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RESEARCH ARTICLE

Seasonal variations in the occurrence of *Golovinomyces orontii* and *Podosphaera xanthii*, causal agents of cucurbit powdery mildew in Northern Italy

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Conidial germination; inoculum dispersion; multiplex-PCR; relative humidity; temperature.

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Abstract

Powdery mildew, caused by *Golovinomyces orontii* and *Podosphaera xanthii*, is a widespread disease that causes important losses in cucurbit production. To determine the aetiology and the epidemiology of cucurbit powdery mildew disease in the North of Italy, observations on the occurrence of the main disease-causing fungal species were conducted during the 2010, 2011 and 2012 growing seasons. Samples of infected leaves of zucchini, melon and pumpkin plants, either from field or greenhouse crops, were collected every 15–18 days from May to September/October. To identify the fungal species, both morphological observations based on the asexual stage and molecular identifications by a Multiplex-PCR reaction with species-specific primers were performed. Climatic parameters of temperature and relative humidity were also monitored. Pearson's correlation coefficient and Principal Component Analysis showed a negative significant correlation between the two species, and a peculiar epidemiological behaviour was also observed: the earlier infections were caused by *G. orontii*, which was the predominant species till the end of June–middle of July. At this time, this species progressively decreased in frequency and was replaced by *P. xanthii* that became the main species infecting cucurbits till the end of the growing season. As the two species have different ecological requirements, these seasonal variations in the cucurbit powdery mildew species composition could possibly be explained by the influence of temperature and relative humidity on the pathogen epidemiology during the growing season but also by the different overwintering strategies adopted by the two species.

Introduction

Cucurbit powdery mildew is probably the most important disease affecting cucurbitaceous plants worldwide. The disease is caused by two obligate biotrophic pathogens: *Podosphaera xanthii* (Castagne) U. Braun and N. Shishkoff (Braun & Takamatsu, 2000) and *Golovinomyces orontii* (Castagne) V.P. Heluta (1988). Both powdery mildews belong to Phylum Ascomycota, Subdivision Pezizomycotina, Class Leotiomycetes, Order Erysiphales and Family Erysiphaceae (Wang *et al.*, 2006; Braun & Cook, 2012). The two species induce the same symptoms, a white powdery fungal growth that covers the entire surface of plants, reducing the photosynthesis and the yield and quality of crops (Pérez-García *et al.*, 2009), and can be distinguished only by the observation of several features of both anamorph and teleomorph stages. Conidia of *P. xanthii* are elliptical or spherical and measure 25–37 × 14–25 μm and have a lateral, often forked, germ tube while those of *G. orontii* are oval or cylindrical and measure 25–45 × 14–26 μm with an apical germ tube (Lebeda, 1983). Also, conidia of *P. xanthii* contain fibrosin bodies, peculiar cell inclusions made of lipids (Kiss et al., 2010) that are refractive when observed at

light microscope in a 3% potassium hydroxide solution (Kable & Ballantyne, 1963). Finally, chasmothecia of the two cucurbit powdery mildew species differ in size and number of asci and ascospores: in *P. xanthii* they are 65–98 μm in diameter and contain one ascus with eight hyaline ascospores while those of *G. orontii* are 80–140 μm in diameter with 10–15 asci containing 2–3 ascospores (Braun & Cook, 2012; Lebeda, 1983).

Differences in ecological requirements are also described: *G. orontii* have a wider range of germination temperature (10–30°C) with an optimum at 25°C with respect to *P. xanthii* that have a range between 20°C and 30°C with an optimum at 22°C (Nagy, 1976). Also, *P. xanthii* tolerates higher moisture content than *G. orontii* during the infection process (Reuveni & Rotem, 1974; Nagy, 1976). Moreover, the two species differ in their geographical distribution: *G. orontii* is stably present in some temperate European countries like Northern Italy (Branzanti & Brunelli, 1987), Germany (Ulbrich & Smolka, 1994), the Czech Republic (Křístková *et al.*, 2009; Lebeda, 1983; Lebeda *et al.*, 2011), Hungary (Nagy & Kiss, 2006), France (Bertrand *et al.*, 1992), Bulgaria (Velkov & Masheva, 2002), Switzerland (Corbaz, 1992) and Ukraine (Tomason & Gibson, 2006), while *P. xanthii* is considered the predominant species in the Mediterranean basin (Bardin *et al.*, 1997). In particular, *P. xanthii* is the only species infecting cucurbits in Spain (del Pino *et al.*, 2002; Fernández-Ortuño *et al.*, 2006), Israel, Turkey (Křístková *et al.*, 2009), Greece (Vakalounakis *et al.*, 1994), Egypt (El-Kazzaz, 1981) and Morocco (Endo *et al.*, 2012). Furthermore, *P. xanthii* is widespread in Germany, the Czech Republic (Lebeda, 1983), Italy (Branzanti & Brunelli, 1987), Bulgaria (Velkov & Masheva, 2002), Hungary (Nagy & Kiss, 2006) and Ukraine (Tomason & Gibson, 2006). Mixed infections have been recorded in the North of Italy (Branzanti & Brunelli, 1992), the Czech Republic, the Netherlands, Great Britain, Germany (Křístková *et al.*, 2009), Bulgaria (Velkov & Masheva, 2002), Hungary (Nagy, 1976) and rarely in France (Bertrand *et al.*, 1992).

Although great efforts have been invested in plant breeding programmes, growers still have important concerns about disease control, and the application of fungicides continues to be the principal practice in the management of powdery mildew in most cucurbit crops (McGrath, 2001; Pérez-García *et al.*, 2009). In Italy, cucurbits are cultivated from the North to the South of the peninsula, covering about 60,000 ha of cultivated surface. Melon is the most important crop in terms of cultivated area and production, followed by zucchini, watermelon and cucumber. In the North of Italy, crops are generally cultivated both in plastic tunnels (from April to June–July) and in open field conditions (from July to September–October), especially in the Lombardy and Emilia-Romagna regions, the most important areas for cucurbit production. In Italy, as in many other countries of the Mediterranean basin, cucurbit powdery mildew is a major problem for these crops (Miazzi *et al.*, 2011) and the occurrence of both powdery mildew species has been documented in the Emilia-Romagna region (Branzanti & Brunelli, 1992). In this area, understanding the epidemiology and the seasonal dynamics of the two species could be very useful to plan an efficient control strategy against cucurbit powdery mildew, especially considering that previous studies have demonstrated a different sensitivity of these species to some fungicides (Bertrand *et al.*, 1992; Sedláková & Lebeda, 2008; Lebeda *et al.*, 2010). The aim of this work was to monitor the occurrence and distribution of the two cucurbit powdery mildew species in distinct locations in the North of Italy during different growing seasons. This should make it possible to obtain important epidemiological data which can be subsequently applied to the rational design of disease management programmes.

Material and methods

Sampling

Powdery mildew-infected leaf samples of different cucurbit crops were collected during the growing seasons of 2010, 2011 and 2012 from both open fields and plastic tunnels on farms located in the provinces of Bologna and Mantova, important areas of cucurbit production in the North of Italy. In particular, farms from Bologna province were located in the surroundings of the city of Bologna with distances between farms from 1 to 8 km. Farms from Mantova were located in the southern part of the province and were about 3–5 km from each other. Crops under plastic tunnels were generally started from plant transplanting in February and ended in July/August. However, in some farms where cultivation was not performed in open field, crops were cultivated in plastic tunnels until September/October. Crops in open field were transplanted and managed independently from crops under tunnel and transplanting was performed in April/May and continued until September/October. As illustrated in Table 1, from April until July/August plastic tunnels and open fields coexist in farms where both cultivation systems were adopted.

Host plants covered some of the major cultivated cucurbit crops: zucchini (*Cucurbita pepo* L.), melon (*Cucumis melo* L.) and pumpkin (*Cucurbita maxima* Duchesne, 1786). Locations of farms subjected to sampling, the corresponding host plants, cultivars and cultivation systems are shown in Table 1. Each season, sampling started at the appearance of the first powdery mildew infections and

Table 1 Locations and crops subjected to sampling in the provinces of Bologna (BO) and Mantova (MN)

Farm code	Location	Geographical coordinates	Host	Cultivar	Cropping method	Cultivation period
BO1	Bologna	44°31′26.68″N	*C. pepo*	Giambo	Tunnel	February–July
		11°23′3.90″E	*C. melo*	Pamir		March–September
BO2	Bologna	44°31′11.74″N	*C. pepo*	Giambo	Tunnel	February–October
		11°23′27.40″E				
BO4	Granarolo	44°32′39.49″N	*C. pepo*	Giambo	Tunnel	February–July
		11°25′2.63″E			Field	April–October
BO6	Granarolo	44°32′35.39″N	*C. pepo*	Giambo	Tunnel	February–July
		11°24′30.19″E		Mikonos	Field	April–October
MN2a	Sermide	44°57′34.35″N	*C. melo*	Harper	Tunnel	February–July
		11°15′23.32″E		Honey Moon		April–September
MN2b	Sermide	44°57′34.35″N	*C. maxima*	Delica	Tunnel	February–October
		11°15′23.32″E				
MN3	Sermide	44°59′11.30″N	*C. melo*	Honey Sweet	Tunnel	February–July
		11°14′4.42″E		Honey Moon	Field	April–September
MN4	Sermide	44°57′55.67″N	*C. melo*	Capitol	Tunnel	February–August
		11°13′42.76″E		Honey Moon	Field	April–September

Suffixes 'a' and 'b' for farm MN2 discriminate different crops sampled in the same farm.

continued till the end of the crop season, usually in October. From each farm, at least 20 leaves showing typical powdery mildew symptoms were randomly collected in either open field or plastic tunnel crops. To monitor the powdery mildew species composition during the crop season, sampling was repeated every 15–18 days on the same plants or, when a crop cycle ended, on plants of surrounding crops in the same farms.

Species identification

To determine the occurrence of the two powdery mildew species in a given sample, a precise identification of the two fungal pathogens was imperative. Species identification was carried out both morphologically, by examination of conidial germination by light microscopy, and by means of a Multiplex-PCR reaction.

The mode of germination is specific for a particular taxon and represents a useful diagnostic tool for the taxonomy of *Erysiphaceae* (Braun *et al.*, 2002; Braun & Cook, 2012). Thus, the mode of germination was used to morphologically identify the two cucurbit powdery mildew species and to assess the percentage of them in each sample. Germination was assessed by taking five groups of four leaves from the pool of 20 collected leaves, and manually shaking them on three dry cavity slides, in order to have repetitions of each count. Cavity slides were used instead of ordinary slides to facilitate the counting of germinated conidia that was limited to the cavity area. The slides were supported by two toothpicks above a wet paper tissue in a Petri dish and incubated for 24 h at 25°C to induce germination (Zaracovitis, 1965). The slides were then observed under a light microscope (200×) and the percentage of conidia belonging to both species was calculated based on the total number of germinated conidia. For each cavity slide, all germinated conidia were counted and, from this count, the percentage of conidia belonging to both species was assessed. Species were identified according to germination criteria described by Nagy (1976) and Lebeda (1983). Barrel-shaped conidia with single germ tubes produced apically were identified as belonging to *G. orontii*, whereas elliptical conidia with germ tubes usually forked and produced laterally were identified as belonging to *P. xanthii*.

For molecular identification of the two species, pieces of the same infected leaves used for morphological identifications were used to corroborate the results. DNA was extracted following the protocol of Doyle & Doyle (1987) with some modifications. Fungal biomass was recovered from infected leaf material by washing with 2 mL of CTAB-0.04% β-mercaptoethanol solution, previously heated at 65°C for 1 h. One mL of the conidial suspension was then deposited in a 2 mL Eppendorf tube, vortexed for 20–30 s and 2.5 µL of proteinase-K (10 mg mL^{-1}) were then added. The suspension was then heated at 65°C for 2 h. Subsequently, 1 mL of a mixture of chloroform-octanol (24:1) was added to the solution and centrifuged for 5 min at 5900 g. After the addition of 5 µL of RNAase (10 mg mL^{-1}) to the supernatant and incubation at 37°C for 30 min, 1 mL of chloroform-octanol was added again and the solution was centrifuged as described above. DNA precipitation was carried out by adding 0.7–0.8 volumes of isopropanol to the supernatant and centrifuging for 20 min at 16100 g. After that, the pellet was dried under vacuum, washed with 500 µL of 70% ethanol and centrifuged for 5 min at 13400 g. Finally, the pellet was air dried

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and re-suspended in 20–50 μL of sterile double distilled water. DNA concentration and A260/A280 ratio were assessed using the Infinite 200 NanoQuant spectrophotometer (Tecan® Group Ltd., Grödig, Austria). Molecular identification of the two pathogens was carried out by Multiplex-PCR. This method is considered a rapid, simple and effective technique to detect and differentiate powdery mildews by using the *Internal Transcribed Spacer (ITS) regions of ribosomal DNA* as target (Chen *et al.*, 2008). Amplification of the *ITS* regions was carried out using a modification of primers S1/S2 and G1/G2 previously described for species identification in sunflower (Chen *et al.*, 2008). After amplification and sequencing of the ITS regions of both species with the universal primer pairs ITS1F/ITS4 (White *et al.*, 1990), some differences in *ITS* sequences of primer binding sites were observed when aligned with sequences of the same species from *Asteraceae* (sequences of both species were submitted to GenBank with Accession Numbers KJ438820 and KJ438821). To improve primer specificity for the species from cucurbits, the reverse primers S2pfc and G2goc were designed. Primer pairs S1/S2pfc and G1/G2goc were used in a Multiplex-PCR reaction for molecular identification of *P. xanthii* and *G. orontii*, respectively (Table 2). PCR reactions were carried out in 25 μL using 2.5 μL of TaKaRa® 10× buffer, 1.5 μL of 25 mM $MgCl_2$, 1.5 μL of 10 mM dNTPs, 1 μL of 10 mM solution of each primer, 0.125 μL of TaKaRa® Taq polymerase (5 U/mL) (Takara® Bio Inc., Otsu, Japan), 14.375 μL of double distilled water and 1 μL of genomic DNA (50–150 ng μL^{-1}). The PCR programme consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 40 s, 62°C for 60 s and 72°C for 1 min and 30 s, and a final extension step of 5 min at 72°C (Chen *et al.*, 2008). To verify reactions, 10 μL of each PCR product were separated on 2% agarose gels in 0.5× TAE buffer, stained with ethidium bromide and visualised and photographed under UV light.

Specificity of the primer pairs designed for diagnostic purposes was tested on DNA of the following phytopathogenic fungi and oomycetes that affect cucurbitaceous plants: *Alternaria* spp., *Dydimella bryoniae*, *Fusarium oxysporum*, *Pseudoperonospora cubensis* and *Sclerotinia sclerotiorum*.

Climate data

Because the two species have different ecological requirements, data of temperature (T) and relative humidity (RH) were obtained from both provinces to observe whether the species behaviour demonstrated during the growing seasons could be related to some changes of these two climatic parameters. In particular, data from the Mantova sampling area were obtained from the database of the Regional Environmental Protection Agency (ARPA) of the Lombardy region (http://ita.arpalombardia.it/meteo/meteo.asp). Values of T were recorded in weather station no. 816 in Sermide (Id sensor no. 8223) while those RH were recorded in the Gonzaga station no. 110 (Id sensor no. 2135). For the Bologna area, the same data were obtained from a thermohygrograph located at the weather station of the experimental farm located in Altedo.

Statistical analysis

Count data obtained from conidial germination tests were analysed both by descriptive and inferential statistics. Because sampling was performed until October in some farms, to even out the sampling dates between farms, only six dates (from the appearance of the first symptoms at the end of May until September) were considered in the analysis. Also, because samples from farms MN4 and BO6 were only collected in 2010 and 2011 years, data from these farms were not included in the analysis.

Correlation analysis using Pearson's correlation coefficient (r) was performed with Statistica 12 (StatSoft Inc., Tulsa, OK, USA) to measure the strength of the association between the two powdery mildew species. Analysis was performed both on data from each year of investigation in each location (province) and on the pooled data. Using the same software, the Principal Component Analysis (PCA) was performed. This technique is able to reduce the number of variables to fewer, simpler ones that may describe much of the variation in the original data. Count data of germinated conidia of both powdery mildew species obtained from the six sampling dates were analysed comparing years and locations. Moreover, a repeated measure ANOVA was performed on *G. orontii*

Table 2 Primers used for multiplex-PCR

Species	Target gene	Primer designation	Sequence (5′→3′)	Fragment size (bp)
G. orontii	ITS	G1[a]	TCCGTAGGTGAACCTGCGGAAGGAT	414
		G2goc	CAACACCAAGCCACACACACGGCG	
P. xanthii	ITS	S1[a]	GGATCATTACTGAGCGCGAGGCCCCG	455
		S2pfc	CGCCACTCTGTCGCGAGATACA	

[a]Primers taken from Chen *et al.* (2008).

data using a linear mixed model. Years and farms were considered as random factors, dates as a repeated factor and data from conidia of *P. xanthii* were used as a covariate variable. Because of counted data, before the analysis square root transformation was applied to the entire counted conidia data set (Zar, 1996). Analysis was performed with SAS software version 9 (Statistical Analysis System Institute, Cary, NC, USA).

Results

Morphological identifications

Samples were taken during the 2010–2012 growing seasons from powdery mildew diseased melon, pumpkin and zucchini plants collected in the provinces of Bologna and Mantova in Northern Italy. Both *G. orontii* (barrel-shaped conidia with apical germination and without fibrosin bodies) and *P. xanthii* (elliptical conidia with fibrosin bodies and lateral germination, in some cases, with forked germ tubes) were found, but variations in their frequency were observed during each growing season. In Bologna province (Fig. 1), during the 3 years of sampling, the first powdery mildew infections were recorded between the last week of May and the first half of June. With the exceptions of farms BO1 and BO2, where in 2011 5% of conidia belonging to *P. xanthii* was recorded, the only species causing the earlier infections was *G. orontii*. From the second half of June, a progressive increase of conidia belonging to *P. xanthii* and a decrease of those of *G. orontii* was observed in all farms. A prevalence of *P. xanthii* conidia (80–100%) was observed in late July–early August in all farms and years of sampling with the only exception of farm BO4 in 2012, when *P. xanthii* was the predominant species already in June (70%). After that period, the predominant conidia found on infected leaves were those of *P. xanthii* that progressively became the only species infecting cucurbits until the end of the crop season in October.

In Mantova province (Fig. 2), during the 3 years of sampling, the first powdery mildew infections appeared at the end of May and observations indicated that the first infections were caused mainly by *G. orontii* that was the predominant species infecting crops until a period that went from the last half of June to the first half of July when *P. xanthii* progressively starts to appear. As observed in Bologna farms, a prevalence of *P. xanthii* conidia (60–100%) was observed in July–August in most farms and years of sampling. After that period, *P. xanthii* became the only species infecting cucurbits until the end of the crop season in October (September for farm MN2a).

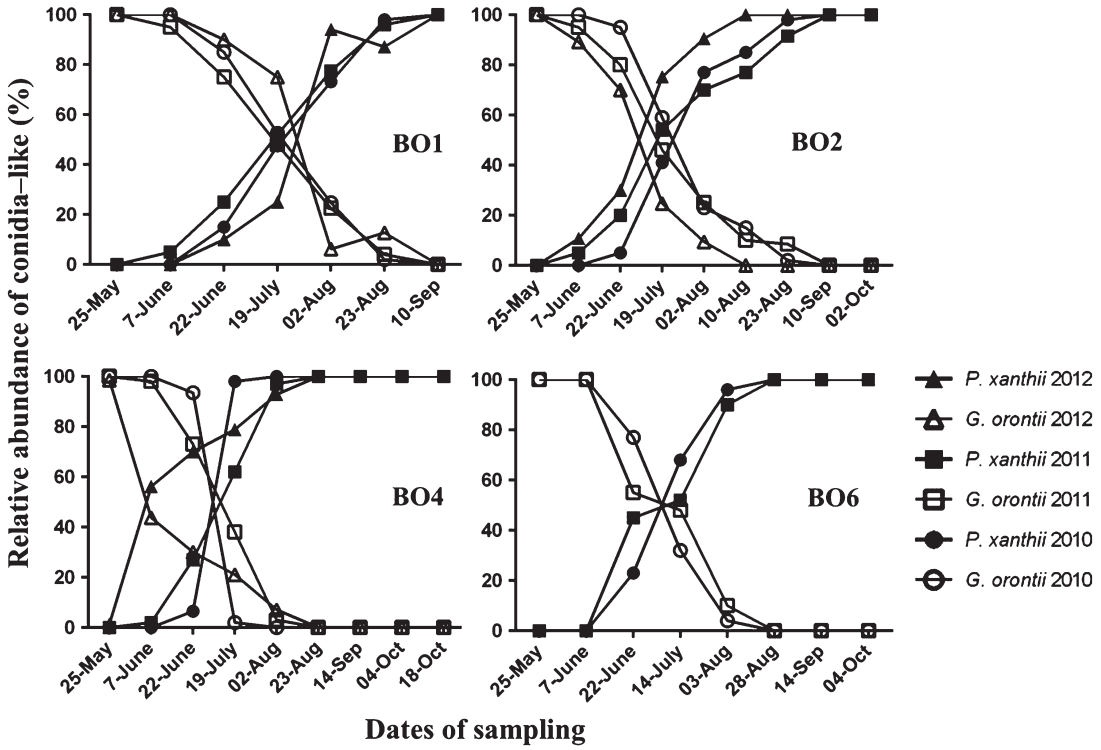

Figure 1 Relative abundance of the two cucurbit powdery mildew fungi assessed as the percentage of germinated conidia belonging to each species in Bologna farms during the 3 years of survey. Samples from farm BO6 were collected only during the 2010 and 2011 growing seasons.

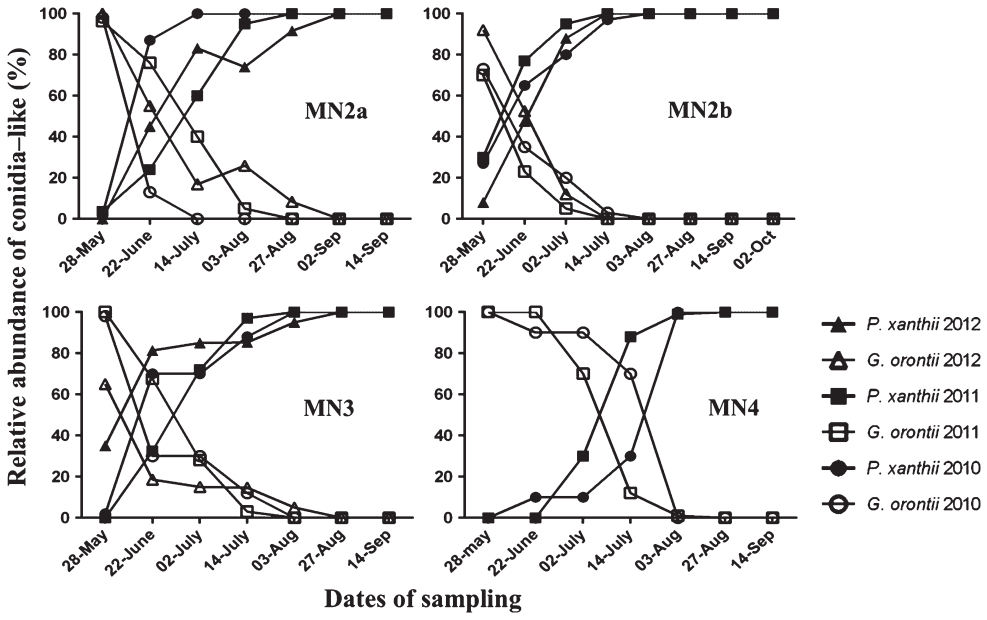

Figure 2 Relative abundance of the two cucurbit powdery mildew fungi assessed as the percentage of germinated conidia belonging to each species in Mantova farms during the 3 years of survey. Samples from farm MN4 were collected only during the 2010 and 2011 growing seasons.

Molecular identifications

A multiplex-PCR assay was designed to detect the presence of both cucurbit powdery mildew species and to confirm the results obtained by microscopic analysis. The *ITS regions of ribosomal DNA* of both species were used as target DNA. Similarly to Chen *et al.* (2008), amplification of such regions of *P. xanthii* and *G. orontii* allowed us to directly identify the two species. Expected fragments of 455 bp and 414 bp were observed for *P. xanthii* and *G. orontii*, respectively. Furthermore, designed primers did not amplify any DNA of the cucurbit pathogens *Alternaria* spp., *D. bryoniae*, *F. oxysporum*, *P. cubensis* and *S. sclerotiorum* (data not shown), confirming the specificity for the *ITS* region of the two powdery mildew species and the robustness of the assay.

The results confirmed the same seasonal trend of the two species observed by conidial germination trials. An example is given in Fig. 3 for farm BO2 for the 3 years of sampling. Double bands, which represent the simultaneous presence of the two species, were obtained in a period that went from the last half of June until the second/third weeks of July that corresponded, in the morphological observations, to about 40–60% of conidia belonging to both species.

Climate data and occurrence of powdery mildew species

In 2010, the lowest values of mean RH were recorded at the end of August in Bologna and in May in Mantova while the highest values of mean T were recorded in July in both provinces (Figs 4a and 5a). In 2011, in the Bologna area, minimum RH values were observed in August while in Mantova mean RH tended to have lower values with a minimum recorded in May. Mean T was the same in both areas, with a maximum recorded in August (Figs 4b and 5b). In 2012, the lowest values of RH were recorded in May in both provinces while the highest T was recorded in June and August in Bologna and Mantova provinces, respectively (Figs 4c and 5c). In Bologna province, *G. orontii* predominated in the first weeks from the appearance of symptoms (25 May–22 June). After that, in the second interval (23 June–02 August), a progressive replacement of *G. orontii* by *P. xanthii* was observed. As illustrated in Table 3, this period was characterised by an increase of mean minimum, mean and mean maximum T. In particular, the most important increase was observed in mean (+3.89°C in 2010, +2.6° in 2011 and + 4.14°C in 2012) and mean maximum T (+4.59°C in 2010, +2.74°C in 2011 and +4.22°C in 2012). In the same period, a decrease of all RH was observed especially for minimum (−7.55% in 2010, −7.02% in 2011 and −4.39% in 2012) and mean (−6.19% in 2010, −7.6% in 2011 and −5.43% in 2012) values. In the third interval, *P. xanthii* replaced *G. orontii* and became the predominant species infecting cucurbits. During this period, with the exception of year 2011, values of both mean (−3.13°C in 2010 and −1.74°C in 2012) and mean maximum T (−3.29°C in 2010 and −2.16°C

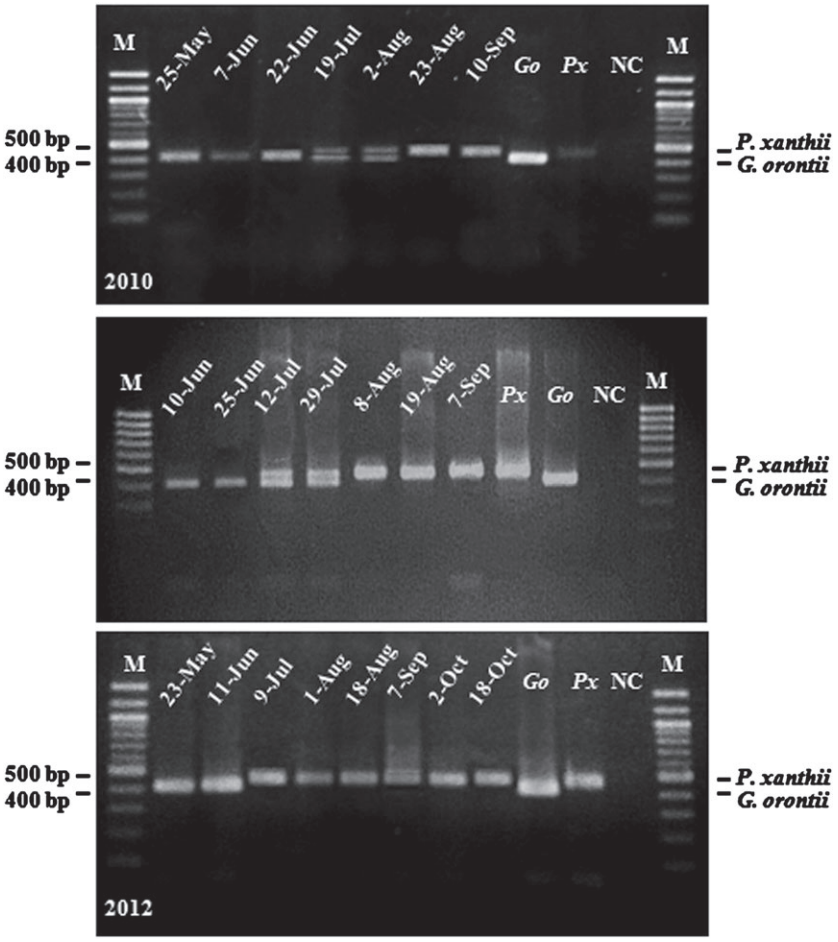

Figure 3 Molecular detection of *Podosphaera xanthii* and *Golovinomyces orontii* by multiplex-PCR. Presence of the two cucurbit powdery mildew species was investigated by multiplex-PCR in all farms sampled during the crop seasons 2010, 2011 and 2012. Representative gels relating to farm BO2 are given. Genomic DNA from *P. xanthii* (*Px*) and *G. orontii* (*Go*) were used as positive controls. M is the molecular size marker 100 bp DNA ladder (New England BioLabs®, Ipswich, UK) used for 2010 and 2012 gels and MassRuler Low Range DNA ladder (Thermo Scientific®, Waltham, MA, USA) used for 2011 gel, and NC is the negative control (no DNA was added).

in 2012) decreased. On the contrary, with the exception of year 2011, an increase of minimum (+6.62% in 2010 and +4.53% in 2012) and mean (+5.7% in 2010 and +6.08% in 2012) RH was recorded. Minor variations were observed in all intervals for both mean minimum T and mean maximum RH values. Similarly, in Mantova province, *G. orontii* was the predominant species from the end of May until the last week of June (first week of July in farm MN4). Also in this area, during the second interval (23 June–14 July), a progressive replacement of *G. orontii* by *P. xanthii* was observed. In this period, an increase of minimum, mean and maximum T was recorded (Table 4). Similarly to Bologna province, the most important increase was observed in mean (+4.65°C in 2010, +3.31° in 2011 and + 3.7°C in 2012) and mean maximum T (+5.11°C in 2010, +3.74°C in 2011 and +4°C in 2012) values while a decrease of mean minimum (−7.76% in 2010, −6.49% in 2011 and −4.24% in 2012) and mean (−3.68% in 2010, −6.03% in 2011 and −3.78% in 2012) RH values was recorded. In the third interval (15 July–14 September), when *P. xanthii* became the predominant species, the overall T values did not change and values of both mean (−2.74°C) and mean maximum T (−2.76°C) decreased substantially only in 2010. Compared to Bologna province, no variations were observed in RH values as an increase of minimum (+5.33%) and mean (+5.16%) RH was recorded only in 2010. Also in Mantova, less variation was observed in all intervals for both mean minimum T and mean maximum RH values.

Statistical analysis

Correlation analysis showed a negative significant correlation between the two species in all years and both

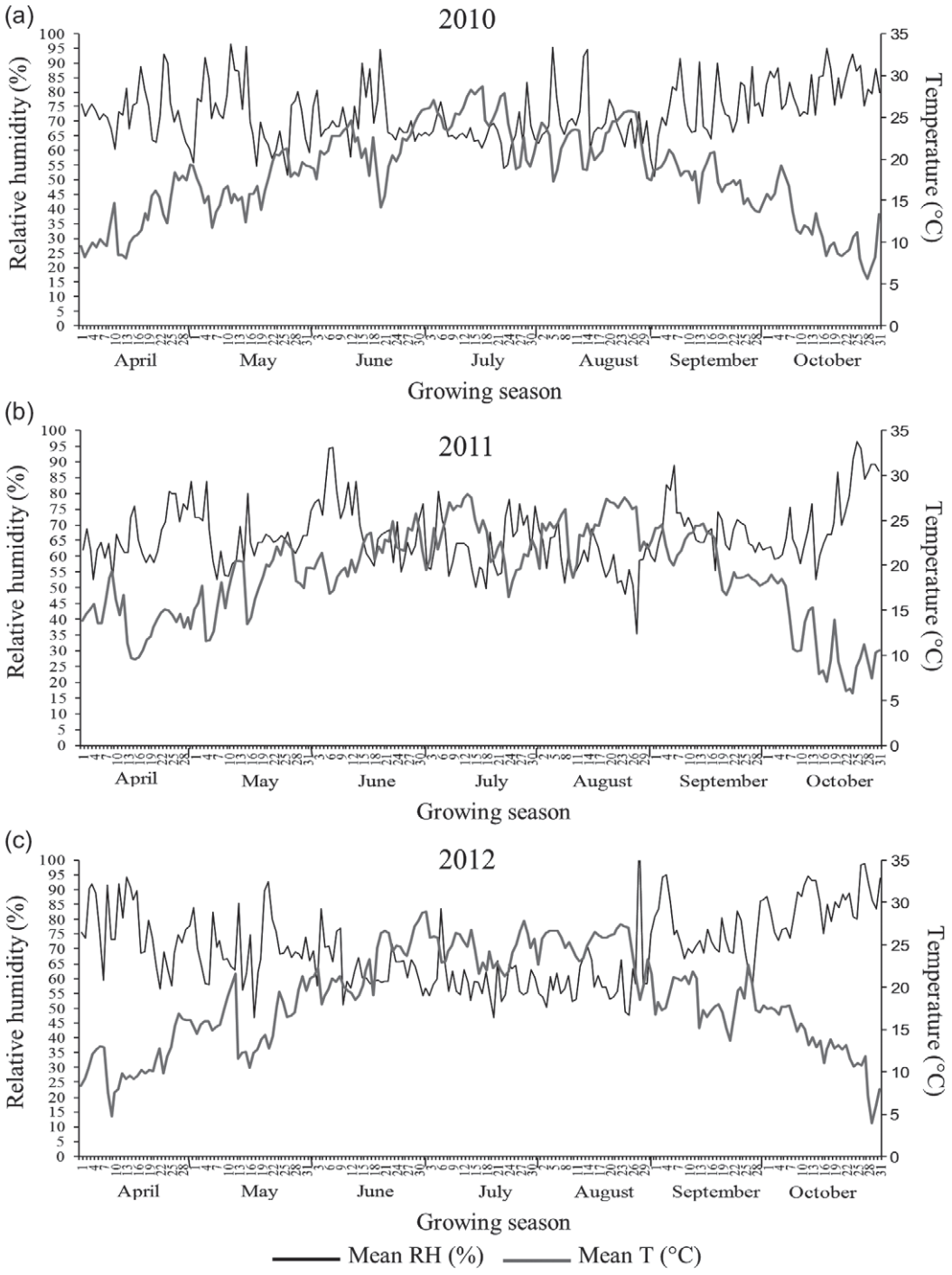

Figure 4 Mean RH (%) and T (°C) measured in Bologna province in the 2010 (a), 2011 (b) and 2012 (c) growing seasons.

provinces (Table 6). Pearson's correlation coefficients between years and sites did not differ statistically using the Student's *t*-test ($P<0.01$). PCA also showed a negative correlation between the two powdery mildew species (Tables 5 and 7): the first component explained the 44.42% of the variability and clearly separated the two species (Fig. 6), while the second component (with 16.42%) indicated an unexpected behaviour of the variable px4. The analysis seems to reveal an interaction between years and farms (Fig. 7a and Fig. 7b) as data

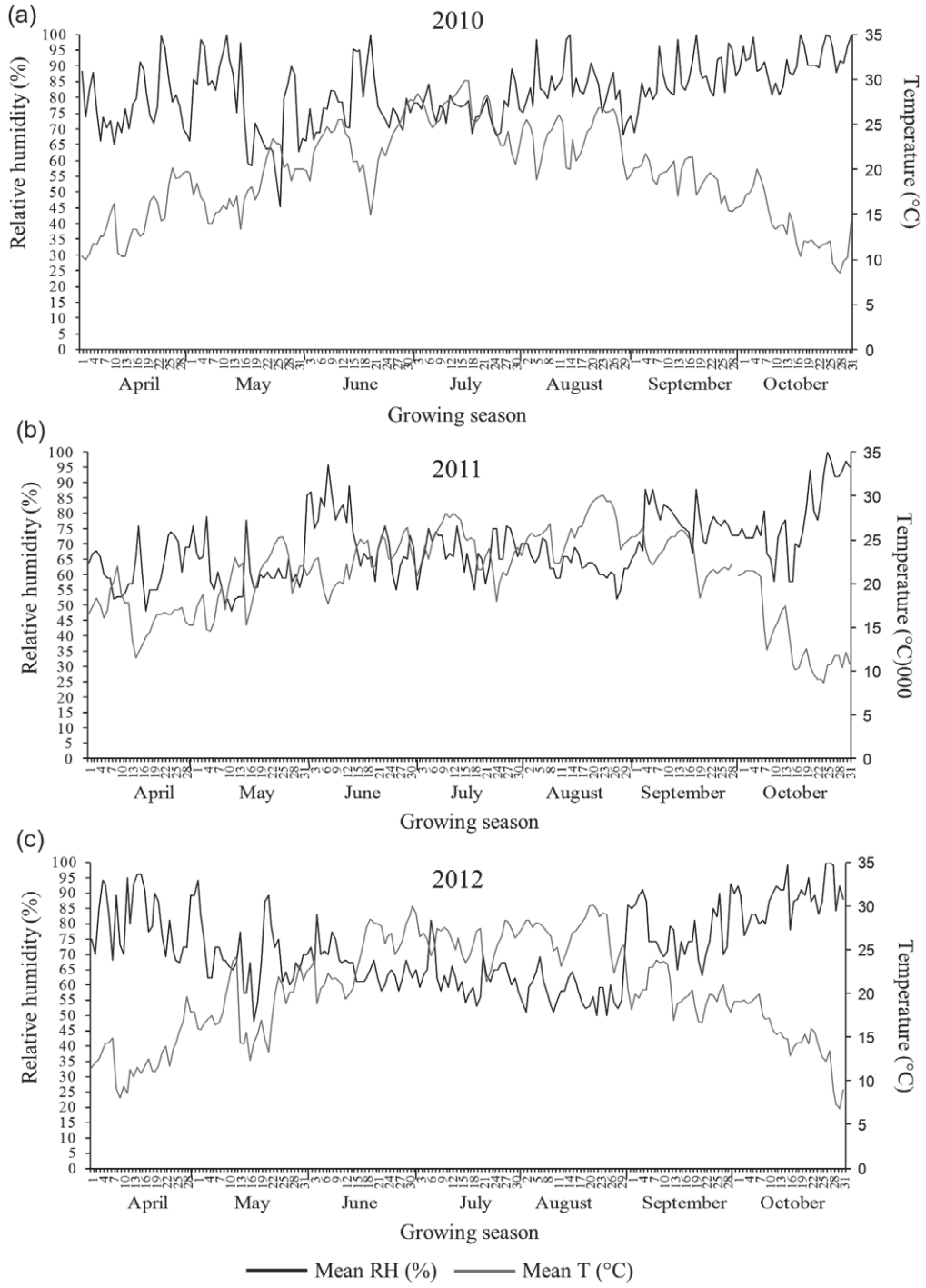

Figure 5 Mean RH (%) and T (°C) measured in Mantova province in the 2010 (a), 2011 (b) and 2012 (c) growing seasons.

of 2010 and 2011 from Bologna farms were separated from data of the same farms in 2012 that clustered with Mantova farms.

Using the linear mixed model procedure, most of the variation in the data was explained by significant differences in sampling dates, locations and, as already suggested by the descriptive analysis of PCA, by an interaction between dates and locations. The influence of *P. xanthii* was not statistically significant when used as a covariate variable (data not shown).

Table 3 Climate data of temperature (T °C) and relative humidity (RH %) recorded in Bologna province during four intervals (interval = period between sampling dates) of the growing seasons of 2010, 2011 and 2012

		Growing season			
Climate parameter	Years	01 April–24 May	25 May–22 Jun	23 June–02 Aug	03 Aug–14 Sep
Mean minimum T (°C)	2010	6.29	14.38	17.24	15.09
	2011	5.63	14.52	16.12	16.60
	2012	4.91	13.55	17.10	16.21
Mean T (°C)	2010	11.01	20.23	24.12	20.99
	2011	11.76	20.36	22.96	23.96
	2012	11.79	20.62	24.76	23.02
Mean maximum T (°C)	2010	15.97	26.04	30.63	27.34
	2011	17.69	26.41	29.15	31.33
	2012	19.13	27.66	31.88	29.72
Mean minimum RH (%)	2010	52.31	46.50	38.95	45.57
	2011	44.17	44.14	37.12	35.23
	2012	37.83	37.07	32.68	37.21
Mean RH (%)	2010	76.61	72.07	65.88	71.58
	2011	71.75	71.49	63.89	62.26
	2012	68.15	65.26	59.83	65.91
Mean maximum RH (%)	2010	94.45	95.86	94.35	94.62
	2011	94.81	96.55	92.02	90.93
	2012	96.20	93.97	91.34	93.72

The first powdery mildew symptoms were recorded at the end of the first interval.

Table 4 Climate data of temperature (T °C) and relative humidity (RH %) recorded in Mantova province during four intervals (interval=period between sampling dates) of the growing seasons of 2010, 2011 and 2012

		Growing season			
Climate parameter	Years	01 April–27 May	28 May–22 Jun	23 June–14 July	15 July–14 Sep
Mean minimum T (°C)	2010	11.68	17.24	21.16	18.68
	2011	13.10	17.36	20.16	19.94
	2012	10.72	17.70	21.05	19.84
Mean T (°C)	2010	15.97	21.35	26.00	23.26
	2011	18.21	21.73	25.04	24.89
	2012	15.01	22.93	26.63	25.13
Mean maximum T (°C)	2010	20.27	25.50	30.61	27.85
	2011	23.19	25.87	29.61	30.07
	2012	19.51	27.92	31.92	30.63
Mean minimum RH (%)	2010	54.12	57.02	49.76	55.09
	2011	34.35	49.04	42.55	38.95
	2012	51.75	42.92	38.68	36.89
Mean RH (%)	2010	77.65	79.68	76.00	81.16
	2011	61.12	73.85	67.82	67.79
	2012	75.53	67.42	63.64	63.50
Mean maximum RH (%)	2010	97.51	98.25	99.52	99.66
	2011	91.28	97.15	96.00	96.48
	2012	95.11	92.38	89.36	91.00

The first powdery mildew symptoms were recorded at the end of the first interval.

Discussion and conclusions

From morphological and molecular observations of powdery mildew diseased plants, temporal variations in the cucurbit powdery mildew species composition were observed. Both Pearson's correlation coefficients and PCA showed a negative correlation between the two species in all 3 years of investigation and in both provinces. The data showed a replacement of species causing powdery mildew on cucurbits during all growing seasons. However, some differences between years and provinces were revealed by PCA. From the observation of Figs 6, 7a and 7b, farms from Bologna grouped together as well as those from Mantova and only in 2012 were samples

Table 5 Results from Principal Components Analysis (PCA)

Principal components	Autovalue	Variance (%)	Cumulative autovalue	Cumulative variance (%)
CP1	5.331	44.421	5.331	44.421
CP2	1.971	16.422	7.301	60.843
CP3	1.110	9.249	8.411	70.092
CP4	0.987	8.222	9.398	78.313

Table 6 Pearson product–moment correlation coefficient (*r*) between *G. orontii* and *P. xanthii* between years and sites

	Years			
Location	2010	2011	2012	Pooled
Bologna	−0.817	−0.8	−0.803	−0.807
Mantova	−0.735	−0.715	−0.777	−0.744

Table 7 Principal Component Analysis (PCA): correlation between variables and components

	Principal components			
Variables	CP1	CP2	CP3	CP4
go1	−0.424	0.493	0.315	−0.419
go2	−0.814	−0.116	−0.172	−0.306
go3	−0.846	0.112	−0.104	0.445
go4	−0.797	−0.317	−0.014	0.172
go5	−0.680	−0.443	0.345	0.041
go6	−0.451	−0.547	0.177	−0.162
px1	0.624	−0.235	0.527	0.156
px2	0.901	−0.134	−0.092	−0.223
px3	0.885	−0.130	−0.195	−0.214
px4	0.129	0.761	−0.078	0.408
px5	0.660	−0.306	0.379	0.374
px6	0.180	−0.585	−0.588	0.190

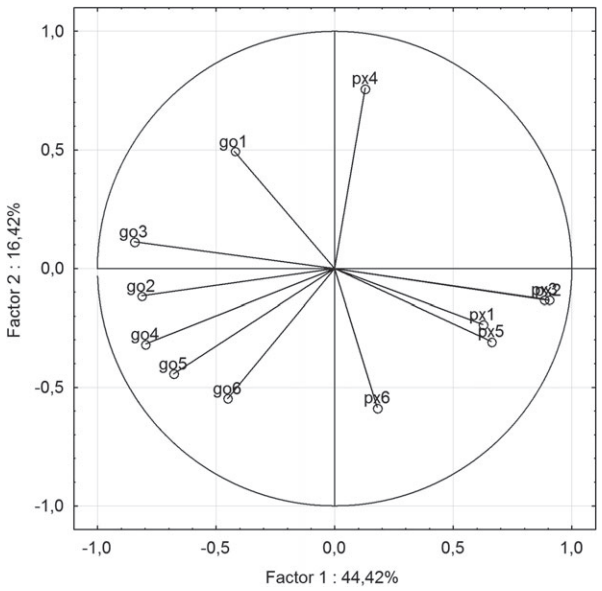

Figure 6 Results from Principal Component Analysis (PCA). A negative correlation between the two species was observed.

from Bologna grouped together with those of Mantova. In general, the species behaviour in Mantova was more homogeneous than Bologna. Again, Fig. 6 suggests a more abundant presence of *G. orontii* in Bologna with respect to Mantova in all six sampling dates. On the contrary, *P. xanthii* was more abundant in Mantova. The same year-locations interaction was confirmed by using the linear mixed model procedure.

According to Gause (1934), species sharing the same resource cannot stably co-exist and this behaviour could be influenced by several factors. First, these seasonal variations seem to be affected by environmental conditions. In particular, *G. orontii* was the only species found in the earlier infections in all locations investigated and remained the predominant species infecting cucurbits until the third week of June. At the same time, *P. xanthii* increased in frequency and started to replace *G. orontii* during a period that went from the last week of June until the beginning of August in Bologna and from the last week of June until the second week of July in Mantova. In all years and in both provinces, these periods were characterised by an increase of all T values, suggesting that *P. xanthii* could be favoured by higher T as already observed by Nagy (1976); Bertrand *et al.* (1992) and Lebeda *et al.* (2011). After that *P. xanthii* replaced *G. orontii* and became the predominant species causing powdery mildew until the end of the crop season in September/October. At that time, T values decreased (with the exception of mean maximum T in Mantova and for all T values in Bologna in 2011), but they remained in any case higher than the first interval (May–June). Development of powdery mildews is affected by RH (Itagaki *et al.*, 2014) and, in particular, sporulation and spore dispersal are favoured by low RH values (Reuveni & Rotem, 1974). This could explain the increase of *P. xanthii* frequency in the intervals 23 June–02 August (Bologna) and 23 June–12 July (Mantova), both characterised by a decrease of all RH values with respect to the previous period when *G. orontii* predominated. Moreover, experimental data confirmed that *P. xanthii* requires higher T and lower RH than *G. orontii* for its development (Ratna Hadi, 2005; Ratna Hadi *et al.*, 2005; Křístková *et al.*, 2009; Lebeda *et al.*, 2009). After that, when *P. xanthii* replaced *G. orontii*, minimum and average RH values increased in 2010 and 2012 in Bologna and in 2010 in Mantova, with a small decrease in the other years. Less variation was observed in this period in both provinces for maximum RH values. Germination,

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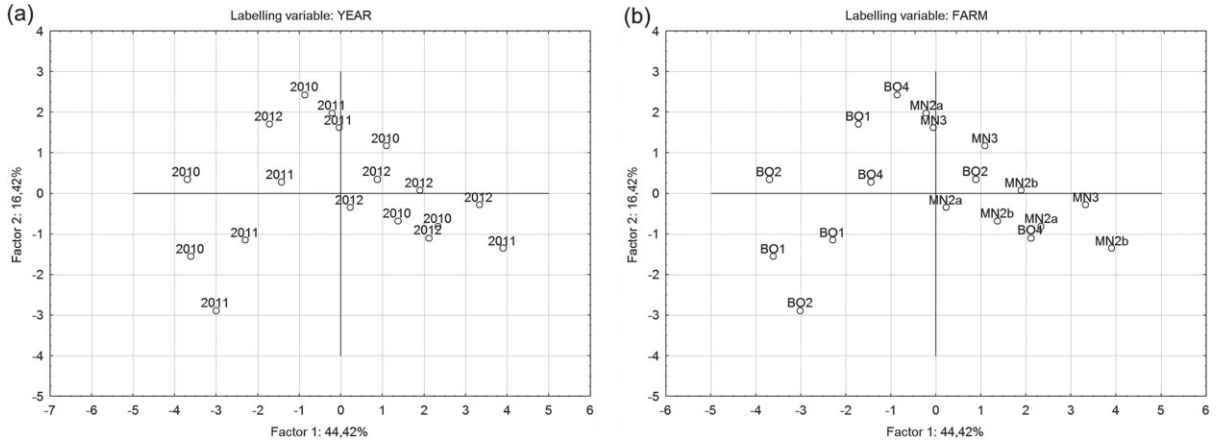

Figure 7 Results from Principal Component Analysis (PCA). Some differences in the epidemiological behaviour of the two pathogens were observed between locations (a) and years (b) of sampling.

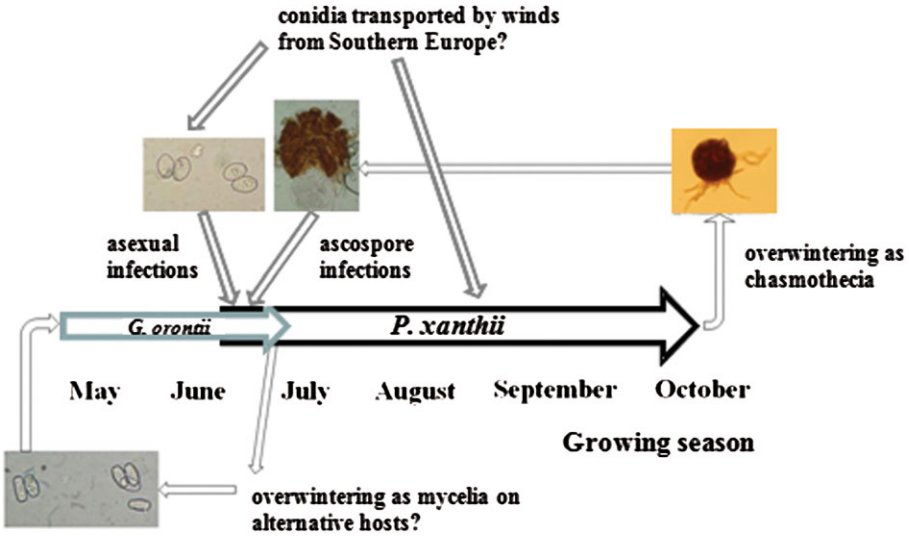

Figure 8 Epidemiology of cucurbit powdery mildew disease in Northern Italy during the growing season. The occurrence of the two pathogens may be affected by their different overwintering strategies. As indicated by question marks, there are a number of questions that remain to be answered.

infection and survival of powdery mildews are favoured by high RH. In particular, *P. xanthii* is found to be more sensitive to moisture than *G. orontii* as turgid conidia of *P. xanthii* can persist for 24 h only in saturated atmosphere of 100% RH (Nagy, 1976) which could explain the predominance of the species until September/October when RH is higher than in early summer. In general terms, the spread and appearance of *P. xanthii* and the replacement of *G. orontii* seem to coincide with an increase of T, a decrease of minimum RH values in June–July and the constant values of maximum RH. However, as already hypothesised by Branzanti & Brunelli (1992), this should be in contrast to the fact that this pathogen is not found in the earlier infection that occurs when all crops are cultivated under plastic tunnels where the values of T are higher than in field conditions. Also, the same species behaviour is observed in farms where the entire crop cycle is conducted under tunnel conditions that generally should have higher values of T and RH than in field conditions. So, climate conditions alone cannot explain the seasonal behaviour of cucurbit powdery mildew species.

Another factor that should be considered is the innate resistance or susceptibility of crops and cultivars. Although monitored crops belonged to different host plants and to different cultivars among the same cucurbit species (Table 1), the seasonal behaviour observed seems to be independent of this factor.

These temporal variations seem to be clearly influenced also by the cultivation systems. It is interesting to note that under plastic tunnels *G. orontii* was progressively replaced by *P. xanthii*, but, in the period when the crop cycles switched to field conditions (end of July–beginning of August), the symptoms were caused by *P. xanthii*. An explanation for this could be that chasmothecia of *P. xanthii* that overwinter in soil and crop residues could play a significant role in initiating these field infections. As a consequence, an important factor to consider is represented by the different overwintering strategies of the two species. A parallel study performed in the same area and farms (Pirondi *et al.*, 2015), showed the occurrence of chasmothecia belonging only to *P. xanthii*, suggesting the different perennation strategies of the two pathogens with *G. orontii* that, alternatively, overwinters in the form of mycelium on alternative hosts, like crops and volunteer plants as hypothesised by Sharma (1989). This finding, together with the fact that the pathogen is adapted to survive at lower temperatures (Nagy, 1976), could explain why this species is responsible for the first powdery mildew symptoms observed in the growing season. *P. xanthii*, on the other hand, overwintering as chasmothecia, seems to initiate the disease cycle only after the breaking of chasmothecia and release of ascospores can act as primary inoculum. Moreover, the same study suggests that the optimal conditions for breaking chasmothecia seem to occur in middle spring, which may explain the delay in the appearance of the pathogen. As the preservation of chasmothecia is facilitated in soil and plant material this could explain the fact that *P. xanthii* predominate in the infections that occur on field crops. Moreover, the fact that in the first part of the crop season (March–July) the crops are all under plastic tunnel can negatively influence the preservation of chasmothecia over the next winter because plants are cultivated on black plastic material that is removed together with crop residues at the end of each season. A similar temporal variation, where epidemiology is influenced by the different overwintering strategies of two species, has been observed in France for *Erysiphe quercicola* and *Erysiphe alphitoides*, the causal agents of oak powdery mildew (Feau *et al.*, 2012).

Powdery mildews are completely dependent on living tissue for survival and for this reason they produce a huge number of spores that are easily dispersed from one susceptible host to another (Brown & Hovmøller, 2002). Therefore, chasmothecia should not be considered the only source of primary inoculum of *P. xanthii* in the North of Italy. In addition, the species produce abundant fungal mass and, like other powdery mildew fungi, conidia can be efficiently disseminated by wind even over very long distances (Bardin *et al.*, 1997; Pérez-García *et al.*, 2009; Miazzi *et al.*, 2011). Thus, as already hypothesised by Křístková *et al.* (2009) and Lebeda *et al.* (2009), cucurbit cultivations in the southern part of Europe could represent an important source of primary inoculum, also considering that *P. xanthii* is the predominant species in the Mediterranean basin where cucurbits are grown year-around and the pathogen is always present on either protected crops or open fields (Bardin *et al.*, 1997; Miazzi *et al.*, 2011). In the South of Italy, because of the different climatic conditions, cucurbit cultivation starts earlier in the season (first transplanting from January on plastic tunnel and from April to May on open fields) than in the North, and powdery mildew infections also appear early. Moreover, in southern Spain, especially in the regions of Andalusia and Murcia, the most important areas of cucurbit production in Europe, cultivation of these crops never stops during the year, and they could therefore be a continuous source of powdery mildew inoculum (A. Pérez-García, personal communication). In these areas, the Sirocco and Libeccio winds that blow in spring-summer from the Southeast and Southwest, respectively, could play a significant role in transporting powdery mildew conidia from these southern areas to the North of Italy. Genetic diversity analyses of *P. xanthii* populations from Northern Italy and Southern Spain could help to answer these questions. Finally, the greater aggressiveness of *P. xanthii* (Chat-Locussol & Lavoy, 1990; McGrath, 2011) could be one more reason to explain its predominance over *G. orontii*.

Currently, the investigated area represents the only case of species replacement of cucurbit powdery mildew-causing fungi, where the two species occur in different periods of the growing season. A similar variation, although not supported by molecular data, was observed in the same area in the mid 1980s (Branzanti & Brunelli, 1992) and, in the same years, a similar behaviour was reported in France (Bertrand *et al.*, 1992). However, mixed infections of the two species were recorded very rarely in this last country (Křístková *et al.*, 2009). In the Czech Republic, where *G. orontii* is considered the predominant species, observations carried out over 5 years indicated that, from the beginning, infections were caused by either *G. orontii* alone or mixed with *P. xanthii* without any temporal succession (Křístková *et al.*, 2009). In Fig. 8, we present a model summarising the epidemiology of cucurbit powdery mildew disease in Northern Italy.

Considering the prevalence during the growing season, the study suggests that *P. xanthii* is the most important causal agent of cucurbit powdery mildew in Northern Italy and, as a consequence, this species should be considered the main target of disease management practices. However, the different occurrence of the two species during the growing season could be very important to

plan new control strategies against the disease. The first reason is that both cucurbit powdery mildew species are highly variable in their pathogenicity and virulence, as evidenced by the existence of a large number of different pathotypes and races (Lebeda *et al.*, 2011). Pathotypes are based on intergeneric and interspecific differences in host–pathogen interactions, e.g. for pathotype determination in *P. xanthii*, two cultivars of the major cucurbit crops are normally used (Bertrand, 1991; del Pino *et al.*, 2002; Lebeda *et al.*, 2008). Races are characterised by the interactions of different isolates of a pathogen with different genotypes of a given host species (Bertrand, 1991; Pitrat *et al.*, 1998; Bardin *et al.*, 1997; Lebeda & Sedláková, 2010). To date, four pathotypes of *G. orontii* and three of *P. xanthii* were identified worldwide (Lebeda *et al.*, 2011; Sedláková *et al.*, 2014). Moreover, two races of *G. orontii* and 31 of *P. xanthii* have been also identified and differentiated on melon (Lebeda *et al.*, 2011; McCreight *et al.*, 2012). This diversity of races is a serious problem for powdery mildew control because it represents a serious limitation for the use of varieties only resistant to a limited number of races. Consequently, although many commercial varieties have been released with resistance to *P. xanthii*, the development of new races of the pathogens continues to be a serious problem for disease management and resistance breeding is necessary.

In addition, as fungicide resistance is known in both powdery mildew species (McGrath, 2001; Sedláková & Lebeda, 2008), they could have a different sensitivity to fungicides. Differences between the two species in sensitivity to benomyl, bupirimate and DMI fungicides were observed in France (Bertrand *et al.*, 1992) and, similarly, Sedláková & Lebeda (2008) observed differences in sensitivity to fenarimol, dinocap and benomyl fungicides in 108 powdery mildew isolates tested. Moreover, isolates of both powdery mildew species resistant to fenarimol, dinocap, benomyl, tiophanate-methyl and azoxystrobin were detected in Czech Republic (Lebeda *et al.*, 2010). The results obtained in this work could be of great relevance if differences between both species in the sensitivity to modern fungicides could be found. Considering the current critical situation in fungicide efficacy against cucurbit powdery mildew, the development of fungicide resistance (Fernández-Ortuño *et al.*, 2006; López-Ruiz *et al.*, 2010; Ishii *et al.*, 2011; Pirondi *et al.*, 2014) and the new limitations on fungicide applications in the European Union, research on sensitivity to QoI, DMI, boscalid, cyflufenamid and quinoxyfen based fungicides in the cucurbit powdery mildew populations from Northern Italy must be carried out in order to obtain information on the sensitivity of both species to fungicides. This information should make it possible to design new disease management programmes and to improve control efficacy.

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