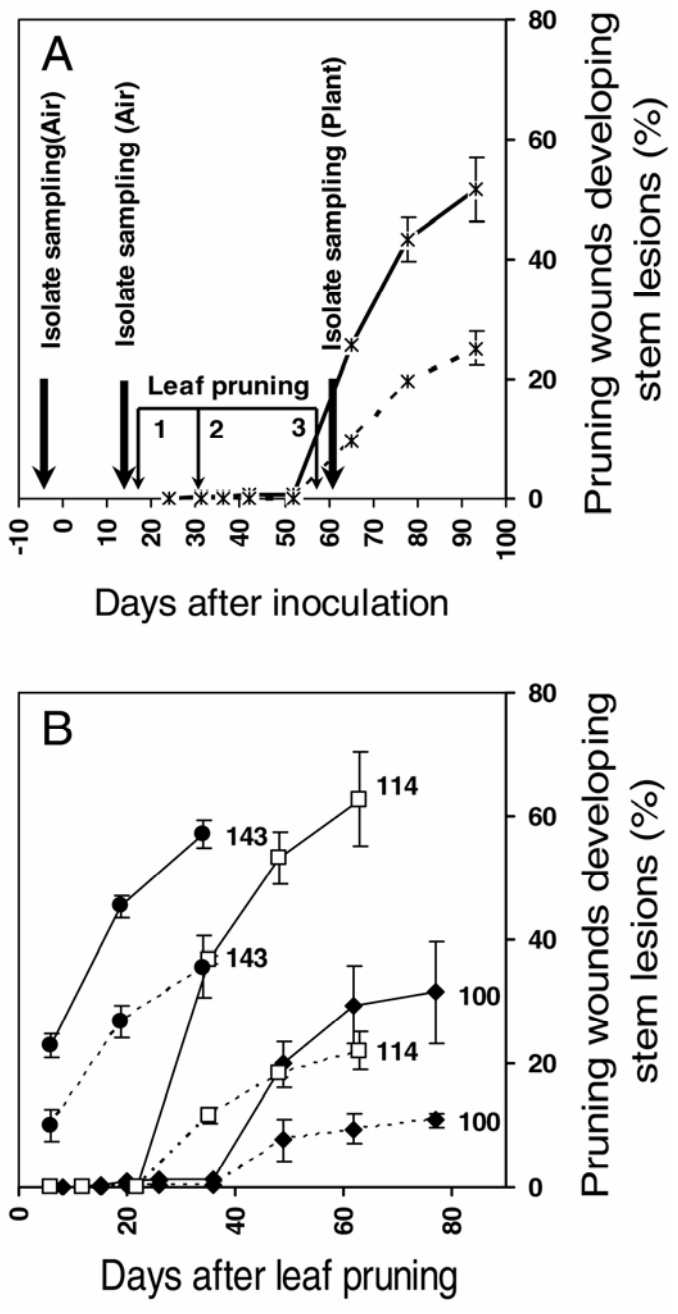

Fig. 1. Kinetics of stem lesion development by *Botrytis cinerea*, as influenced by relative humidity, on tomato plants grown in quasi-commercial conditions in four glasshouse compartments. **A,** Overall incidence of stem lesions. **B,** Incidence of lesion development on three successive cohorts of pruning wounds generated 100 (♦), 114 (□), and 143 (●) days after sowing. Climate differentiation was initiated (9 days after one single event of artificial plant inoculation) to distinguish two humid (—) and two control (----) compartments. Error bars indicate the standard error of the mean.

Inoculum production and inoculation. We used two monoconidial strains of *B. cinerea*, BC1 and I11, isolated from glasshouse-grown tomatoes, in Plougastel, France in 1989 and Berre l'Etang, France in 1991, respectively. These strains were selected for their difference in aggressiveness on tomato plants in preliminary studies (following a protocol identical to that described for the present study in Materials and Methods). Isolates were maintained in stock cultures stored at –20°C in glycerol-phosphate buffer 0.06 M (50/50, vol/vol). Inoculum was produced in petri dishes on potato dextrose agar (PDA; Difco Laboratory, Detroit) medium incubated under cool white fluorescent light (14-h photoperiod, 65 µmole m^{-2} s^{-1}) in a growth chamber at 18°C (night) and 23°C (day). Conidia were collected by washing 10-day-old cultures in 10 ml of water. The suspensions were filtered through 30 µm mesh filters to remove mycelial fragments, and then vortexed for 1 min with 5 g of glass beads (2.5 mm diameter) to separate the spores. The final concentration of the suspensions was adjusted to 10^7 conidia ml^{-1} with the aid of a hemacytometer.

In each compartment, the first plant of each row on the northern side near the fans was inoculated 84 days after sowing. Six leaves were removed from the lower part of each plant, leaving 5-mm petiole stubs on the stems to promote infection by *B. cinerea.* To inoculate the plants, 20 µl of inoculum were applied on each petiole stub (2×10^5 spores per wound). In each compartment, strain BC1 was used to inoculate the first plant of the three western rows and strain I11 to inoculate the first plant of the three eastern rows.

Monitoring development of gray mold. Following inoculation, the plants were misted 20 to 30 s per hour for 4 days and the amplitude of vent opening was reduced to 20% of its full potential in all compartments in order to maintain relative humidity above 75 to 80% to promote disease development. At the first observation of sporulation on the inoculated pruning wounds (9 days after inoculation), two distinct microclimate regimes were established in the compartments. In compartments A and B (considered control compartments), all measures initially undertaken to increase relative humidity were discontinued. In contrast, high relative humidity was maintained during 7 weeks in compartments C and D (considered "humid" compartments) to ensure conditions conducive to disease development. During this period, the average of the relative humidity values recorded in these compartments was 85.4% (with a range of 80 to 91%) compared with 78.8% (with a range of 70 to 85%) in compartments A and B. Temperature was maintained between 18 and 22°C in all compartments.

Three cohorts of pruning wounds were created during the experiment by removing three leaves on each plant at 16 and 30 days after inoculation (DAI) and 5 leaves at 59 DAI (Fig. 1A). Each time, 0.5-cm-long petiole stubs were left to promote infection. The plants were examined individually at weekly intervals and the incidence of stem lesion development was recorded independently for each of the three cohorts of pruning wounds.

To compare the development of epidemics on each cohort of pruning wounds in the control and the humid compartments, we computed the areas under the disease progress curves (AUDPC) as described by Hausbeck et al. (9) for individual plants. We also

combined the data of the three cohorts to assess the cumulative incidence of stem lesions for each plant over the whole experiment. For all analyses, we used the analysis of variance (ANOVA) module of Statistica Kernel version 5.5 software (Statsoft Inc., Tulsa, AZ). Each compartment was considered as a block and the individual plant as a replication.

Quantification of airborne inoculum. Thirty petri plates (90 mm diameter) containing a *Botrytis*-selective medium (12) were placed regularly on the ground in each glasshouse compartment 4 days before inoculation (DBI) and at 14 DAI (Fig. 1A). Petri plates were exposed continuously for 24 h in the glasshouse, and the number of developing *B. cinerea* colonies was recorded after 10 days incubation at 18 to 23°C (light period of 14 h). For each compartment, the numbers of colonies per petri plate were subjected to nonparametric ANOVA (test of Kruskal and Wallis, Statistica software) to assess for possible uneven distributions of airborne inoculum in the North-South direction (parallel to the rows of plants) and in the East-West direction (perpendicularly to the rows).

Isolate collection. For the petri plates exposed 4 DBI, an arbitrary sample of 20 *B. cinerea* colonies isolated from the selective medium in each compartment was transferred on PDA medium. Fourteen days after inoculation with BC1 and I11, an arbitrary sample of 90 colonies per compartment was transferred on PDA medium. Sixty days after inoculation, 60 isolates were collected from diseased plants in each compartment (Fig. 1A). A sterile cotton bud was applied on the youngest sporulating lesions (located on the highest pruning wounds of the plant) and then deposited on PDA medium. Ten isolates were sampled per row, each on a different plant whenever possible.

After single-spore isolation, the monoconidial isolates were subcultured on PDA medium. After 10 days incubation, mycelia and conidia were harvested by adding 1 ml of sterile distilled water in the plates and by scraping a sterile cotton bud on the medium. The fungal material was then lyophilized and stored at –20°C prior to DNA extraction. In order to conserve each isolate, the inoculum-carrying cotton bud was placed in a 2-ml tube containing glycerol-phosphate buffer 0.06 M (50/50, vol/vol) and stored at –20°C.

DNA extraction and isolate genotyping with microsatellite markers. Genomic DNA was extracted from 15 mg of lyophilized fungal material in 96-well plates according to the Dneasy Plant extraction Kit (Qiagen, Chatsworth, CA). Stock DNA of two internal reference strains S1-4639 and S1-10877 was extracted following the protocol described by Möller et al. (18). Amplifications reactions of the nine microsatellite loci characterized for *B. cinerea* by Fournier et al. (6) were performed in a final volume of 20 µl in the presence of 1× *Taq* polymerase buffer, 3 mM $MgCl_2$, 0.06 units of *Taq* DNA polymerase (Promega, Madison, WI), 0.2 µM of the reverse primer, 0.07 µM of the forward primer, 0.13 µM of the forward IRD-700 or IRD-800 labeled primers (MWG-Biotech, Courtaboeuf, France), 0.25 mM dNTP (Roche, Penzbeg, Germany), and 30 ng of genomic DNA. The amplification reaction for each primer pair was conducted separately using a PTC100 thermocycler (MJ Research). After an initial preheat for 3.5 min at 95°C, 35 cycles were performed with the following profile: 1 min of denaturation at 94°C, 1 min of annealing at 50°C for the microsatellite loci Bc1, Bc3, Bc6, Bc9, 53°C for the microsatellite loci Bc2 and Bc5, 59°C for the microsatellite loci Bc4, Bc7, Bc10, and 1 min of extension at 72°C with a final step of 5 min at 72°C. The IRD-700-labeled PCR products were mixed with a two fold concentration of IRD-800-labeled PCR products, heated at 95°C for 5 min, chilled on ice and then separated using a 6.5% KB Plus polyacrylamide gel (LI-COR Biosciences, Lincoln) on a LI-COR IR^2 sequencer under the following conditions: 1,500 V, 40 Watts, 40 mA, and 45°C for 1.5 h. Each gel contained a molecular weight standard (50 to 350 bp, ScienceTec, Les Ulis, France) to analyze microsatellite sizes using $SAGA^{GT}$ software (LI-COR) and the two internal reference strains to ensure constancy of allele sizing across the gels.

Population genetic analyses. The allele sizes of the nine microsatellite loci were combined into a multilocus haplotype. Isolates with haplotypes identical to BC1 or I11 were called type BC1 and type I11, respectively. The other isolates were called unknown. The frequency of each haplotype was calculated for each sampling date in the different glasshouse compartments. Analyses were performed both on populations with all isolates and on populations without the two inoculated strains BC1 and I11 (i.e., unknown isolates).

To compare genetic diversity between populations, the mean number of alleles per microsatellite locus (N_a) was calculated. As this parameter is strongly dependent on sample size, it was estimated by a jackknife subsampling procedure (over 1,000 replicated subsamples of the smallest sampling size of the populations) using GenClone (2). Haplotypic diversity for a population is composed of richness and evenness (8). Haplotypic richness was estimated by the number of haplotypes both in the entire population (g_{obs}) and in a subsample of this population (g_{exp}) as previously described when sample size differ, using the GenClone procedure. Indices of haplotypic diversity were determined by computing the G index of Stoddart and Taylor (26) and the Shannon-Wiener's index H' using the formula $H' = \{-\Sigma i\ [p_i \times \log_{10}(p_i)]\}$ in which p_i is the relative frequency of the ith haplotype using the software GenoDive (17). The evenness, indicator of how evenly the haplotypes are distributed within the populations, was calculated by scaling the index of Stoddart and Taylor by the number of expected haplotypes as recommended by Grünwald et al. (8) when sample sizes differ. Indices of haplotypic diversity and evenness were estimated using a jackknifing approach using 1,000 permutations with subsampling to match the size of the smallest size population.

To estimate the genetic differentiation between the populations, pairwise Weir and Cockerham's F_{ST} values (29) were calculated using the Genetix software (version 4.02 available online from Genetix website). Significance level for the population pairwise values was determined after 10,000 permutations.

Isolate aggressiveness. The aggressiveness of BC1, I11 and 15 unknown isolates was tested on tomato plants cv. Palmiro. The 15 unknown isolates were selected as follows: five collected from the air 4 DBI, five from the air 14 DAI, and five from diseased plants 60 DAI. A suspension of conidia was applied on two pruning wounds per plant (0.5 cm petiole stubs) at 10^4 conidia per wound using a micropipette. Three plants were inoculated for each isolate and three independent repetitions of the assay were conducted. Plants were incubated in a growth chamber (21°C, relative humidity > 80%). The length of stem lesions was monitored daily for 7 days after inoculation. To take into account the kinetics of disease development for each isolate, we computed the AUDPC as

$$\text{AUDPC} = [Y_1/2 + \sum_{2}^{n-1} Y_j + Y_n/2][I]$$

where Y_j was the observed lesion length at the jth observation time, n was the total number of observations, and I the interval between each observation. These values were computed for individual pruning lesions for $n = 7$ observations dates at daily intervals during the period from 1 to 7 DAI. For a given repetition of the assay, we computed an average AUDPC for each isolate. To facilitate the comparison of aggressiveness among isolates, in relation to reference strain BC1, a relative aggressiveness index (in percent) was computed as the ratio between the average AUDPC of the tested isolate and that of strain BC1, multiplied by 100. Two statistical analyses were performed, using the ANOVA module of Statistica software. In the first analysis, the relative aggressiveness indices of all isolates were compared, considering the average values for each of the three assays as replications. In

the second analysis, we compared the aggressiveness of the three groups of isolates (air 4 DBI, air 14 DAI and diseased plants 60 DAI), using the three independent repetitions of the assay as blocks and the five isolates per group as replications.

TABLE 1. Estimated daily deposition of *Botrytis cinerea* airborne inoculum per square meter in four tomato glasshouse compartments

Sampling date[w]	Compartments[x]			
	A	B	C	D
4 DBI	396[y]	553	412	629
14 DAI	687	849	975	482
	$P = 0.000$[z]	$P = 0.000$	$P = 0.000$	$P = 0.038$

[w] Sampling took place 4 days before inoculation (DBI) and 14 days after inoculation (DAI) of plants with *B. cinerea.*

[x] Four adjacent 60 m^2 compartments. Starting 9 DAI, climate was differentiated between compartments A and B (control) and C and D (humid).

[y] Estimate based on the average number of *B. cinerea* colonies recorded on 30 plates placed regularly on the ground of each compartment and maintained open for 24 h.

[z] *P* value of test comparing values over time in each compartment.

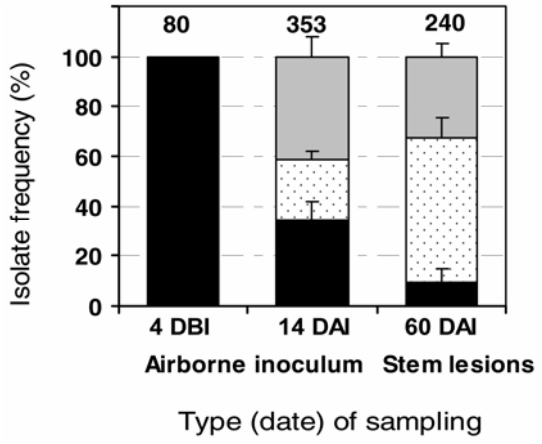

Fig. 2. Frequency distribution of microsatellite profiles of 673 isolates of *Botrytis cinerea* sampled from the air of four glasshouse compartments 4 days before inoculation (DBI) or 14 days after inoculation (DAI) with strains BC1 and I11 of *B. cinerea*. Isolates also were collected from infected pruning wounds on noninoculated plants at 60 DAI. Three classes of isolates are distinguished: those with a microsatellite pattern identical to that of strain BC1 (dots) or strain I11 (gray), and those with a microsatellite profile different from either BC1 or I11 (black). The number above each bar indicates the total number of isolates genotyped at each sampling date. Error bars indicate the standard error of the mean.

Sporulation on stem lesions on the plants inoculated with strains BC1 or I11 was assessed 7 days after inoculation (three plants per assay; three independent assays). Conidial suspensions were obtained by collecting stem lesions in flasks containing 5 ml of alcohol (20%) with Tween 80 (0.05%). Conidial concentration was determined with a hemacytometer under a microscope. The data were analyzed with Statistica software, using the three repetitions of the assay as blocks and the individual plants as replications.

RESULTS

Characterization of natural populations of *B. cinerea* present in the air of the glasshouse before inoculation. Four days before plants were artificially inoculated in the glasshouse, *B. cinerea* was detected in the air of all compartments (Table 1). No symptoms of gray mold were found in the glasshouse at that time. Colony counts on the deposition plates varied among sampling sites within a compartment, but statistical analysis did not reveal any systematic directional effect on the distribution ($P > 0.050$ both for North-South and for East-West effects), suggesting the absence of preferential deposition zones in the compartments.

Characterization of a sample of 80 isolates (20 per compartment) with nine microsatellite markers revealed 63 distinct haplotypes, all different from either of the two strains used for the artificial inoculation (Fig. 2). Most haplotypes (53 haplotypes) were observed only once. Among the 10 haplotypes represented by two or more isolates at 4 DBI, only four (H235, H237, H238, and H239) were present simultaneously in two compartments (Fig. 3).

Kinetics of disease development in the glasshouse. The first symptoms of gray mold were observed simultaneously in all compartments 3 days after pruning wounds were inoculated on one plant in each row. The pathogen first colonized the short petiole stubs that remained on the stems after leaf removal. By 9 DAI, when climate differentiation was established to distinguish two humid and two control compartments, 89 to 97% of these petiole stubs were infected and sporulation was observed. Despite the difference in climate regimes, the incidence of stem lesions continued to increase in all compartments, and reached 94 to 100% of the inoculated petiole stubs by 16 DAI. During the remaining time of the experiment, the overall cumulative incidence of stem lesions on noninoculated plants increased faster in the more humid compartments (Fig. 1A). This difference in the kinetics of disease development was statistically significant ($P < 0.001$) based on ANOVA on the AUDPC values. The kinetics of disease development also differed strongly ($P < 0.001$) according

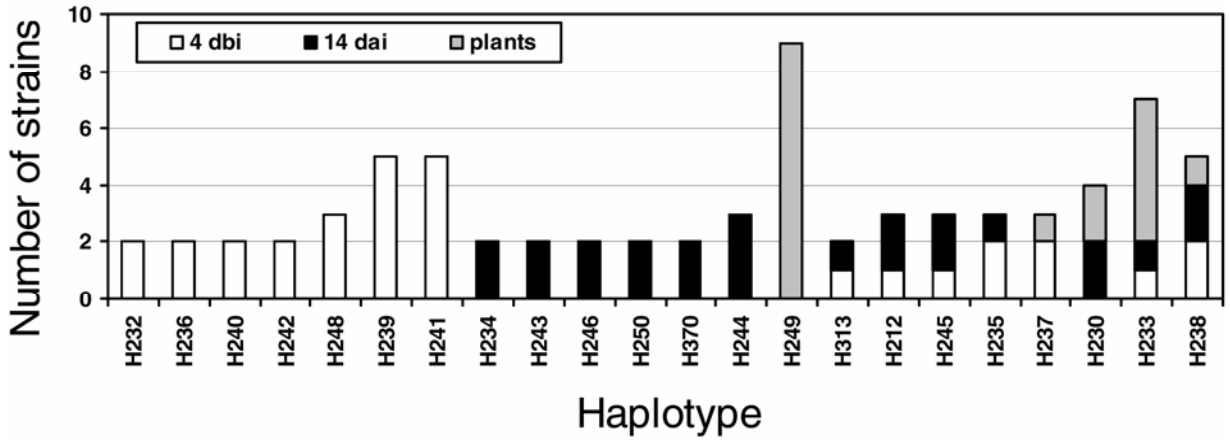

Fig. 3. Distribution of haplotypes of the unknown isolates of *Botrytis cinerea* (isolates with microsatellite profiles different from the introduced strains BC1 or I11) sampled from the air 4 days before or 14 days after a single event of plant inoculation, or isolated from stem lesions 60 days after inoculation. Haplotypes represented by a single isolate (151 out of 173) are not shown.

to compartment humidity when examining separately the three cohorts of pruning wounds (Fig. 1B). For both climate regimes, the time elapsed between leaf removal and detection of the first stem lesions decreased with increasing age of the plant at the date of leaf pruning (Fig. 1B). It was less than 48, 38, and 6 days, respectively, for the three successive cohorts of pruning wounds generated when plants were aged 100, 114, and 143 days after sowing.

Characterization of airborne populations of *B. cinerea* after plant inoculation. With the exception of one compartment, the density of airborne *B. cinerea* inoculum was significantly greater ($P < 0.001$) at 14 DAI than at 4 DBI (Table 1).

In comparison with the previous sampling date, a marked change in the haplotypic diversity of the population was detected at 14 DAI. Among 353 isolates characterized from the air at 14 DAI (*ca.* 90 per compartment), only nine were haplotypes already observed at 4 DBI (Fig. 3), and 232 (65%) had a profile identical to either of the two strains used for artificial inoculation (Fig. 2). The 121 isolates with profiles distinct from BC1 and I11 exhibited 110 haplotypes, of which 100 were represented by a single isolate (Fig. 3). Among 10 haplotypes observed more than once at 14 DAI, only two (H234 and H238) were detected in more than one compartment.

The frequency of isolates with haplotypes identical to either strain BC1 or I11 varied from 50% in compartment D to 83% in compartment C. Isolates with BC1 haplotypes and isolates with I11 haplotypes were present in similar proportions in two of the compartments (47%/53% and 45%/55% for BC1/I11 in compartments B and D, respectively), but I11 haplotypes were clearly dominant in compartments A and C (39%/61% and 23%/77% for BC1/I11, respectively).

As observed for the previous sampling date, colony counts on the deposition plates at 14 DAI varied among the 30 sampling sites of each compartment. No systematic directional effect was detected in a North-South direction ($P > 0.050$), but a significant effect was observed in the East-West direction for compartment D ($P = 0.006$), with higher colony counts in the deposition plates located in the central pathway (6.2 colonies per plate on average) compared to those located in the western and eastern pathways (3.4 and 3.5 colonies per plate, respectively). The isolates with an unknown haplotype appeared scattered across the compartments as exemplified by compartment C (Fig. 4). In contrast, isolates with the microsatellite profile of BC1 tended to be more frequent in the western than in the eastern pathway. A symmetrical pattern was observed for isolates with the I11 haplotype (Table 2).

Characterization of *B. cinerea* populations on diseased plants. Further change in the haplotypic diversity of the population of *B. cinerea* was observed between 14 DAI and 60 DAI, when 240 isolates were collected from stem lesions. In these samples (60 per compartment), only 23 isolates had haplotypes different from either BC1 or I11 (Fig. 2), mostly concentrated in compartments A and C (Fig. 5). These 23 isolates exhibited 10 different haplotypes, of which 7 were represented by single isolates, and three (H230, H233, and H249) were detected in two compartments (Fig. 3). Four haplotypes had been observed previously in samples from the air at 4 DBI or 14 DAI (Fig. 3).

On average in the glasshouse, 58 and 33% of isolates from stem lesions had microsatellite profiles identical to those of strains BC1 and I11, respectively (Fig. 2). This uneven proportion of BC1 and I11 haplotypes was particularly marked in three of the four compartments (Fig. 5). Within a compartment, haplotypes identical to BC1 tended to be more frequently observed on plants in the western rows (Table 2, Fig. 5), where the first plant had been inoculated with strain BC1 (Fig. 4), and a symmetrical situation was observed for I11.

Genetic diversity within populations. Haplotypic richness (g_{exp}) dropped sharply after inoculation both in populations sampled from the air at 14 DAI (28.90) and from the plants at 60 DAI (6.68), compared with the population sampled from the air at 4 DBI ($g_{exp} = 63.00$; Table 3). However, the mean numbers of alleles per microsatellite locus (N_a), observed in the two populations collected from the air, were quite similar (8.78 and 7.35 alleles, respectively, at 4 DBI and 14 DAI). In contrast, it was sharply lower in the population sampled on plants at 60 DAI (3.52 alleles) (Table 3). Haplotypic diversity values, estimated using Stoddart and Taylor's (G) and Shannon-Wiener's (H') indices, were also lower in *B. cinerea* populations collected after

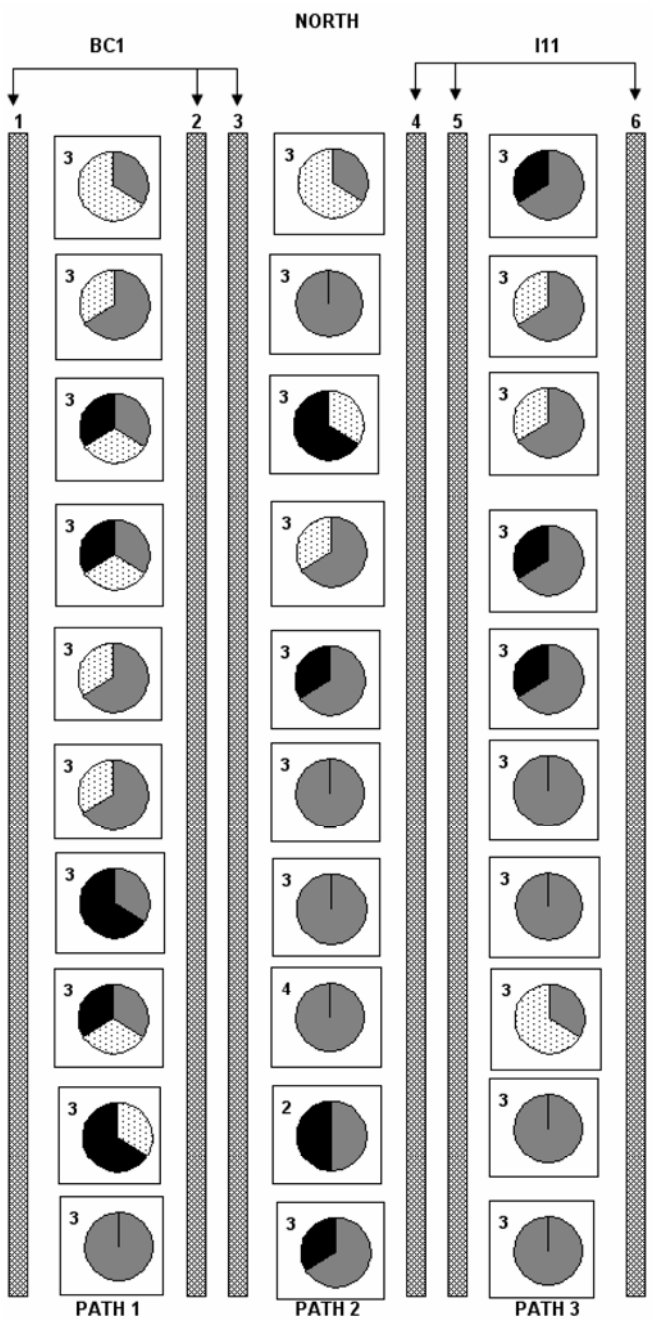

Fig. 4. Example of the spatial distribution of haplotypes among isolates of *Botrytis cinerea* sampled from the air 14 days after inoculation in one of four glasshouse compartments (compartment C). Isolates were collected from 30 deposition plates placed at regular intervals on the ground in three pathways. The rows of tomato plants, numbered from 1 to 6, are represented as gray vertical bands. The first plant of each row was inoculated with either strain BC1 or I11, as indicated over the bands. For each square representing a sampling site, the number on the upper left corner indicates the number of isolates that were genotyped. Three categories of microsatellite profiles are distinguished: similar to BC1 (dots), similar to I11 (gray), or unknown (black).

inoculation than in the 4 DBI population (Table 3). Evenness (G/g_{exp}) values decreased from the 4 DBI populations to the two others (airborne 14 DAI and plantborne 60 DAI), as a result of the domination of the two strains, BC1 and I11, after their inoculation on plants (Table 3, Fig. 2).

The populations of unknown isolates collected from the air at 4 DBI and 14 DAI had quite similar mean numbers of alleles per microsatellite locus (5.87 and 6.48 alleles, respectively) when compared at the smallest population size (n = 23) (Table 3). In contrast, it sharply decreased in the population sampled on plants at 60 DAI (3.56 alleles). Indices of haplotypic richness, diversity, and evenness were also quite similar for the two populations of *B. cinerea* sampled from the air (Table 3), but were sharply reduced for the isolates collected from plants.

TABLE 2. East-west distribution (percentage of total) of haplotypes of strain BC1 and haplotypes of strain I11 among isolates of *Botrytis cinerea* sampled from the air and from stem lesions on tomato plants in four glasshouse compartments

	Isolates from airborne inoculum 14 DAI[w]			Isolates from stem lesions 60 DAI[w]	
Sample (location)	I11	BC1	Sample (location)	I11	BC1
Path 1 (west)[x]	22.2 ± 1.0[y]	53.0 ± 6.1	Row 1 (west)[x,z]	13.7 ± 3.4[y]	21.6 ± 4.3
Path 2 (central)	27.3 ± 4.3	35.2 ± 6.4	Row 2	9.1 ± 4.3	22.9 ± 1.9
Path 3 (east)	50.5 ± 4.4	11.8 ± 4.5	Row 3	9.2 ± 3.3	16.2 ± 2.4
			Row 4	17.6 ± 4.6	14.3 ± 1.5
			Row 5	24.8 ± 1.8	13.6 ± 2.1
			Row 6 (east)	25.6 ± 5.7	11.4 ± 2.9
Total 3 paths	100.0	100.0	Total 6 rows	100.0	100.0

[w] DAI: days after inoculation.
[x] Figures 4 and 5 provide a description of pathways and plant rows.
[y] Data are means of four replications ± standard error of the mean.
[z] The first plant of each row was inoculated once with either strain BC1 (rows 1 to 3) or I11 (rows 4 to 6).

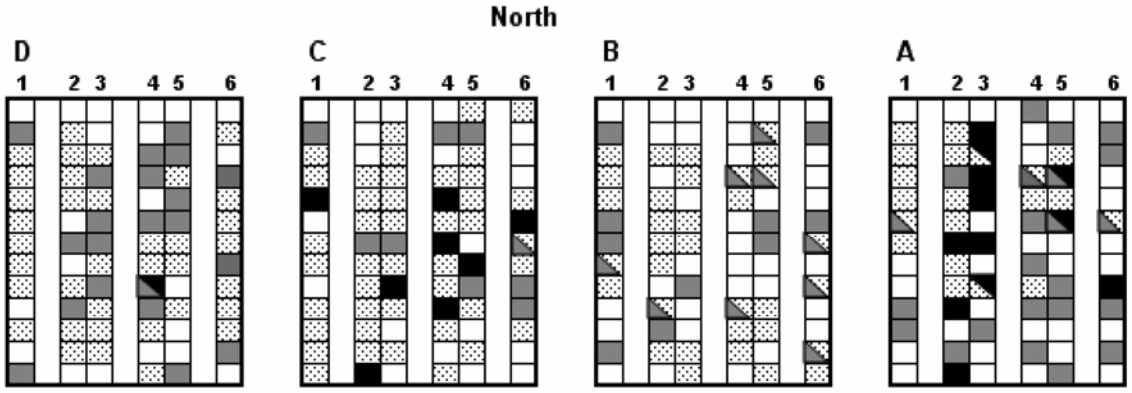

Fig. 5. Spatial distribution of haplotypes among isolates of *Botrytis cinerea* collected from stem lesions on tomato plants in four glasshouse compartments (A to D). Plants were distributed along six rows (each plant represented by a square) separated by three pathways. In each compartment, the first plant of each row was inoculated with either strain BC1 (rows 1, 2, and 3 in the northwest) or strain I11 (rows 4, 5, and 6 in the northeast). Three categories of microsatellite profiles are distinguished: similar to BC1 (dots), similar to I11 (gray), or unknown (black). A split square indicates the haplotype of two isolates collected from the same plant.

TABLE 3. Measures of haplotypic and gene diversity in the populations of *Botrytis cinerea* sampled from the air and from stem lesions in a tomato glasshouse

		Richness			Diversity		
Isolates[w]	Sample size	g_{obs}[x]	g_{exp}[y]	N_a[y]	G[z]	H'[z]	Evenness[z]
All isolates							
4 DBI	80	56 (7)	63.00 (0.00)	8.78 (0.00)	45.71	1.75	0.73
14 DAI	353	103 (9)	28.90 (0.12)	7.35 (0.02)	4.25 (3.36–5.50)	0.95 (0.81–1.10)	0.15 (0.13–0.16)
Plants	240	6 (6)	6.68 (0.04)	3.52 (0.01)	2.24 (1.97–2.51)	0.45 (0.38–0.51)	0.35 (0.26–0.46)
Unknown isolates							
4 DBI	80	56 (7)	21.03 (0.01)	5.87 (0.02)	19.37 (15.11–23.00)	1.31 (1.23–1.36)	0.92 (0.76–1.00)
14 DAI	121	103 (7)	22.57 (0.02)	6.48 (0.02)	22.24 (19.59–23.00)	1.35 (1.31–1.36)	0.98 (0.93–1.00)
Plants	23	6 (4)	10.00 (0.00)	3.56 (0.00)	4.52	0.81	0.45

[w] Populations with all isolates and populations without the two introduced strains BC1 and I11 (unknown isolates). Air sampling took place 4 days before inoculation (DBI) and 14 days after artificial inoculation (DAI) of plants with *B. cinerea.*
[x] Number of haplotypes specific to each sampling date (in brackets, number of haplotypes shared with other populations). The sum of these two numbers gives the number of haplotypes observed in each population.
[y] Number of expected haplotypes (g_{exp}) and allelic richness (N_a) compared for each population at the smallest population size (n = 80 isolates for the populations with all isolates and n = 23 for populations of the unknown isolates) using GenClone based on 1,000 permutations (standard errors are given in brackets).
[z] Stoddart and Taylor's index (G); Shannon-wiener's index (H'); Evenness (G scaled by the number of expected haplotypes). Indices estimates were calculated using a jackknifing procedure with subsampling at the size of the smallest population for comparative purpose (software GenoDive). Numbers in parentheses indicate the lower and upper bounds of the 95% confidence interval around the average for the 1,000 replicated subsamples.

Genetic diversity among populations. A significant F_{ST} value of 0.159 ($P < 0.05$) was observed between the two *B. cinerea* populations collected from the air. Furthermore, a significant differentiation was observed between the populations sampled from diseased plants and those from the air (F_{ST} values of 0.313 and 0.100, respectively, before and after inoculum introduction; $P < 0.05$ for both comparisons).

Significant ($P < 0.05$) F_{ST} values were measured between the three populations of unknown isolates. The lowest genetic differentiation was assessed between the two populations sampled from air ($F_{ST} = 0.006$). When airborne and plant populations were compared, almost 13% of genetic differentiation was observed (F_{ST} values of 0.136 and 0.131, respectively, before and after inoculum introduction).

Compared aggressiveness of isolates. Following the observation of the prevalence of BC1 and I11 haplotypes among isolates collected from the air at 14 DAI and from stem lesions on plants (Fig. 2), it was hypothesized that their aggressiveness on tomato may be higher than that of the other isolates collected during this study. It was further hypothesized that isolates found on stem lesions may be more aggressive than isolates only observed in the air spora. In three independently repeated bioassays for 15 isolates, a wide range of aggressiveness levels was observed (Table 4). Individually, the isolates with an unknown haplotype were significantly less aggressive than strain BC1, but not than strain I11. On average, isolates collected from stem lesions were significantly more aggressive than those collected from the air at 14 DAI but not at 4 DBI (Table 4). However, there was no evidence of a relationship between aggressiveness and prevalence among isolates with unknown haplotypes collected from plants. This is exemplified by isolate 98, representative of an abundant haplotype (H249; Fig. 3) whose aggressiveness was similar to that of isolate 35, representing a rare haplotype H238 (Table 4).

As haplotypes similar to I11 were slightly more prevalent than those of BC1 in the air samples taken 14 DAI, despite a lower aggressiveness of I11 on tomato, their sporulation was compared on tomato plants used in the aggressiveness bioassays. Seven days after inoculation, average spore production was 2.2×10^6 and 9.1×10^5 spores per stem lesion for strains BC1 and I11, respectively ($P = 0.055$), with average lesion length of 63.5 ± 3.5 and 20.5 ± 2.2 mm, respectively.

DISCUSSION

Our study led to the observation of a rapid and sharp change in the genetic diversity of *B. cinerea* populations, following the one-time inoculation of two strains with known microsatellite profiles on tomatoes in four separate glasshouse compartments. The haplotypes of the two introduced strains became dominant among airborne inoculum within 2 weeks after inoculation. Together, they later represented 91% of the isolates on stem lesions following the epidemic development of the disease in the crop, resulting in the observation of the lowest genetic diversity for this population. The lower F_{ST} estimates among the two populations sampled after inoculation was also associated with the gradual domination of the two introduced strains. The development of sporulating lesions on inoculated pruning wounds showed that one first disease cycle occurred on these plants. The occurrence of at least one additional cycle was supported by the detection of the haplotypes of the introduced strains on sporulating lesions on plants that were not artificially inoculated. The overwhelming abundance of strains BC1 and I11 on plant lesions 60 days after their introduction in the four compartments suggests an important role of endogenous inoculum in our experimental system and is compatible with the hypothesis of a polycyclic development of *Botrytis* epidemics in tomato greenhouses. The abundance of certain haplotypes on a sampling site appears to be quite original in comparison with results of previous studies on population of *B. cinerea* based on strain characterization with a variety of neutral molecular markers, showing a high level of diversity (1,4,13, 19,31). On vegetables grown in commercial greenhouses, Alfonso et al. (1) and Moyano et al. (19) reported that the genetic diversity of *B. cinerea* was much higher within a greenhouse site than between greenhouses (assessed on 65 and 44 isolates in each study, respectively). In the tomato greenhouses, the genetic diversity within subpopulation accounted for 96% of total genetic diversity (19). The highest level of diversity was reported for a rose glasshouse in the Netherlands. Using random amplified polymorphic DNA analysis, Kerssies et al. (13) observed that 29 isolates sampled in a single rose glasshouse in The Netherlands were all genetically different. In all these greenhouses, the scarcity or absence of recurrent isolates suggested that polycyclic epidemics may not have occurred before sampling was carried out.

By contrast, these studies suggested the prevalence of inoculum intrusions into the greenhouses from a pool of genetically diverse inoculum present outside. Indeed, in addition to (endogenous) spores produced on diseased plants within a crop, the airborne spora of *B. cinerea* over a canopy may include incoming exogenous inoculum carried from a variety of places and hosts. By using selenate-resistant strains of *B. cinerea*, Korolev et al. (14) demonstrated that exchange of inoculum between the outside and the inside of the glasshouse was possible. Kerssies et al. (13) hypothesized that *B. cinerea* inoculum in rose glasshouse originates from many sources in their vicinity. The hypothesis of spore migration, and penetration into the greenhouses, is supported in some studies by the lack of clear geographical differentiation reported among populations of *B. cinerea* collected in different greenhouses (1,25). Alfonso et al. (1) suggested that a continuous gene flow must occur between vegetable greenhouses in southeastern Spain. Results obtained in our study also support the hypothesis of spore migration if one considers the isolates with haplotypes different from the two strains used for plant inoculation (unknown isolates). Most of the haplotypes observed in the first air sampling were absent among the 121 unknown isolates collected in the second air sampling 18 days later and significant

TABLE 4. Aggressiveness on tomato (cv. Palmiro) of reference strains BC1 and I11 of *Botrytis cinerea* and 15 isolates sampled from the air and stem lesions in a tomato glasshouse

Strains/ isolates	Origin[w]	Haplotypes	Relative aggressiveness index[x]	
			Individual[y]	Group average[z]
BC1	Reference	"Type BC1"	100 a	
I11	Reference	"Type I11"	33 cd	
35	Plants	H238	24 cd	
67	Plants	H231	49 c	
93	Plants	H233	69 b	39.7 a
96	Plants	H230	30 cd	
98	Plants	H249	27 cd	
242	Air 4 DBI	H240	28 cd	
246	Air 4 DBI	H239	26 cd	
255	Air 4 DBI	H232	31 cd	33.6 a
268	Air 4 DBI	H248	43 c	
273	Air 4 DBI	H242	39 c	
327	Air 14 DAI	H238	4 d	
351	Air 14 DAI	H244	19 cd	
381	Air 14 DAI	H212	18 cd	18.9 b
456	Air 14 DAI	H329	32 cd	
488	Air 14 DAI	H332	22 cd	

[w] Air sampling took place 4 days before inoculation (DBI) and 14 days after inoculation (DAI) of plants with *B. cinerea*. Isolates were collected from stem lesions 60 DAI.

[x] Within column, numbers followed by the same letter are not significantly different according to the Newman and Keuls test ($P = 0.050$).

[y] Average from three plants per isolate per bioassay (three independent assays).

[z] Average from five isolates per group (three independent assays).

genetic differentiation (6%) was observed among the two *B. cinerea* populations of unknown isolates. As the vents of the glasshouse were open for several hours daily, a common practice at that season in commercial tomato production, these isolates presumably represented the exogenous airborne inoculum that entered the glasshouse. These results are compatible with the hypothesis that entry of spores into the glasshouse is probably an important phenomenon and that air spora in the glasshouse is periodically supplied with new exogenous inoculum coming from an extremely diverse metapopulation. The periodicity of the replacement of the exogenous inoculum in a greenhouse structure remains a fundamental unanswered question. Is there a continuous arrival of inoculum inside a greenhouse or an intermittent entry linked to variable environmental and mesoclimatic factors that take place outside?

One striking result of our work is that the numerous and genetically diverse unknown isolates that were examined in this study had a limited contribution to epidemic development. Isolates collected from infected lesions on tomato plants 60 DAI were mainly identified as the two introduced strains BC1 and I11. Several hypotheses may be proposed to explain this phenomenon. One hypothesis could be that naturally occurring airborne isolates in our study differed strongly with the two introduced strains in terms of their aggressiveness on tomato. Although *B. cinerea* is considered to have a very wide host range (11), a possible host specialization has sometimes been suggested. The finding of a population of unknown isolates sampled from plants with a low genetic diversity, compared with isolates sampled from the air, would be consistent with this hypothesis. Characterization of populations of *B. cinerea* in France and Chile with neutral molecular markers revealed some level of genetic differentiation between isolates coming from different hosts (7,20,28). In contrast, Ma and Michailides (16) observed that 234 isolates of *B. cinerea* collected from five hosts in California were not clustered based on their source hosts. In our study, we compared directly the aggressiveness to tomato of the two introduced strains, and 15 other isolates. The high level of aggressiveness of strain BC1 compared to all other strains was compatible with its strong epidemiological impact in the glasshouse. However, the concomitant dominance of strain I11 could not be solely explained in terms of aggressiveness. One trait common to both strains BC1 and I11 is their high sporulation ability on tomatoes, which resulted in representing 24 and 41%, respectively, of total airborne inoculum recorded on deposition plates in the compartments 14 DAI. The resulting high inoculum pressure, relative to that of other airborne isolates, may have contributed to amplify the epidemic impact of the two introduced strains. Another possible hypothesis to explain the limited epidemiological impact of strains from the naturally incoming air spora could also be that their aggressiveness (and possibly viability) was reduced by aging and by encountering deleterious environmental conditions during air travel. Conidia of *B. cinerea* can be damaged by different physical stresses encountered during air travel, such as low humidity and extremes of temperature or exposure to sunlight, as reviewed by Holz et al. (10) and Williamson et al. (30).

Another significant result of our study concerns the effect of climatic conditions on the development of gray mold in the glasshouse. We observed differences in the kinetics of disease development in the two types of microclimates that were maintained in the compartments of the experimental glasshouse. These results provide a formal validation at crop level of previously observed conducive effects of high relative humidity on the infection of detached stems pieces and potted tomato plants in growth cabinets (24). In contrast, the differences in microclimate in our glasshouse compartments did not appear to influence the structure of the *B. cinerea* population, as the dominance of the two introduced strains BC1 and I11 was similarly high in all compartments.

In conclusion, this study showed both a high level of genetic diversity among naturally occurring isolates of *B. cinerea* and a strong epidemiological impact of endogenous inoculum. As the work was conducted in the somewhat controlled environment of an experimental glasshouse, the question of the relative contribution of endogenous and exogenous inoculum in the air spora of a greenhouse merits further attention in the more complex context of commercial production. Understanding this phenomenon may have important consequences for devising disease control strategies and extending their durability. For example, if endogenous inoculum is predominant, farmers should devote effort on the elimination of inoculum sources inside the glasshouses. Such practices could include the systematic removal of dead plants and plant debris or appropriate treatment of individual lesions before sporulation of *B. cinerea*.

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