Compared dynamics of grey mould incidence and genetic characteristics of Botrytis cinerea in neighbouring vegetable greenhouses

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COMPARED DYNAMICS OF GREY MOULD INCIDENCE AND GENETIC CHARACTERISTICS OF *BOTRYTIS CINEREA* IN NEIGHBOURING VEGETABLE GREENHOUSES.

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SUMMARY

Production of vegetables in southern France often relies on groups of greenhouses located in close vicinity. These crops are commonly affected by grey mould caused by *Botrytis cinerea*, a fungus known for its ability to produce abundant airborne inoculum. Possible exchange of inoculum could affect the epidemics developing in neighbouring greenhouses. To test this hypothesis, grey mould incidence was assessed in four successive crops in six unheated polyethylene tunnels located in the region of Avignon. On lettuce, the incidence was similar for a given harvest date in two tunnels where this crop was grown four times consecutively. In the four other tunnels, lettuce was grown in alternation with tomato. No grey mould developed on tomato and disease incidence was low on lettuce. One hundred and seventy four strains collected from lettuce in two tunnels were investigated for their genetic diversity, genetic structure and their mating type. Both known mating types of *B. cinerea* were observed in the

tunnels but MAT1-1 was prevalent. The gene diversity of *B. cinerea* strains was similar in both tunnels. However haplotypic diversity and linkage disequilibrium were substantially higher in one tunnel. We hypothesize that this situation is related to differences in microclimatic conditions in the tunnels. It highlights a possible interest in individualizing disease management in the different tunnels of a given farm.

Keywords: tunnel, inoculum, tomato, lettuce

INTRODUCTION

In south-eastern France many vegetables, berries and flowers are grown under greenhouse. According to the 'Agreste' statistics of the French Ministry of Agriculture (Anonymous, 2014) for the 'Provence-Alpes-Côte-d'Azur' region, located on the east side of the Rhône Valley and bordered by the Mediterranean Sea, there are 2500 hectares of greenhouses. The greenhouses constitute shelters in which climatic conditions foster plant growth but also the development of diseases. One of the most damageable plant pathogens for crops grown under greenhouse (lettuce, tomato, strawberry among others) is *Botrytis cinerea*. This ascomycete fungus is the causal agent of grey mould. The symptoms of this disease are necrotic lesions on stems, leaves and fruits that make the products unmarketable and that may even lead to plant death.

Grey mould epidemics can be induced by soilborne or airborne inoculum. *B. cinerea* inoculum can be present in the soil in the form of sclerotia, conidia (mitotic spores) or mycelium on plant debris (Coley-Smith, 1980; Raposo *et al*., 2001; Strømeng *et al*., 2009; Leyronas *et al*., 2014a). *B. cinerea* is considered to lack host specialization (Leyronas *et al*., 2014b) and soilborne propagules are likely to carry over the inoculum to successive susceptible crops. However *B. cinerea* is considered to be predominantly airborne disseminated. Indeed, when climatic conditions are favourable, the lesions formed on aerial parts of plants become covered

with large amounts of conidia (Nicot *et al*., 1996) that can be easily dispersed by wind (Jarvis, 1962; Harrison and Lowe, 1987). These conidia can serve as primary inoculum since they are present almost all the time in the air (Leyronas and Nicot, 2013). They also play a major role in the rapid colonization of neighbouring plants when conditions are favourable (Holz *et al*., 2004) and they can spread outside of the greenhouse. Although greenhouses are often regarded as quasi-closed systems, exchange of *B. cinerea* inoculum can occur between the inside and the outside of a greenhouse through vents (Korolev *et al*., 2006; Leyronas *et al*., 2011). The dissemination of *B. cinerea* airborne inoculum is also likely to occur on a larger scale. Strains of *B. cinerea* showing an identical haplotype (based on 9 microsatellites markers) were collected in tomato greenhouses located 23 km apart in northern Algeria (Adjebli *et al.*, 2014) and 220 km apart in southern France (Bardin *et al*., 2014). The hypothesis that airborne conidia have been carried by air masses between these greenhouses appears relevant since it is known that long distant dispersal of fungal spores can spread diseases on regional and continental scales (Brown and Hovmøller, 2002). The absence of differentiation of *B. cinerea* populations between several vegetable greenhouses reported by Alfonso *et al.* (2000) in southern Spain could be due in part to such dissemination, resulting in the homogenisation of airborne inoculum.

In horticultural farms of south-eastern France, several vegetable greenhouses are usually located in close vicinity. Since this region is predominantly scoured by a strong northern wind (Mistral), the greenhouses are aligned, side by side, in a north-south direction. Groups of greenhouses are often protected by a high cypress hedge to attenuate strong air currents that may damage the structures. Species susceptible to grey mould are often cultivated concomitantly in these adjacent greenhouses or in rotation in the same greenhouse. Control methods are mainly based on fungicidal treatments applied on aerial parts of plants but this can be complicated by the appearance of resistant strains (Leroux, 2004). Moreover the cryptic

species *B. pseudocinerea* (Walker *et al.*, 2011) causing the same symptoms of grey mould is naturally resistant to the hydroxyanilide fungicide fenhexamid (Leroux *et al.*, 1999; 2002). Climate management inside greenhouses, particularly the control of temperature coupled to relative humidity, also constitutes a tool to reduce disease outbreaks (Jewett and Jarvis 2001; Dik and Wubben, 2004). In the Mediterranean climate, there may be wide thermic amplitude between night and day, inducing dew formation on plants and enhancing grey mould development. Heating can prevent dew formation in glasshouses equipped with heating system. In non-heated polyethylene greenhouses (tunnels), climate is only minimally controlled through the opening of lateral and top vents.

In order to reduce the risk of fungicide resistance appearance and to reduce the cost of chemical and energy inputs in greenhouses, the cultural practices to control grey mould may be enhanced. The objective of this study was to test the hypothesis that *B. cinerea* inoculum and grey mould epidemics are similar in neighbouring greenhouses where a same crop is grown. To this aim, we monitored grey mould incidence in six neighbouring tunnels in south-eastern France over four successive crops grown during a year-and-a-half-period. A collection of *B. cinerea* isolates sampled from diseased lettuce was genetically characterized and its diversity was assessed over time and space.

MATERIALS AND METHODS

Experimental set up

From 2006 to 2008, vegetable crops were grown in six experimental unheated polyethylene greenhouses (tunnels numbered from T1 to T6). These tunnels were built in 2006 specifically for this experiment, on a plot located at the Plant Pathology research unit of the French National Institute of Agricultural Research (INRA) in southern France (lat. 43.93N; long. 4.88E). All

100 the tunnels were oriented north-south. Tunnel T1 was the most eastern and T6 was the most
101 western of the group. The tunnels each covered an area of 128 m² and were located 4 m apart.
102 In T1 and T6 four successive butterhead lettuce crops (cv. Faustina and Leandra) were
103 grown (Table 1). In T2, T3, T4 and T5, two lettuce crops (cv. Leandra) were grown in rotation
104 with two tomato crops (cv. Swanson). During lettuce cropping the tunnels each contained 14
105 rows of 93 plants with a density of 12 lettuces per square meter. The crops were conducted on
106 a micro-perforated polyethylene mulch mat. Irrigation was realised by sprinkling. The plants
107 were efficiently protected against downy mildew with fungicide treatments applied until the
108 18-leaf stage according to common practice in commercial production. During tomato
109 cropping, the tunnels each contained 8 rows of 29 plants that were drip irrigated. Throughout
110 all experiments, the climate inside the tunnels was minimally controlled through the opening of
111 lateral vents. The vents and doors of all tunnels were kept fully open during the warm months
112 (from early May to mid-September) and closed during the cold season (from early November
113 to end of February). During the intermediate periods, the opening of the vents was adjusted
114 daily according to the inside temperature.
115 Throughout the growing seasons, the crops were examined daily for the presence of
116 pests and diseases. In the tomato crop, axillary shoots were removed twice a month and fruits
117 were harvested once a week starting in late May. Lettuce harvest was completed within one day
118 at the end of each cropping season (Table 1). After each lettuce and tomato culture, the plant
119 residues were removed with the mulch mat and the soil in the tunnels was tilled and left fallow
120 until the next crop.
121
122 **Grey mould incidence at harvest and isolate collection**
123 On lettuce crops, grey mould incidence was evaluated at harvest by recording the presence of
124 grey mould symptoms on every plant (1302 per tunnel). To account for possible spatial

variability, each tunnel was divided in four sectors. Mean grey mould incidence and its associated standard error were calculated for each tunnel. On tomato crops, the presence of grey mould was monitored once-a-week during the growing season.

Isolates of *B. cinerea* were sampled on lettuce plants at harvest time in T1 and T6, by rubbing dry sterile cotton buds on sporulating lesions. A total of 40 samples were collected throughout each tunnel. The cotton buds were then stored at -20°C until isolate purification. All isolates used in this study were purified and single-spored in a classical way (Leyronas *et al.* 2012) prior to their genotypic characterization.

Isolate genotyping

The isolates were genotyped following the protocol described by Leyronas *et al*. (2014b). In short, genomic DNA was extracted from aliquots of 15 mg lyophilized fungal material 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*. (2014b). The size of the microsatellites were determined with a Megabace sequencer (Amersham Pharmacia) and Genetic Profiler software (Amersham Biosciences) was used for the microsatellite size analysis. Complete microsatellite size profiles (referred to as "haplotypes" hereafter) were obtained for 174 isolates. Hereafter, characterized isolates from tunnels T1 and T6 will be referred to as “T1 strains” and “T6 strains”, respectively.

Genetic diversity of *B. cinerea* strains

Several indices of genetic diversity were used to compare *B. cinerea* strains collected from tunnels T1 and T6 at different dates. Unbiased gene diversity (Hnb) and allelic richness were computed with the Genetix software (Nei, 1978). The number of different multilocus haplotypes (MLH) was computed with GenClone 1.0 software (Arnaud-Haond and Belkhir,

2007) and was used to calculate the haplotypic diversity (computed as the ratio of (number of distinct MLH - 1) over (sample size - 1)) which estimates the proportion of haplotypes present in a group and takes a value of 1 when a group is composed exclusively of unique haplotypes (Arnaud-Haond *et al.*, 2007). The program Multilocus 1.3b was used to calculate the standardized version of index of association (r_D) that is a measure of the multilocus linkage disequilibrium (Agapow and Burt, 2001). It varied between 0 (complete panmixia) and 1 (complete clonality).

Mating types of *B. cinerea* strains

Mating types were utilized as an additional marker to compare the strains collected from tunnels T1 and T6. We used the primers BcMAT1alpha-for and BcMAT1alpha-rev, and BcMAT2HMG-for and BcMAT2HMG-rev described by Duarte (2008). PCR amplification was carried out in a 10 µL reaction mixture, containing 10 ng of genomic DNA, 5 µL of master mix (Qiagen), 1 µL of solution Q, 1.6 µL of water and 0.2 µL of each primer (10 µM). The targeted fragments were amplified as follows: activation of Taq polymerase at 94°C for 15 min; 40 cycles of denaturation at 94°C for 30 sec, hybridization at 51°C for 1.5 min and extension at 72°C for 1.5 min. A final extension phase was performed at 72°C for 10 min. PCR products were observed under UV light on a 1% agarose gel electrophoresis containing 0.5 µg/L of ethidium bromide). Strains SAS56 and SAS405 of *B. cinerea* were used as reference for MAT1-1 and MAT1-2, respectively.

Genetic differentiation between strains from tunnels T1 and T6

The genetic differentiation between strains collected from the two tunnels at different harvest times was assessed with pairwise Weir and Cockerham's F_{ST} indices and with a hierarchical

analysis of molecular variance (AMOVA) performed with version 3.0 of Arlequin software (Excoffier *et al.*, 2005).

Genetic structure of *B. cinerea* populations

The genetic structure of the populations was determined with version 2.3.4 of Structure software (Falush *et al.*, 2003). Since there was no geographical barrier between tunnels T1 and T6, the model with admixture was chosen. We tested values of clusters (K) between 1 and 9, with 10 replicates for each K. Each simulation consisted of 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 of ancestry of a strain in a cluster was greater than the arbitrary threshold of 0.80, this strain was considered to be unambiguously assigned to this cluster.

Statistical analyses

Statistical analyses were performed with StatView (version 5, SAS Institute). Non-parametric tests were used to determine significant differences between tunnels for disease incidence, unbiased gene diversity and allelic richness (Mann-Whitney) and between sampling dates for the distribution of strains among genetic clusters in each tunnel (Kruskal-Wallis). Exact tests of Fisher were realised to determine if there was a link between the distribution of *B. cinerea* isolates among mating types and the tunnel they had been collected from at each harvest date. The tests were computed with the GENEPOP version 3.1c, Prog. STRUC with 500,000 iterations (Raymond and Rousset, 1995). Statistical inferences were made at the 5 % level of significance, unless indicated otherwise.

199 **RESULTS**

200 **Grey mould incidence on crops**

201 No symptom of grey mould developed on tomato plants during the two growing seasons of the
202 study. In contrast, the incidence of grey mould on lettuce in the six tunnels ranged from 0.2 to
203 25.9% (Fig. 1). Lower incidence values were recorded at harvest time in tunnels with an
204 alternation of lettuce and tomato crops (T2 to T5) compared to tunnels with a succession of
205 lettuce crops (T1 and T6). However, such comparison can only be made in February 2008
206 because in 2007 the harvest time of T2, T3, T4 and T5 occurred one month before that of T1
207 and T6. Disease incidence was never significantly different (P>0.99) between T1 and T6. Its
208 variation followed the same pattern in both tunnels (ρ=1.0, P=0.08) over the four lettuce crops,
209 with a noticeable peak in March 2007.

210

211 **Spatial and temporal variability of *B. cinerea* genetic characteristics**

212 Among the 174 isolates fully genotyped, four (three collected from T1 and one from T6) carried
213 a private allele on microsatellite locus BC6 and were identified as *B. pseudocinerea* (Walker *et*
214 *al.*, 2011). They were removed from further analysis. We thus compared the indices of genetic
215 diversity based on the haplotypes obtained for the 170 remaining strains considered to be *B.*
216 *cinerea* (85 in each tunnel). There was no significant difference between strains collected from
217 T1 and those from T6 when considering unbiased gene diversity and allelic richness (P =0.38
218 and P =0.79, respectively) for each of the nine microsatellite loci (Table 2). When considering
219 the four sampling dates taken together, the strains collected from T1 and T6 had similar gene
220 diversity and mean number of alleles per locus (Table 3). However, T1 strains had higher
221 haplotypic diversity and lower linkage disequilibrium than T6 strains.

222 When considering the four sampling dates successively, a different variation pattern was
223 observed in the two tunnels for global unbiased gene diversity (Fig. 2A) but not for haplotypic

diversity (Fig. 2B). Only 2 MLHs were shared by T1 strains and T6 strains (accounting for 2.3% of the total number of strains) with a single copy of these MLHs observed in each tunnel: one associated with 2 strains collected in March 2007 in T1 and T6; one associated with two strains collected in May 2007 in T1 and in June 2006 in T6.

Inside a given tunnel certain MLHs were observed repeatedly at different dates (Table 4). The number of such strains was higher in T6 (5 MLHs representing 31.7 % of all T6 strains) than in T1 (2 MLHs representing 10.5 % of all T1 strains).

Linkage disequilibrium decreased over the study period in T1 and reached a value close to zero indicating the occurrence of substantial level of recombination (Fig. 2C). In contrast, it increased to 0.26 (March 2007) in T6, reflecting a substantial level of clonality.

The *B. cinerea* strains collected in T1 and T6 had predominantly only the MAT1-1 allele (51.7 and 59.1% respectively). Some strains had both alleles MAT1-1 and MAT1-2 (10.8 and 9.4% respectively in T1 and T6). The distribution of strains among mating types varied over time in both tunnels (Fig. 3). There was no relationship between the distribution of strains among mating types and the tunnel of origin on June 2006, March and May 2007 (P>0.5). However in February 2008, the distribution of strains among mating types was not independent of their tunnel of origin (P=0.0004).

Spatial and temporal genetic differentiation between *B. cinerea* strains in tunnels

The global degree of genetic differentiation between T1 strains and T6 strains was very low and not significant (F_{ST} <0.001, P=0.99). When considering each date, F_{ST} values between T1 and T6 strains were also very low (<0.01) and not significant (P>0.40). These results are consistent with those of AMOVA analysis showing that only 0.21% (P=0.40) of genetic variation occurred between T1 and T6 strains (Table 5). In addition to this absence of spatial genetic differentiation, no temporal genetic differentiation was observed between sampling

dates for strains inside a given tunnel (F_{ST} values low <0.05 and not significant). These results are consistent with those of AMOVA analysis showing that no genetic variation occurred between dates in the tunnels (Table 5).

Spatial and temporal variation of *B. cinerea* genetic structure

Bayesian clustering was carried out using 114 MLH (clones were removed from each date in each tunnel for the analysis) in order to assess the genetic structure of *B. cinerea* strains sampled from T1 and from T6. The highest probability given by the maximum likehood distribution was obtained for K=5. The percentage of strains assigned to the 5 genetic clusters and of strains not assigned (P<0.80) at each date is shown in Figure 3. The differences in the distribution of strains in the genetic clusters between T1 and T6 at each sampling date were not statistically significant (P=0.93; 0.57; 0.68; 0.41 for June 2006, March 2007, May 2007 and February 2008, respectively). Inside each tunnel, the distribution of strains among the five genetic clusters seemed to vary over time but there was no significant difference between sampling dates in T1 (P=0.34) nor in T6 (P=0.78).

DISCUSSION

This study is the first attempt to assess the variability of grey mould incidence and the variability of *B. cinerea* strains genetic diversity in six neighbouring tunnels in which lettuce and tomato were cultivated. Based on monitoring of grey mould over four successive crops and on the genetic characteristics of 170 *B. cinerea* strains collected from lettuce, our results supported the hypothesis that grey mould epidemics and the genetic characteristics of *B. cinerea* strains were similar in neighbouring greenhouses where a same plant species was grown. The similar variation of disease incidence in the tunnels monitored in the present study is consistent with results recently reported in another region (Leyronas *et al*., 2014a). The absence of genetic

differentiation of *B. cinerea* strains between both tunnels is consistent with the weak association between population structure and geography, even when assessed on large spatial scales (Fournier and Giraud, 2008; Walker *et al*., 2014). The absence of temporal genetic differentiation of *B. cinerea* strains in each tunnel of the present study is also consistent with the absence of genetic differentiation over time reported by Alfonso *et al.* (2000) in vegetable greenhouses in southern Spain and the stability of genetic structure reported by Walker *et al*. (2014) for strains collected from crops grown in open fields and in greenhouses. The higher abundance of mating type MAT1-1 in both tunnels and the existence of strains showing both mating types are consistent with results previously reported (Beever and Weeds, 2004; Wessels *et al*., 2013).

However, the results of the present study also raise several questions. The first intriguing result is the lower haplotypic diversity observed in the present study (0.55 and 0.65; Table 3) compared to that of strains collected from four successive lettuce crops in another region (0.80; Leyronas *et al*., 2014a). Since the values of gene diversity indices and the numbers of alleles per locus were in the same range in both regions, we expected similar values of haplotypic diversity. The differences found between the two regions may have been induced by differences in local climatic conditions. Indeed, humid periods known to be favourable to *B. cinerea* development (Eden *et al*., 1996) often occur in the Alenya region (on the Mediterranean coast near the Spanish border) due to winds blowing from the sea, while they are much less frequent in Avignon (Rhône Valley). These climatic differences are likely to be the cause of (i) the lower disease incidence observed in this study for lettuce grown in Avignon compared to that in the region of Alenya (Leyronas *et al*., 2014a), and (ii) the absence of disease on tomato in the present study whereas, in similar experimental set up in Alenya, tomato crops are regularly attacked (Leyronas *et al*., 2014b). It could be further speculated that the generally less favourable conditions of Avignon may have allowed only a few strains to multiply, thus

reducing haplotypic diversity. Microclimatic differences might also explain the differences observed between tunnels T1 and T6 in the present study. Although no specific climatic data were collected inside the tunnels, we suspect that conditions were drier in tunnel T6 where lower haplotypic diversity and higher linkage disequilibrium were observed. During the spring of 2006 and of 2007, the averages of the daily relative humidity recorded at the local meteorological station of the Meteo France network (located at INRA-Avignon) were 57.3% and 68.6%, respectively, with minimal values of 34% and 41%, respectively. Tunnel T6 benefited of the longest daily exposure to sunshine and was noticeably warmer than T1, which was rapidly shaded by neighbouring tunnels. These differences in temperature, combined with overall low values of ambient relative humidity, are believed to have created drier conditions inside T6. Further work is needed to investigate possible links between local climatic conditions and the genetic diversity of *B. cinerea*.

Another surprising result of the present study is that tunnels T1 and T6 shared only two haplotypes despite their spatial proximity, the long periods when all vents and doors were kept open and the daily passage of workers inside each tunnel. A higher number of shared haplotypes was expected since Adjebli *et al*. (2014) reported 9 haplotypes shared by tomato greenhouses located 15-23 km apart. The low number of shared haplotypes in the present study may have been caused by limited exchange of air between the tunnels due to their north-south orientation (parallel to the dominant winds) and to the presence of cypress hedges. Another explanation for the differences between the present study and that of Adjebli *et al*. (2014) may originate from differences in plant architecture and sites of *Botrytis* sporulation between the lettuce and tomato crops. In the tomato study, sporulating lesions were observed mostly on stems, enabling the pathogen to be easily dispersed by air currents toward other greenhouses (Adjebli *et al*., 2014). In contrast, sporulating lesions on lettuce were observed under older leaves, near the ground, suggesting that release of inoculum from diseased plants into the air may have been limited

until harvest time. Evidence for this phenomenon is the peak in disease incidence observed in T1 and T6 in March 2007 following the harvest four weeks earlier in tunnels T2-T5 (Fig. 1) for which up to 5% of the plants showed sporulating lesions. Possible exchange of airborne inoculum between tunnels at that particular time was fostered by the circulation of workers and the opening of doors to facilitate harvest operations (Hausbeck and Pennypacker, 1991).

In conclusion, in tunnels, often regarded as quasi-closed systems, some exchange of *B. cinerea* airborne inoculum may happen, and particularly at harvest time due to the handling of diseased plants. Thus it is likely that inoculum released at harvest time may impact the susceptible crops in neighbouring tunnels. Nevertheless, this study also showed that each tunnel harboured a high proportion of strains with specific characteristics and that there were differences in the level of haplotypic diversity, a situation which could have implications for disease management such as the choice of anti-*Botrytis* fungicides and the timing of their application. A low level of haplotypic diversity results from the multiplication of specific strains in a tunnel. If some of these strains happen to be resistant to certain fungicides, a treatment against grey mould may be adequately efficient in one tunnel but less in another. Furthermore, a possible difference in aggressiveness between haplotypes (a point which remains hypothetical at this time) could result in differences in disease severity in tunnels with similar microclimatic environment, with consequences on the timing of intervention by the grower. Altogether, these considerations point to a possible interest in individualizing disease management in the different tunnels of a given farm.

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Table 1. Successions of lettuce and tomato crops in the six experimental tunnels.

Dates	T1 and T6	T2, T3, T4 and T5
5April to 8 June 2006 20 April to 5 October 2006	Lettuce (cv. Faustina)	 Tomato (cv. Swanson)
30 November to 22 February (T2-T5) or 19 March 2007 (T1, T6)	Lettuce (cv. Leandra)	
3 April to 31 May 2007 26 April to 19 July 2007	Lettuce (cv. Leandra)	 Tomato (cv. Swanson)
15 November 2007 to 19 February 2008	Lettuce (cv. Leandra)	

Table 2. Allelic richness (AR) and unbiased gene diversity (Hnb) of *B. cinerea* strains collected from tunnels T1 and T6, at each of 9 microsatellite loci.

	BC1		BC2		BC3		BC4		BC5		BC6		BC7		BC9		BC10	
	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb	AR	Hnb
T1	15	0.90	12	0.88	13	0.87	3	0.25	11	0.88	18	0.83	10	0.85	8	0.50	14	0.89
T6	16	0.92	14	0.91	13	0.87	4	0.34	12	0.83	14	0.88	9	0.80	7	0.46	13	0.90

Table 3. Genetic characteristics of *B. cinerea* strains collected from tunnels T1 and T6.

	Sample size	Gene diversity	Mean number of alleles per locus	Number of distinct haplotypes	Haplotypic diversity	r_d[a]
T1	85	0.76 (0.22)	11.60	56	0.65	0.09 **
T6	85	0.77 (0.21)	11.33	48	0.55	0.12 **

a: linkage disequilibrium

**: statistically highly significant (P<0.01)

Table 4. Frequency distribution of *B. cinerea* haplotypes common to several dates in T1 and T6.

	MLH code	Total number of copies	June 2006	March 2007	May 2007	February 2008
T1	a	2	1		1	
	b	7		5	1	1
T6	c	2		1	1	
	d	3	1			2
	e	3			1	2
	f	8	3	4		1
	g	11		3	2	6

Table 5. Hierarchical analysis of molecular variance (AMOVA) with the origin of strains (T1 *vs* T6) as grouping factor.

Sources of variation	d.f	Sum of squares	Variance components	Percentage of variation	*P* value
Between T1 and T6 strains	1	3.89	0.007	0.21	0.40
Between dates	6	20.90	-0.0003	-0.01	0.47
Within dates	106	369.78	3.48	99.8	0.29

Fig. 1. Incidence of grey mould in Tunnels 1 to 6. Four successive lettuce crops were grown in T1 and T6 (plain black and grey bars); two lettuce crops in rotation with two tomato crops were grown in the four other tunnels (bars with patterns). Error bars indicate the standard error of the mean

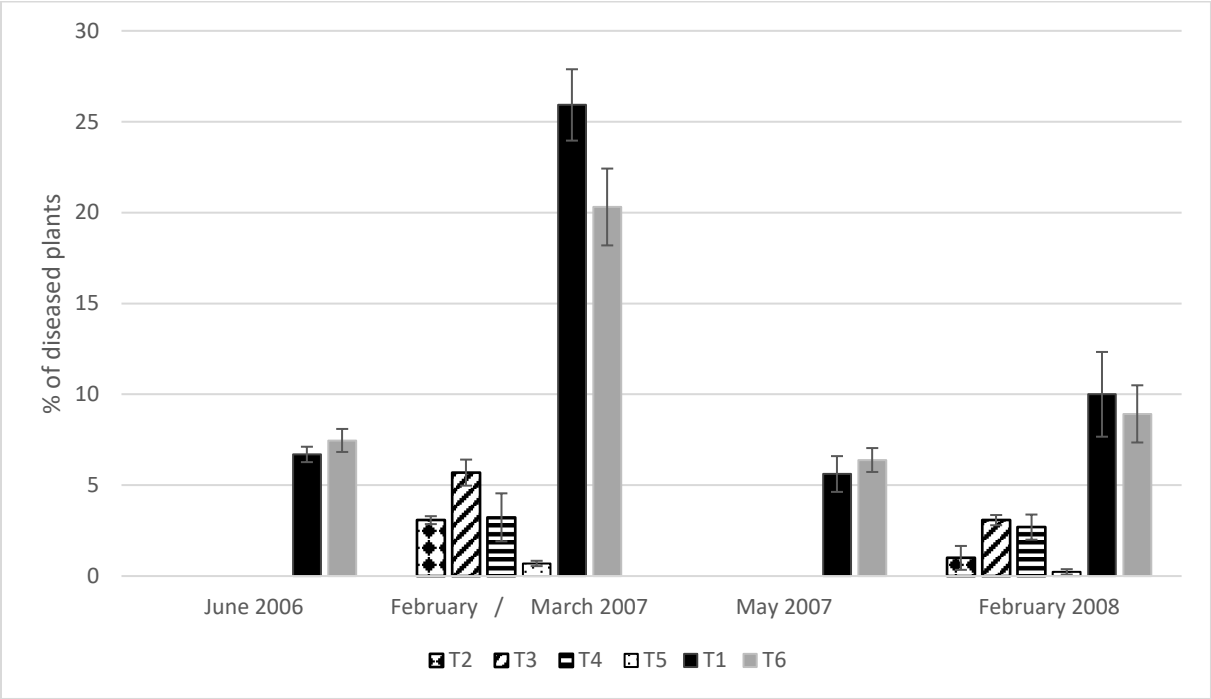

Fig. 2. Temporal variability of genetic characteristics (A: unbiased gene diversity, B: haplotypic diversity, C: linkage disequilibrium) of *B. cinerea* inoculum over four successive lettuce crops in tunnels T1 (black points) and T6 (light grey points).

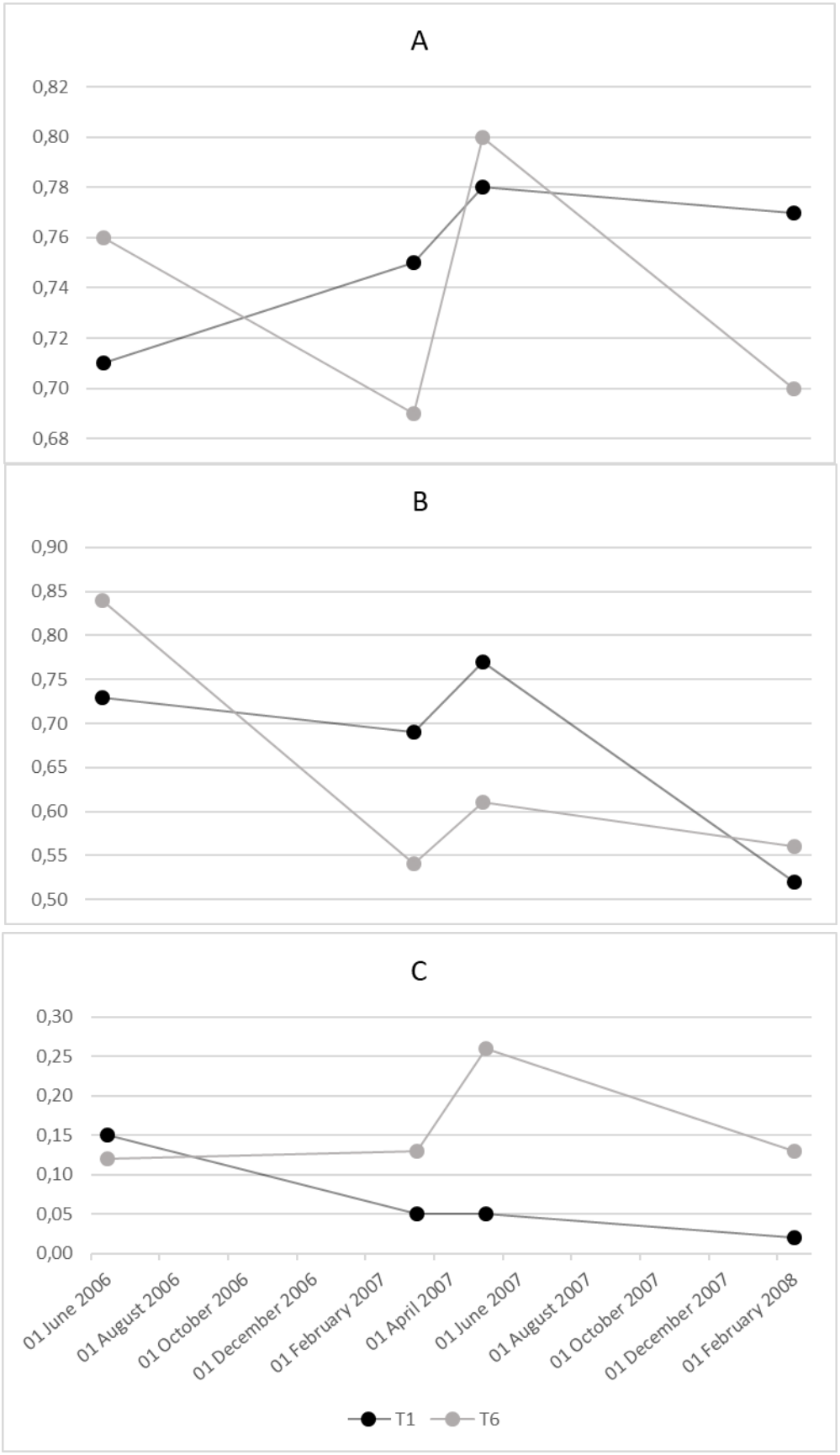

Fig. 3. Distribution of *B. cinerea* strains among 5 genetic clusters (a to e; na : not assigned) at each harvest date in tunnels T1 (A) and T6 (B).

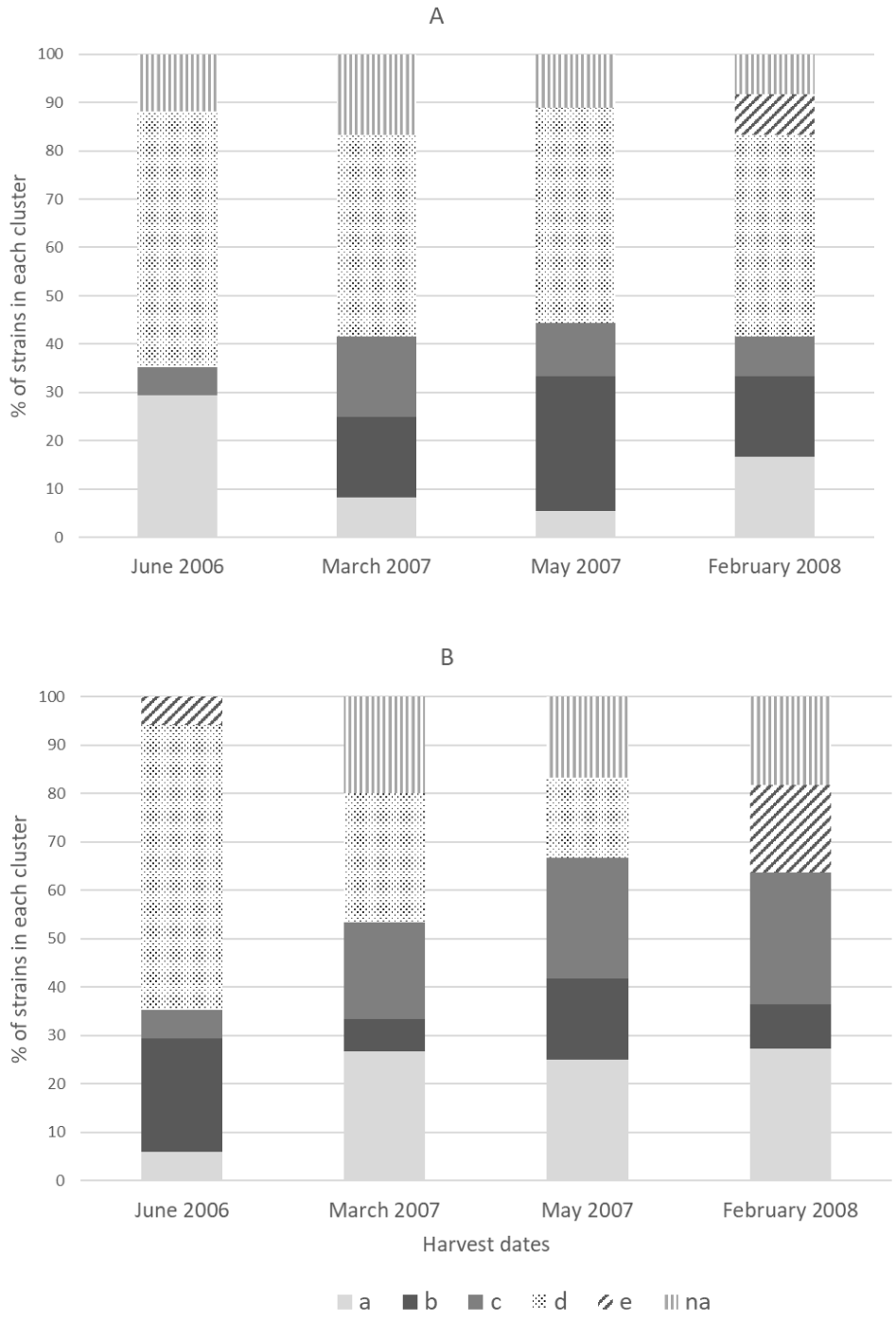

Fig. 4. Distribution of *B. cinerea* strains collected in Tunnels T1 (A) and T6 (B) among mating types MAT1-1 (light grey), MAT1-2 (dark grey) and MAT1/1-2 (checkered pattern).