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**Informative title:** Flow of *Botrytis cinerea* inoculum between lettuce crop and soil.

C. Leyronas <sup>a\*</sup>, M. Duffaud <sup>a</sup>, L. Parès <sup>b</sup>, B. Jeannequin <sup>b</sup>, P.C. Nicot <sup>a</sup>

a : INRA, UR0407, Pathologie Végétale, F-84140 Montfavet, France

b : INRA, UE411, Domaine Expérimental Alénia Roussillon, F-66200 Alénia, France

\*corresponding author: christel.leyronas@avignon.inra.fr

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**Abstract**

*Botrytis cinerea* causes grey mould, a disease common on many economically important crops. Although much attention is paid to the airborne inoculum of this fungus, as it sporulates abundantly in favourable conditions, knowledge on the abundance and genetic characteristics of soilborne inoculum could help improve control strategies. In this study, the soilborne inoculum of *B. cinerea* was quantified in two greenhouses at different times surrounding the cultivation of four successive lettuce crops. Between 0 and 1177 CFU of *B. cinerea* per gram of soil were recorded. There was no significant correlation between abundance of soilborne inoculum and subsequent disease incidence on lettuce ( $P=0.11$ ). Sixty-five strains collected from diseased plants and sixty-six strains collected from the soil

were investigated for their genetic diversity. The soil strains showed lower genetic diversity

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than the lettuce strains when considering the unbiased gene diversity within the nine microsatellite loci, the mean number of alleles per locus and the haplotypic diversity. The genetic differentiation between lettuce and soil strains decreased over three successive lettuce crops. In the same time, the genetic structure of the two groups of strains tended to become similar. These results are consistent with the hypothesis of a flow of inoculum between the lettuce crop and the soil, and vice-versa. Our study shows that grey mould management should pay more attention to the inoculum of *B. cinerea* present in the soil.

### **Introduction**

Grey mould is a common disease on many economically important crops worldwide. It is caused by the fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana*), an ascomycete able to attack more than two hundred species. When conditions are favourable, the fungus produces lesions and sporulates abundantly on aerial parts of plants (Epton & Richmond, 1980; Nicot *et al.*, 1996). Conidia are released by a twisting of the conidiophore during variation of air humidity or by other mechanic forces related to wind or splash, and are easily disseminated by air currents (Jarvis, 1962; Harrison & Lowe, 1987). Conidia are considered as short-lived propagules and their survival is highly dependent on temperature, moisture and exposure to sunlight (Holz *et al.*, 2004). In the epidemiology of grey mould, airborne inoculum can serve as primary inoculum as it is present almost all the time in the air (Leyronas & Nicot, 2013). Conidia also play a major role in the rapid colonization of neighbouring plants when conditions are favourable (Holz *et al.*, 2004). In studies dealing with *B. cinerea* epidemiology a lot of attention is paid to airborne inoculum and much effort has been dedicated to monitoring this type of inoculum in order to prevent grey mould outbreaks (Johnson & Powelson, 1983; Blanco *et al.*, 2006; Rodríguez-Rajo *et al.*, 2010).

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Besides its abundant airborne inoculum, *B. cinerea* can also be found in the soil. Conidia may become buried in the soil and survive under favourable conditions of temperature and humidity. Moyano & Melgarejo (2002) reported a survival period for conidia in soil of one month at 22°C but only a one-week at 40°C. The fungus can also form sclerotia which are considered as the most important structures involved in the survival of *Botrytis* species (Coley-Smith, 1980). When fertilized with microconidia they can form apothecia leading to the production of ascospores. Moreover conidiophores can be formed directly on sclerotial tissue, producing numerous conidia. Both types of spores produced by sclerotia can be wind dispersed and may induce grey mould epidemics in neighbouring crops. Sclerotia are considered as a common form of conservation of *B. cinerea* in European regions with cold winters (Strømeng *et al.*, 2009) but not in southern countries (Raposo *et al.*, 2001), where sclerotia and apothecia are never observed in nature. *B. cinerea* can also survive as mycelium in plant debris (Coley-Smith, 1980). The mycelium usually hidden and protected inside the plant tissue produces conidia once it is wet (Yunis & Elad, 1989) thus increasing the inoculum pressure. Strømeng *et al.* (2009) reported up to 137,200 conidiophores produced per square meter of plant debris and 2,000 conidiophores produced per square meter of soil, in which respectively 53 and 43% were produced by mycelium rather than by sclerotia. Furthermore, direct infection by hyphae can also occur (Verhoeff, 1980). It is recommended to remove plant debris between cropping periods (Dik & Wubben, 2004), but some may remain on the soil and eventually become buried, for example by cultivation. Despite its possible epidemiological importance, little is known about the abundance and the role of soilborne inoculum in the development of grey mould.

As *B. cinerea* is mostly considered as an airborne pathogen, practices to reduce grey mould incidence on major crops are largely based on fungicidal treatments applied on aerial parts of plants (Leroux, 2004) and little is done to reach and treat the inoculum located in the soil.

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Nevertheless, soil sanitation measures have been described for the control of various soilborne diseases. The efficacy of solarisation, which consists in increasing soil temperature with the help of a polyethylene soil cover, has been shown against several soil fungi, weeds and nematodes (Stapleton & DeVay, 1986). Biofumigation with a cruciferous cover crop (mustard in general) ploughed under immediately after flowering has been shown to release biocidal compounds against fungi, bacteria and nematodes (Vig *et al.*, 2009). No information is available on a possible effects against *B. cinerea*.

In the South of France many vegetable species cultivated in soil under non-heated greenhouses are affected by grey mould. Contaminated plant debris and sclerotia from one crop may be released in the soil and constitute a potentially harmful reservoir of inoculum for the next. To investigate this issue, we characterized soilborne inoculum of *B. cinerea* in two unheated greenhouses, in which successive crops of lettuce were grown. The specific objectives of this study were to i)- evaluate the abundance of *B. cinerea* inoculum in the soil over time, ii)- determine if there was a relationship between this abundance and the incidence of grey mould on lettuce, iii)- compare the genetic characteristics of soilborne inoculum and inoculum sampled from lettuce plants.

## Material and methods

### *Experimental set up*

The experimental work was conducted from 2008 to 2010 in two unheated polyethylene greenhouses (tunnels named T5 and T7) located at the Alenya-Roussillon experimental domain of the French National Institute of Agricultural Research (INRA) in southern France (lat. 42.64N; long. 2.98E). Four successive lettuce crops were grown in these tunnels, with two butterhead flat leaf varieties: Fidel and Arcadia (Rijk Zwaan) (Table 1). Both tunnels contained 28 rows of 150 lettuce plants with a planting density of 14 plants per square meter.

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The soil was covered with a micro-perforated polyethylene mulch and the plants were sprinkle-irrigated. At harvest, leaf trimming was carried out inside the tunnels and the plant residues were removed immediately, together with the polyethylene mulch. The root residues were rotovated and buried in the soil at a depth of 25 cm. No fungicide was ever applied to the soil. A new mulch was placed over the soil before each new lettuce planting. The plants were efficiently protected against downy mildew with fungicide treatments applied until the 18-leaf stage according to common practice in commercial production. Minimal control of the climate inside the tunnels was obtained by passive ventilation through temperature-based opening of lateral and top vents.

As a sanitation practice in tunnel T5, a mustard crop (*Brassica juncea*, cv 1420) was sown on 23 April 2009. It was grown until flowering and then comminuted and buried on 19 June. In tunnel T7 the soil was left bare (fallow) during that time. In both tunnels the soil was then watered and covered with polypropylene mulch, and subjected to solarisation between 19 June and 20 September 2009.

#### *Soil sampling*

Soil sampling was carried out a few days before lettuce planting, just after lettuce harvest and before and after sanitation practices in both tunnels (Table 1). Each tunnel was divided in 4 sectors of equal area and in each sector, four soil samples were taken randomly with a 2 cm diameter auger at a depth of 0-30 cm. The four samples from a given sector were bulked and then stored at -20°C until analysis.

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### *Quantification of soilborne inoculum*

In each soil sample, a 1 g subsample was taken, examined to detect eventual sclerotia or plant debris and then suspended in 10 mL of sterile water. Two dilutions (10 and 100-fold) were carried out. Three 500  $\mu$ L aliquots of the initial suspension and of each of the two dilutions were then plated on Petri plates containing the semi-selective medium Botrytis Spore Trap Medium (BSTM) (Edwards & Seddon, 2001). The Petri plates were sealed and incubated at 20°C in daylight for 15 days to allow the development of fungal colonies. The colonies showing a mycelium resembling *B. cinerea* were individually sub-cultured on Potato Dextrose Agar (PDA) and their identity was confirmed 7 days after transplanting by observing characteristic conidiation of *B. cinerea*. The results were then expressed as numbers of Colony Forming Units (CFU) per gram of soil. For a given date, a mean value of soilborne inoculum abundance and a standard error were calculated with the values of the four soil samples.

### *Evaluation of grey mould incidence on the lettuce crops*

Incidence of grey mould in a tunnel was assessed immediately at harvest on the basis of presence or absence of typical *B. cinerea* conidiation on 150 plants sampled in a regular fashion in each of four sectors. Thus, a total of 600 plants was examined per tunnel. Mean grey mould incidence and its associated standard error were calculated for each tunnel.

### *Collection of isolates for genotyping*

Isolates were collected on lettuce plants of tunnel T7 during harvest by rubbing sterile cotton buds on sporulating lesions. Isolates were also taken from soil samples by rubbing dry sterile cotton buds on sporulating colonies obtained in the soilborne inoculum quantification process described above. The cotton buds were stored at -20°C until isolate purification. All isolates

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were purified and single-spored in a classical way (Leyronas *et al.*, 2012) prior to their genotypic characterization. Hereafter these characterized single spore isolates will be referred to as "lettuce strains" and "soil strains".

#### *Isolate genotyping.*

Isolates from 8 combinations of dates and origin were genotyped (Table 1). Genomic DNA was extracted from aliquots of 15 mg lyophilized fungal material (harvested from two-week old cultures on Potato Dextrose Agar) in 96-well plates, following the Dneasy Plant extraction Kit protocole (Qiagen). The nine microsatellite markers designed for *B. cinerea* by Fournier *et al.* (2002) were amplified following the protocol described by Leyronas *et al.* (2014). To determine the size of the microsatellites, the PCR products were diluted and multiplexed prior to scanning with the help of a Megabace sequencer (Amersham Pharmacia). Genetic Profiler software (Amersham Biosciences) was then used for the microsatellite size analysis. Complete microsatellite size profiles (referred to as "haplotypes" hereafter) were obtained for 67 lettuce strains and 66 soil strains.

#### *Genetic diversity of strains collected from soil and from lettuce*

Several indices of genetic diversity were used to compare lettuce and soil strains. The software FSTAT version 2.9.3 (Goudet, 1995) was used to compute allelic richness (corrected for the smallest sample size) and unbiased gene diversity per locus. Unbiased gene diversity (Hnb) and allelic richness (average over the nine loci) were computed separately for the soil strains and for the lettuce strains with the Genetix software (Nei, 1978). The number of different multilocus haplotypes (MLH) was computed with GenClone 1.0 software (Arnaud-Haond & Belkhir, 2007) and was used to calculate the haplotypic diversity (computed as  $[\text{number of distinct MLH} - 1] / [\text{sample size} - 1]$ ), which estimates the

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proportion of haplotypes present in a population and takes a value of 1 when a population is composed exclusively of unique haplotypes (Arnaud-Haond *et al.*, 2007). We also used the evenness index, whose value tends to 1 when haplotypes have a similar abundance (equitable distribution of clones) (Arnaud-Haond *et al.*, 2007).

The program Multilocus 1.3b (Agapow & Burt, 2001) was used to calculate the standardized version of the index of association  $r_d$ . This index is a measure of the multilocus linkage disequilibrium, which varies between 0 (complete panmixia) and 1 (no recombination). The null hypothesis of complete panmixia was tested by permuting alleles (microsatellite sizes) among strains, independently for each microsatellite marker (1000 permutations).

#### *Genetic differentiation between lettuce strains and soil strains*

In order to assess the genetic differentiation between soilborne inoculum and lettuce inoculum, pairwise Weir and Cockerham's  $F_{ST}$  values were calculated with Arlequin version 3.0 (Excoffier *et al.*, 2005) between strains collected from soil before a lettuce croplanting, from lettuce at harvest and from soil after lettuce harvest. ARLEQUIN was also used for a hierarchical analysis of molecular variance (AMOVA) among soil and lettuce strains.

#### *Genetic structure of *B. cinerea* strains*

The software Structure version 2.3.4 (Falush *et al.*, 2003) was used to determine the genetic structure of lettuce and soil strains taken together. The model with admixture was chosen since there was no geographical barrier between lettuce and soil strains. The tested numbers of clusters (K) varied from 1 to 9, with 10 replicates for each value of K. Each simulation consisted in 100,000 Monte-Carlo Markov Chain iterations preceded by a burn-in period of 200,000 iterations. The most probable structure was determined by computing the posterior probability for each K using the distribution of maximum likelihoods. When the probability

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of ancestry of a strain in a cluster was greater than the arbitrary threshold of 0.8, this strain was considered to be unambiguously assigned to this cluster.

#### *Statistical analysis*

Statistical analysis were performed with StatView (version 5, SAS Institute). Non-parametric tests were used to determine significant differences between soilborne inoculum abundance before and after soil disinfestation (Wilcoxon), between unbiased gene diversity and allelic richness of lettuce and soil strains (Mann-Whitney) and correlations between grey mould incidence on crops at harvest and abundance of soilborne inoculum (Spearman test). Statistical inferences were made at the 5 % level of significance, unless otherwise indicated.

#### **Results**

##### *Grey mould incidence on lettuce and abundance of *B. cinerea* soilborne inoculum.*

In both tunnels, the second lettuce crop was more attacked than the first and the fourth crop was more attacked than the third (Fig. 1). The plants were typically attacked at the collar by *B. cinerea* and we hardly ever observed any infection from the top downward (two or three plants per tunnel per season). The presence of *Rhizoctonia* and *Sclerotinia* was occasionally observed at harvest and their incidence was recorded (data not shown). As it was minor and there was no significant correlation between the incidence of *B. cinerea* and either of these two pathogens we assumed that they did not affect the analyses for *Botrytis*. Collapsed *Botrytis*-infected plants were occasionally observed but this was always linked to a co-infection with *Sclerotinia sp.*

Although detectable amounts of soilborne inoculum could be quantified in all but one sampling date, an increase in disease incidence could not systematically be linked to an increase in soilborne inoculum. The amount of soilborne inoculum before crop planting was

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not highly correlated to subsequent disease incidence on the plants (Spearman's  $Rho = 0.80$ ;  $P = 0.11$ ).

The sanitation practices during summer 2009 did not seem to have a significant impact on soilborne inoculum abundance in tunnel T7 (test of Wilcoxon;  $P = 0.06$ ). In tunnel T5, the statistical test could not be realised because no inoculum was detected in most samples taken before soil disinfestation. However, in both tunnels, there was a sharp decrease in disease incidence after summer and thus after soil disinfestation.

#### *Comparison of genetic diversity of lettuce strains and soil strains*

Among the 67 genotyped lettuce strains and 66 soil strains, two lettuce strains carried the private allele at microsatellite locus BC6 associated with the cryptic species *B. pseudocinerea* (Walker *et al.*, 2011) and were removed from further analyses. We thus compared the indices of genetic diversity based on the haplotypes obtained for the 131 remaining strains considered to be *B. cinerea* (65 lettuce and 66 soil strains). The allelic richness and the gene diversity per locus were not significantly different (test of Mann-Whitney) between lettuce and soil strains ( $P = 0.3518$  and  $P = 0.1840$ , respectively) (Table 2). However, the lettuce strains showed higher genetic diversity than soil strains when considering the unbiased gene diversity within the 9 loci, the mean number of alleles per locus and the haplotypic diversity (Table 3). The evenness index was similar in lettuce strains and in soil strains indicating a similar distribution of haplotypes in both groups of strains. Seven MLH were shared by lettuce strains and soil strains, representing a total of 30 strains. Four MLH were found both in lettuce and in soil sampled just after lettuce harvest. Others were shared by lettuce and soil strains sampled several months apart (Table 4). The  $r_d$  values estimating linkage disequilibrium were highly significant both for lettuce and soil strains (Table 3) thus rejecting the null hypothesis of random mating in both groups of strains.

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### *Genetic differentiation between lettuce strains and soil strains.*

The global degree of genetic differentiation between lettuce and soil strains was significant but very low ( $F_{ST} = 0.03$ ,  $P < 0.01$ ). This result is consistent with the result of AMOVA analysis showing that only 2.75% ( $P < 0.001$ ) of genetic variation occurred between lettuce and soil strains. The greatest variation (93.47%,  $P = 0.10$ ) occurred within sampling dates inside each group (Table 5).

In order to determine a possible relationship between the strains present on diseased lettuce and soilborne inoculum, pairwise  $F_{ST}$  were calculated between strains sampled from soil before crop planting, from lettuce at harvest, and from soil after harvest (Table 6). In spring 2009, moderate and highly significant  $F_{ST}$  were encountered between strains sampled from lettuce and strains sampled from soil before and after the crop. In winter 2009, a significant  $F_{ST}$  value was also encountered between strains collected on lettuce and strains collected in soil before lettuce planting but its value was low (Table 6). In spring 2010 there was no genetic differentiation between strains collected on lettuce and those collected in soil before or after the crop. The genetic differentiation between the soilborne inoculum and the inoculum sampled from lettuce plants seemed to decrease progressively over the three successive lettuce crops.

### *Comparison of the genetic structure of lettuce strains and soil strains*

Bayesian clustering was carried out using 98 MLH (clones were removed from each date for the analysis) in order to assess the genetic structure of *B. cinerea* strains sampled from lettuce and from soil (only in tunnel 7). The highest probability given by the maximum likelihood distribution was obtained for  $K=4$ . The percentage of strains assigned to the 4 genetic clusters and of strains not assigned ( $P < 0.80$ ) at each date is shown in Table 7. Before and after the first lettuce crop (February and April 2009) the soils strains were mainly assigned to one

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cluster (cluster 2). The lettuce strains (April 2009) were distributed among the four clusters, but few were assigned to cluster 2 (Table 7). After one month of fallow and two months of solarisation, a quarter of the soil strains were not clearly assigned to a cluster. The strains collected from the lettuce in December 2009 were also partly not assigned to a cluster (13%) whereas those collected from soil just after harvest were again mostly assigned to cluster 2, as in the previous year. Finally, strains collected from the third lettuce crop in 2010 and those from soil just after that harvest were assigned to the same 3 clusters, with comparable proportions for lettuce and for soil strains.

## Discussion

This study is the first attempt to characterize the inoculum of *B. cinerea* and to investigate its genetic diversity in soil. Based on the genetic structure and differentiation of 66 strains collected from the soil and 65 strains collected from diseased plants, it provided evidence for flows of inoculum between the crop and the soil where it is grown.

The first key result of the present study is the nearly consistent presence of viable propagules of *B. cinerea* in the soil of two greenhouses studied during four consecutive lettuce crops. Based on dilution plating of soil samples, inoculum levels from 0 to 1177 propagules per gram were found in the first 30 cm of soil. These results raise the question of the nature of this inoculum, provided that sclerotia were never observed despite careful examination of the soil samples. Considering that large pieces of plant debris were not present in our soil samples, our hypothesis is that conidia or minute pieces of mycelium-carrying plant debris may have been present in the soil. The operations related to harvest, including the handling of lettuce heads and removal of senescent and diseased leaves, are likely to have released both conidia (abundantly present on some of the plants) and contaminated plant debris on the soil. As the mulch was removed immediately after harvest and discarded, the soil surface may thus

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have been contaminated by the deposition of conidia and pieces of colonized plant debris. Subsequent soil cultivation would then have buried this inoculum, which could have possibly survived until the next soil sampling date, provided that temperatures remained favourable (Moyano & Melgarejo, 2002). This hypothesis is consistent with the previous observation of non-sclerotial inoculum in soil samples taken from strawberry fields in Norway (Strømeng *et al.*, 2009). These soilborne propagules can potentially serve as primary inoculum for grey mould epidemics on either subsequent lettuce or other susceptible crops grown in rotation with lettuce, such as tomato (Leyronas *et al.*, 2014). The inoculum present near the surface of the soil could infect directly the aerial parts of susceptible plants in contact with the soil. The inoculum buried more deeply in the soil could possibly also play a role in disease development, when moved to the surface by soil cultivation. Although not much is known about the possible epidemiological relevance of root colonization of susceptible plants by *B. cinerea*, it could be hypothesized that it may play a role in its endophytic development reported for a variety of plants, including lettuce (Sowley *et al.*, 2010).

The second key result of the present study is evidence for flows of *B. cinerea* inoculum between the lettuce crops and the soil. Direct evidence of inoculum flow was provided by the observation of seven haplotypes shared between soil and lettuce, amounting to 23% of the total number of strains. Moreover the genetic differentiation between soilborne inoculum and the inoculum sampled from lettuce plants (assessed by  $F_{ST}$  values) decreased with the four consecutive lettuce crops over the course of the study. This was paralleled by an increasing similarity of the genetic structure of the two groups of stains. Based on our data it may also be possible to propose a hypothesis on the general direction of these flows of inoculum, mainly from diseased plants to the soil. The absence of correlation between the abundance of soilborne inoculum before the planting of the lettuce crop and the incidence of grey mould at harvest suggests that other sources of inoculum may also have contributed to the epidemics in

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the two greenhouses. Air is one likely additional source of inoculum as it has been shown that exchange of inoculum can occur between the inside and the outside of a greenhouse through vents (Korolev *et al.*, 2006; Leyronas *et al.*, 2011; Bardin *et al.*, 2014). The airborne conidia that deposited in the tunnels may have been collected by the mulch covering the soil and carried by sprinkler irrigation water to zones that retain humidity, such as the collar area of the plants and the underside of the basal leaves. The hypothesis of a contribution of airborne inoculum to disease development in our study is further supported by the generally higher genetic diversity observed for strains collected from lettuce than for those from soil (Table 3) and by the significant genetic variation among dates (Table 5)... Formal assessment of such a hypothesis would be possible in further work by setting up concomitant quantification and genetic characterization of airborne and soilborne inoculum. Such studies could also help to assess a possible role of airborne inoculum abundance in the higher incidence of disease observed in spring than in winter on both years of our study (Fig. 1). Other hypotheses to explain these consistent differences could be the occurrence of more favourable environmental conditions for *Botrytis* development in spring than in winter, as the development of the fungus is known to be strongly reduced for temperatures below 10°C (see for example Sosa-Alvarez *et al.*, 1995). Temperature differences may explain in part our 2009 results, as the mean temperatures in February and April were 8.8°C and 13.4°C, respectively (platform CLIMINRA, US1116, AGROCLIM, F-84000 Avignon). Differences in temperatures were not as contrasted between December 2009 (8.1°C) and March 2010 (9.4°C) and point to another possible source of variability, as two different lettuce varieties were used (Table 1).

The lack of efficiency of soil solarisation to reduce soilborne inoculum of *B. cinerea* in tunnel 7 came as a surprise. In south-eastern Spain, solarisation has been reported as an efficient method to reduce soilborne inoculum in the summer (Lopez-Herrera *et al.*, 1994; Moyano &

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Melgarejo, 2002). In our study, soil temperature (not recorded) may not have been sufficiently increased within the 30 cm layer that was sampled. Although the sanitation practices did not reduce soilborne inoculum, they appeared to induce substantial changes in the genetic structure of soilborne strains, affecting their distribution among genetic clusters. This was supported by the significant genetic differentiation ( $F_{ST} = 0.07$ ;  $P < 0.01$ ) measured among soilborne strains before and after the sanitation measures. As far as we know, there is no literature dealing with the effect of soil sanitation practices on the genetic structure of soilborne fungal strains. However, Gelsomino & Cacco (2006) reported evidence for an influence of soil solarisation on the diversity of soilborne bacteria by promoting changes in species composition and in species richness. We hypothesize that in our study, the fungal strains showing higher resistance to heat may have been selected by solarisation, thus changing the genetic structure of soilborne inoculum. If those selected strains had overall lower aggressiveness on lettuce than the initial population, the reduction in observed disease despite the lack of reduction in inoculum abundance could thus be explained. Future work should focus on a phenotypic characterization of strains to test these hypotheses.

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## References

Agapow PM, Burt A, 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes* **1**, 101-2.

Arnaud-Haond S, Belkhir K, 2007. Genclone: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes* **7**, 15-17

Arnaud-Haond S, Duarte CM, Alberto F, Serrao EA, 2007. Standardizing methods to address clonality in population studies. *Molecular Ecology* **16**, 5115-39.

Bardin M, Decognet V, Nicot PC, 2014. Remarkable predominance of a small number of genotypes in greenhouse populations of *Botrytis cinerea*. *Phytopathology* doi: 10.1094/PHYTO-10-13-0271-R

Blanco C, de los Santos B, Romero F, 2006. Relationship between concentrations of *Botrytis cinerea* conidia in air, environmental conditions, and the incidence of grey mould in strawberry flowers and fruits. *European Journal of Plant Pathology* **114**, 415-25

Coley-Smith JR, 1980. Sclerotia and other structures in survival. In: Coley-Smith JR, Verhoeff K, Jarvis WR (eds) *The biology of Botrytis*. 85-114. Academic Press, London UK

Dik AJ, Wubben JP, 2004. Epidemiology of *Botrytis* diseases in greenhouses. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds) *Botrytis: biology, pathology and control*. 319-31. Kluwer Academic Publishers, Dordrecht, The Netherlands.

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Epton HAS, Richmond DV, 1980. Formation, structure and germination of conidia. In : Coley-Smith JR, Verhoeff K, JarvisWR (eds) *The biology of Botrytis*. 41-84. Academic Press, London UK

Excoffier L, Laval G, Schneider S, 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47-50.

Edwards SG, Seddon B, 2001. Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia. *Letters in Applied Microbiology* **32**, 63-66

Falush D, Stephens M, Pritchard JK, 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**, 1567-87

Fournier E, Giraud T, Loiseau A, Vautrin D, Estoup A, Solignac M, Cornuet JM, Brygoo Y, 2002. Characterization of nine polymorphic microsatellite loci in the fungus *Botrytis cinerea* (Ascomycota). *Molecular Ecology Notes* **2**, 253-55.

Gelsomino A, Cacco G, 2006. Compositional shifts of bacterial groups in a solarized and amended soil as determined by denaturing gradient gel electrophoresis. *Soil Biology and Biochemistry* **38**, 91-102

Goudet J, 1995. FSTAT (Vers.1.2): a computer program to calculate F-statistics. *Journal of heredity* **86**, 485-86

This article is protected by copyright. All rights reserved.

Harrison JG, Lowe R, 1987. Wind dispersal of conidia of *Botrytis* spp. pathogenic to *Vicia faba*. *Plant Pathology* **36**, 5-15.

Holz G, Coertze S, Williamson B, 2004. The ecology of *Botrytis* on plant surfaces. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds) *Botrytis: biology, pathology and control*. 9-27. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Jarvis WR, 1962. The dispersal of spores of *Botrytis cinerea* Fr. in a raspberry plantation. *Transactions of the British Mycological Society* **45**, 549-59.

Johnson KB, Powelson ML, 1983. Analysis of spore dispersal gradients of *Botrytis cinerea* and gray mold disease gradients in snap beans. *Phytopathology* **73**, 741-746

Korolev N, Katan T, Elad Y, 2006. The use of selenate-resistant strains as markers for the spread and survival of *Botrytis cinerea* under greenhouse conditions. *Phytopathology* **96**,1195-1203

Leroux P, 2004. Chemical control of *Botrytis* and its resistance to chemical fungicides. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds) *Botrytis: biology, pathology and control*. 195-222. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Leyronas C, Bryone F, Duffaud M, Troulet C, Nicot PC, 2014. Assessing host specialization of *Botrytis cinerea* on lettuce and tomato by genotypic and phenotypic characterization. *Plant Pathology* DOI: 10.1111/ppa.12234

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Leyronas C, Duffaud M, Nicot PC, 2012. Compared efficiency of the isolation methods for *Botrytis cinerea*. *Mycology: An International Journal on Fungal Biology* **3**, 221-225

Leyronas C, Fatnassi H, Bardin M, Boulard T, Nicot PC, 2011. Modelling *Botrytis cinerea* spore exchanges and production in unheated greenhouses. *Journal of Plant Pathology* **93**, 407-14

Leyronas C, Nicot PC, 2013. Monitoring viable airborne inoculum of *Botrytis cinerea* in the South-East of France over 3 years: relation with climatic parameters and the origin of air masses. *Aerobiologia* **29**, 291-99

López-Herrera CJ, Verdú-Valiente B, Melero-Vara JM, 1994. Eradication of primary inoculum of *Botrytis cinerea* by soil solarisation. *Plant Disease* **78**, 594-97

Moyano C, Melgarejo P, 2002. Survival of *Botrytis cinerea* in soil in South-Eastern Spain. *Journal of Phytopathology* **150**, 536-40.

Nei M, 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583-90

Nicot PC, Mermier M, Vaissière BE, Lagier J, 1996. Differential spore production by *Botrytis cinerea* on agar medium and plant tissue under near-ultraviolet light-absorbing polyethylene film. *Plant Disease* **80**, 555-58

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Raposo R, Gomez V, Urrutia T, Melgarejo P, 2001. Survival of *Botrytis cinerea* in southeastern Spanish greenhouses. *European Journal of Plant Pathology* **107**, 229-36.

Rodríguez-Rajo FJ, Jato V, Fernández-González M, Aira MJ, 2010. The use of aerobiological methods for forecasting Botrytis spore concentrations in a vineyard. *Grana* **49**, 56-65

Sosa-Alvarez M, Madden LV, Ellis MA, 1995. Effects of temperature and wetness duration on sporulation of *Botrytis cinerea* on strawberry leaf residues. *Plant Disease* **79**, 609-15

Sowley ENK, Dewey FM, Shaw MW, 2010. Persistent, symptomless, systemic and seed-borne infection of lettuce by *Botrytis cinerea*. *European Journal of Plant Pathology* **126**, 61-71

Stapleton JJ, DeVay JE, 1986. Soil solarization: a non-chemical approach for management of plant pathogens and pests. *Crop Protection* **5**, 190-98.

Strømeng GM, Hjeljord LG, Stensvand A, 2009. Relative contribution of various sources of *Botrytis cinerea* inoculum in strawberry fields in Norway. *Plant Disease* **93**, 1305-10

Verhoeff K, 1980. The infection process and host pathogen interactions. In : Coley-Smith JR, Verhoeff K, Jarvis WR (eds) *The biology of Botrytis*. 153-181. Academic Press, London UK

Vig AP, Rampal G, Singh T, Arora S, 2009. Bio-protective effects of glucosinolates-A review. *LWT-Food Science and Technology* **42**, 1561-72.

This article is protected by copyright. All rights reserved.

Walker AS, Gautier A, Confais J, Martinho D, Viaud M, Le Pêcheur P, Dupont J, Fournier E, 2011. *Botrytis pseudocinerea*, a new cryptic species causing gray mold in French vineyards in sympatry with *Botrytis cinerea*. *Phytopathology* **101**, 1433-45

Yunis H, Elad Y. 1989. Survival of *Botrytis cinerea* in plant debris during summer in Israel. *Phytoparasitica* **17**, 13-21

Table 1: Chronology of cropping practices and collection of *B. cinerea* samples from soil in two experimental tunnels (T5 and T7).

Table 2: Allelic richness based on a minimum sample size of 39 strains (AR) and unbiased gene diversity (H<sub>nb</sub>) for each microsatellite locus in *B. cinerea* strains sampled from lettuce and from soil.

Table 3: Genetic diversity and linkage disequilibrium of strains sampled from lettuce and from soil.

Table 4: Occurrence of haplotypes of *B. cinerea* shared by the samples taken from lettuce and soil over the duration of the study.

Table 5: Hierarchical analysis of molecular variance (AMOVA) with strains' origin (lettuce vs soil) as grouping factor.

Table 6: Assessment of genetic differentiation among *B. cinerea* strains according to sampling date.

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Table 7: Distribution of lettuce and soil strains collected in tunnel T7 among the four genetic clusters determined by Bayesian computation.

Figure 1: Incidence of grey mould (green bars, left axis) and abundance of soilborne *B. cinerea* inoculum (black line, right axis) over 4 successive lettuce crops. A : in tunnel 5. B: in tunnel 7. Error bars indicate the standard error of the mean.

Table 1

Cropping practices		Dates of soil sampling
Tunnel T5	Tunnel T7	
Lettuce cv. Fidel 1 Oct. 08 to 19 Feb. 09	Lettuce cv. Fidel 1 Oct. 08 to 19 Feb. 09 <sup>a</sup>	24 Feb. 09 <sup>a</sup>
Lettuce cv. Fidel 3 March 09 to 22 Apr. 09	Lettuce cv. Fidel 3 March 09 to 22 Apr. 09 <sup>a</sup>	23 April 09 <sup>a</sup>
Mustard crop 5 May 09 to 18 June 09	Fallow 23 April 09 to 18 June 09	5 May 18 June 09
Solarisation 18 June 09 to 21 Sept. 09	Solarisation 18 June 09 to 21 Sept. 09	21 Sept. 09 <sup>a</sup>
Lettuce cv. Arcadia	Lettuce cv. Arcadia	4 Dec. 09 <sup>a</sup>

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7 Oct. 09 to 2 Dec. 09	7 Oct. 09 to 2 Dec. 09 <sup>a</sup>	
Lettuce cv. Fidel	Lettuce cv. Fidel	1 April 10 <sup>a</sup>
11 Jan. 09 to 30 March 10	11 Jan. 09 to 30 March 10 <sup>a</sup>	

a: lettuce and soil isolates sampled in T7 at these dates underwent genotyping. For all the other dates we only carried out a quantification of CFU in soil and an evaluation of grey mould incidence.

Table 2

locu s	BC1		BC2		BC3		BC4		BC5		BC6		BC7		BC9		BC10	
	A R	H nb	A R	H nb	A R	H nb	A R	H nb	A R	H nb	A R	H nb	A R	H nb	A R	H nb	A R	H nb
lett uce	16 .4	0. 88	11 .7	0. 86	10 .7	0. 86	2. 9	0. 46	9. 9	0. 80	14 .6	0. 86	9. 9	0. 87	7. 8	0. 53	10 .7	0. 85
soil	12 .0	0. 84	12 .0	0. 90	9. 0	0. 77	2. 0	0. 33	8. 0	0. 84	9. 0	0. 66	8. 0	0. 83	5. 0	0. 19	12 .0	0. 85

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Table 3

Strains origin	Number of isolates	Hnb <sup>a,f</sup>	Mean number of alleles per locus	Number of distinct MLH <sup>b</sup>	Haplotypic diversity	Evenness	r <sub>d</sub> <sup>c,d,e</sup>	
lettuce	<b>total</b>	<b>65</b>	<b>0.77</b> <b>(0.16)</b>	<b>10.8</b>	<b>52</b>	<b>0.80</b>	<b>0.84</b>	<b>0.15</b> <b>**</b>
	April 2009	24	0.75 (0.13)	7.3	21	0.56	0.46	0.23 <b>**</b>
	Dec. 2009	27	0.72 (0.20)	6.7	22	0.80	0.67	0.08 <b>**</b>
	March 2010	14	0.73 (0.23)	6.1	12	0.84	0.54	0.29 <b>**</b>
soil	<b>total</b>	<b>66</b>	<b>0.68</b> <b>(0.25)</b>	<b>8.5</b>	<b>39</b>	<b>0.58</b>	<b>0.85</b>	<b>0.10</b> <b>**</b>
	Feb. 2009	8	0.35 (0.23)	2.0	5	0.57	0	0.16 NS
	April 2009	13	0.56 (0.24)	4.0	11	0.83	0.55	0.25 HS
	Sept. 2009	24	0.72 (0.20)	6.3	15	0.60	0.52	0.04 *
	Dec. 2009	6	0.61 (0.26)	3.0	5	0.80	0	-0.005 NS

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April 2010	15	0.59 (0.24)	3.1	7	0.42	0.62	0.12 **
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a : unbiased gene diversity (standard deviation in brackets)

b : multilocus haplotype

c : linkage disequilibrium

d : not significant (NS), significant (\*), highly significant (\*\*) at the 5% level

e: computed with clone-corrected data (data set with only one example of each haplotype) to remove bias of overrepresentation of clones.

Table 4

Haplotypes	Total number of copies observed in the study	Origin and time of sampling							
		Soil Feb. 2009	Lettuce Apr. 2009	Soil Apr. 2009	Soil Sept. 2009	Lettuce Dec. 2009	Soil Dec. 2009	Lettuce March 2010	Soil Apr. 2010
A <sup>a</sup>	3							2	1
B	3		1	1				1	
C	4		3	1					
D	4					1			3

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E	5	1	1			3			
F	5		2			2			1
G	6					3	1		2

a : The letters used to name the haplotypes were assigned arbitrarily

Table 5

Sources of variation	d.f	Sum of squares	Variance components	Percentage of variation	<i>P</i> value
Between lettuce and soil strains	1	9.58	0.09	2.75	<0.0001
Between dates	6	27.98	0.12	3.78	0.0008
Within dates	90	287.53	3.19	93.47	0.1084

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Table 6

Sampling date	24 Feb. 2009	22 Apr. 2009	23 Apr. 2009	21 Sept. 2009	2 Dec. 2009	4 Dec. 2009	30 Mar. 2010	1 Apr. 2010
Origin of samples	soil	lettuce	soil	soil	lettuce	soil	lettuce	soil
F <sub>ST</sub>	0.150**	0.133**	0.070**	0.025*	0.023	0.056	0.030	
					NS	NS	NS	

\* Significant ( $P < 0.05$ ), \*\* highly significant ( $P < 0.01$ ), NS not significant at the level of 5%

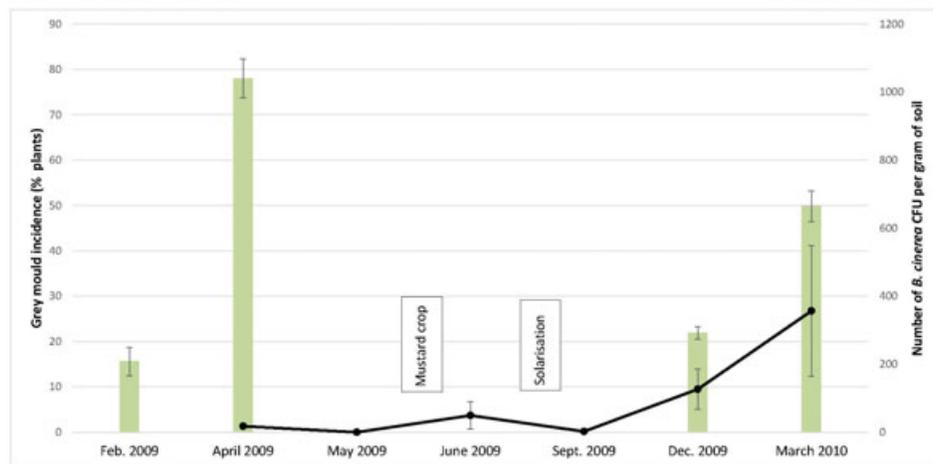
Table 7:

	Origin and time of sampling									
Date	Feb. 2009	Apr. 2009	Apr. 2009	Summer 2009	Sept. 2009	Dec. 2009	Dec. 2009	Mar. 2010	Apr. 2010	
Soil occupation	Soil	Lettuce	Soil	Fallow+ solarisation	Soil	Lettuce	Soil	Lettuce	Soil	
Genetic structure <sup>a</sup>										

a :  : cluster 1,  : cluster 2,  : cluster 3,  : cluster 4,  : not assigned

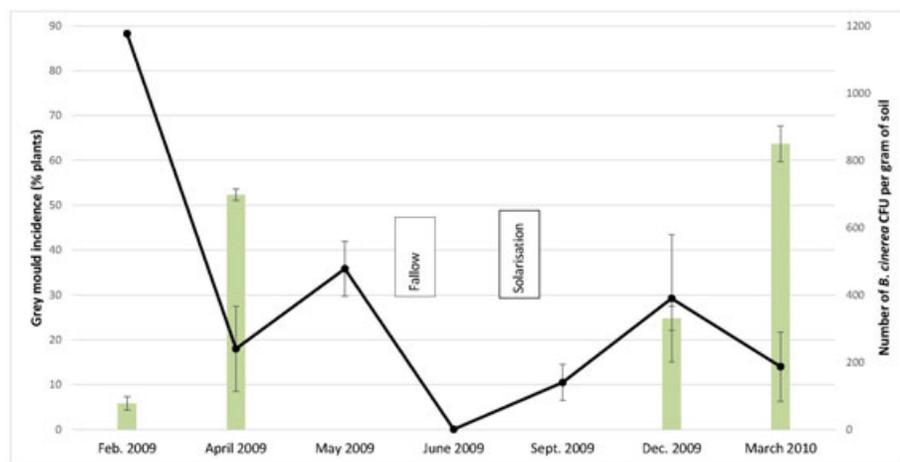
Figure 1

A



1

B



(Soil sample missing on Feb. 09 in T5 ; single soil sample on Feb. 09 in T7, no standard error of the mean)

2

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