OCCURRENCE OF CHASMOTHECIA AND MATING TYPE DISTRIBUTION OF PODOSPAERA XANTHII, A CAUSAL AGENT OF CUCURBIT POWDERY MILDEW IN NORTHERN ITALY

A. Pirondi1, A. Pérez-García2, I. Portillo1, G. Battistini1, C. Turan1, A. Brunelli1 and M. Collina1

1 Dipartimento di Scienze Agrarie, Università degli Studi di Bologna, viale G. Fanin 46, 40127 Bologna, Italy
2 Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora”, Universidad de Málaga, Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Departamento de Microbiología, Universidad de Málaga, Bulevar Louis Pasteur 31 (Campus Universitario de Teatinos), 29071 Málaga, Spain

SUMMARY

Powdery mildew is a widespread disease that causes important losses to cucurbit production. The main agents of the disease are Podosphaera xanthii and Golovinomyces orontii. To determine the occurrence of chasmothecia as overwintering forms of both fungal species in northern Italy, powdery mildew-infected samples from cultivated cucurbits were collected in different locations of the provinces of Bologna and Mantova during 2010, 2011 and 2012. Only the sexual stage of P. xanthii was found, indicating that in northern Italy, contrary to what reported from other areas of the Mediterranean basin, the pathogen overwinters as chasmothecia. In parallel, to determine the frequency and distribution of both MAT 1-1-1 and MAT 1-2-1 P. xanthii idiomorphs, a multiplex-PCR with MAT idiomorph-specific primers was carried out on 147 mononidial isolates obtained from infected leaf samples. The obtained frequencies were tested for random mating. Results showed a MAT ratio that tended to 1:1, supporting the finding of the sexual stage thus suggesting the occurrence of actively mating populations and that sexual reproduction plays a significant role in the life cycle of P. xanthii in this area. The lack of G. orontii chasmothecia suggests that this species might have alternative overwintering strategies.

Key words: ascospore viability, multiplex-PCR, powdery mildew, sexual recombination.
only a preliminary study conducted in the same area by Branzanti and Brunelli (1987) recorded chasmothecia of *P. xanthii*. Additional and more recent information could be very useful to understand the epidemiology of the two fungal species, also considering that ascospores could act as a primary inoculum source. Furthermore, the increased genetic diversity resulting from sexual reproduction represents an advantage for the pathogens that could rapidly evolve in response to agricultural practices, for example with new combinations of virulence genes and resistance to fungicides (McGrath, 1996).

The aim of this work was to monitor the occurrence of the sexual stage of both powdery mildew species and to investigate the spatial distribution of MAT idiomorphs to obtain useful information on the impact of sexual reproduction in these two pathogens.

Powdery mildew-infected leaf samples were collected during the growing seasons of 2010, 2011 and 2012 in eight farms located in the provinces of Bologna and Mantova (Fig. 1). Crops included the main cultivated cucurbits: *Cucumis pepo* (in farms BO1, BO2, BO4, BO6 and MN1), *Cucumis melo* (in farms BO1, MN2, MN3 and MN4), *Cucumis sativus* (in farm MN1), *Cucurbita maxima* (in farm MN2), and *Citrullus lanatus* (in farm MN3).

Because chasmothecia formation is favoured by host senescence, low nutritive condition of the host, dry atmosphere, and low temperatures (Yarwood, 1935; McGrath, 1994) and they also begin to form in older areas of infection late in the season, when temperatures are low and conidial production slows down or ceases (Agrios, 1988, Alexopoulos, 1962; McGrath, 1994), sampling was carried out from late August until October of each growing season. Data of temperature (T), relative humidity (RH) and rainfall that occurred in the three months of sampling during 2010, 2011 and 2012 were obtained from the weather station of the experimental farm AX located in Altedo (Fig. 1) and from the database of the Regional Environmental Protection Agency (ARPA) of Lombardy. Both infected senescent leaves and soil under the plants were collected. With the aim of obtaining monoconidial isolates, powdery mildew-infected leaves were also randomly collected during the entire growing season (from May to October).

Chasmothecia were extracted following the methods used by Pearson and Gadoury (1987), Cortesi et al. (1995) and Portillo (2010) with some modifications. Infected senescent leaves were randomly collected from 15 plants of each field. About 50 g of leaves from each plant were placed in 500 ml flasks and distilled water was added to completely cover them. The flasks were then carefully shaken manually for three min, to avoid rupturing the chasmothecia, and the resulting suspension was filtered through a pile of four stacked test sieves of 10, 30, 60 and 170 mesh (Retsch, Germany), corresponding to mesh diameters of 2000, 600, 250 and 90 µm, respectively. To better clean the chasmothecia and to facilitate their movement through the last two sieves, the sieve pile was washed under running water. The last two sieves were then washed with distilled water and the chasmothecia suspension was filtered through filter paper. Filters with chasmothecia were then placed in a 90 mm Petri dish, air-dried for at least 24 h and stored at 4°C. For extracting chasmothecia from soil, the protocol was essentially the same as described above. The only difference was that 10 g of soil collected under the same infected senescent plants used for leaf extraction were placed directly on the column and washed with running water. Presence of chasmothecia on Petri dishes was finally verified under a stereo microscope. Chasmothecia were then picked up with the aid of a needle and deposited in a drop of water on a microscope slide, covered with a 22×22 mm cover slip and observed under a light microscope. Observation of the morphological features of chasmothecia, i.e. one ascus with eight ascospores in *P. xanthii* or 10-15 asci containing 2-3 ascospores in *G. orontii* (Lebeda, 1983; Braun and Cook, 2012) was possible after rupture, obtained by pressing the needle on the cover slip.

To verify the overwintering efficacy of powdery mildew as chasmothecia, 20 zucchini plants of cv. Afrodit were transplanted in the field and grown through holes in plastic mulch in August 2012 in the experimental farm AX (Fig. 1). Untreated plants that were naturally infected by *P. xanthii* at that time of the growing season were allowed to overwinter in the field. Species identification was based on the observation of the position of the germ...
tube: lateral, often forked in *P. xanthii* and apical in *G. orontii* (Lebeda, 1983; Braun and Cook, 2012). About 40 powdery mildew-infected leaves were randomly collected, spores were recovered and placed onto two glass slides per leaf. Germination was induced using the method described by Zaracovitis (1965) and, for each slide, 50 conidia were counted. In April-May 2013, leaves from the same plants were collected and chasmothecia were extracted as described above. As infection from ascospores is difficult to achieve *in vitro* (McGrath, 1994), the potential capability of chasmothecia to produce infections was assessed by determining the viability of the ascospores by the fluorescein diacetate (FDA) staining method, successfully used to assess the viability of *Erysiphe necator* ascospores (Portillo et al., 2012). FDA (Sigma-Aldrich, USA) stock solution was prepared as described by Miller et al. (1994) and used together with a phosphate buffer (Colgan and Claridge, 2002). Chasmothecia were collected from Petri dishes, deposited in a drop of FDA solution on a microscope slide, broken as described above, then observed in a Nikon Eclipse TE2000-E fluorescent microscope equipped with a digital camera DXM1200F (Nikon, Japan).

From each location, several monoconidial isolates of *P. xanthii* were obtained: with an ethanol-disinfected eyelash one single conidia was taken from an infected leaf under the stereomicroscope (4×) and inoculated on zucchini cotyledons cv. Giambo maintained *in vitro* as described by Álvarez and Torés (1997). Isolates were initially maintained by transferring a single conidium to a fresh cotyledon every seven days. The process was repeated three times to obtain a pure monoconidial colony. The identification of the isolates as *P. xanthii* was carried out by observing the position of the germ tube according to Zaracovitis (1965). A fungal mass was then harvested from a monoconidial colony with a sterile pipette tip, deposited in a two ml eppendorf tube and used for DNA extraction using the cetlytrimethylammonium bromide (CTAB) method (Stewart and Via, 1993), as modified by Robinson et al. (2002) and already used for this pathogen by Vela-Corcía et al. (2014).

DNA concentration and A260/A280 ratio were assessed using the Infinite 200 NanoQuant spectrophotometer (Tecan Group, Austria).

A multiplex-PCR reaction was developed for identifying idiomorphs. Based on the sequences of *P. xanthii* idiomorphs *MAT*1-1-1 (GenBank accession No. HQ171903.1) and *MAT*1-2-1 (HQ171900.1) identified by Brewer et al. (2011), primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 were designed (Table 1). Oligonucleotides were synthesized by Invitrogen (Life Technologies, USA). PCR reactions were conducted in a vol. of 25 µl using 5 µl of Promega GoTaq Green Buffer 5×, 1 µl of 10 mM dNTPs, 2 µl of 25 mM MgCl₂, 1 µl of each primer (10 mM), 0.125 µl of GoTaq flexi DNA polymerase (5 u/µl) (Promega, USA), 11.875 µl of sterile water and 1 µl of DNA sample. PCR conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 30 sec, 52°C for 30 sec

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**Table 1.** Primer designed to amplify *MAT* idiomorphs by multiplex-PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer designation</th>
<th>Sequence (5’→3’)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MAT</em>1-1-1</td>
<td>aboxF2</td>
<td>GGCTTCAGAAGTATGTCATG</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>aboxR2</td>
<td>CCGCAGAAATTATAGACCAC</td>
<td></td>
</tr>
<tr>
<td><em>MAT</em>1-2-1</td>
<td>hmgF2</td>
<td>AAGGCTAAGCATGGAGAAAC</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>hmgR2</td>
<td>CCGTAAACGATAACGCGGAT</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Number of isolates of *P. xanthii* identified for each *MAT* idiomorph in the Bologna and Mantova provinces during the 2010, 2011 and 2012 growing seasons. Frequencies of *MAT*1-1-1 and *MAT*1-2-1 idiomorphs both from each year of sampling and from the total count were tested for random mating by the chi-squared test (χ²) to show deviations from the 1:1 ratio.

<table>
<thead>
<tr>
<th>Year of collection</th>
<th>Province</th>
<th><em>MAT</em>1-1-1</th>
<th><em>MAT</em>1-2-1</th>
<th>Total no. of isolates</th>
<th>χ²b</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>Bologna</td>
<td>11</td>
<td>16</td>
<td>39</td>
<td>0.641</td>
</tr>
<tr>
<td></td>
<td>Mantova</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>17 (0.44)</td>
<td>22 (0.56)</td>
<td>39</td>
<td>0.641</td>
</tr>
<tr>
<td>2011</td>
<td>Bologna</td>
<td>8</td>
<td>14</td>
<td>22</td>
<td>0.320</td>
</tr>
<tr>
<td></td>
<td>Mantova</td>
<td>15</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>23 (0.46)</td>
<td>27 (0.54)</td>
<td>50</td>
<td>0.320</td>
</tr>
<tr>
<td>2012</td>
<td>Bologna</td>
<td>13</td>
<td>17</td>
<td>20</td>
<td>1.724</td>
</tr>
<tr>
<td></td>
<td>Mantova</td>
<td>11</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>24 (0.42)</td>
<td>34 (0.58)</td>
<td>58</td>
<td>1.724</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>64 (0.44)</td>
<td>83 (0.56)</td>
<td>147</td>
<td>2.456</td>
</tr>
</tbody>
</table>

a *MAT* frequencies of each idiomorph are indicated in parentheses.

b differences between *MAT* frequencies were not statistically significant for P < 0.05.
and 72°C for 60 sec, and a final extension step at 72°C for 5 min. PCR products were separated on 1.5% agarose gels in 0.5× TAE buffer, stained with ethidium bromide and visualized under UV light. Before proceeding to amplification of the whole isolates, the two amplified fragments (each one from a MAT idiomorph) were purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare Bioscience, USA) and sequenced in both directions (Macrogen Europe, The Netherlands) to confirm the amplification of the correspondent idiomorph. MAT frequencies obtained from each year of sampling were tested for random mating by the chi-squared ($\chi^2$) test using the GraphPad Prism 6.01 software (GraphPad Software, USA).

Based on morphological observations, from all the three years of sampling, 53 monoconidial isolates obtained from samples collected from the end of May until the end of June were identified as *G. orontii* while 147 isolates from samples collected from late June-early July until September-October showed morphological features corresponding to *P. xanthii*. Differences in the occurrence of the two powdery mildew species during the growing season had already been observed in the same area during the ’80s (Branzanti and Brunelli, 1987, 1992). Isolates of *P. xanthii* and the corresponding sampling dates are shown in Supplementary Table S1. Considering that isolates of *G. orontii* were mainly found for a few weeks, we suppose that *P. xanthii* could be the main agent of the cucurbit powdery mildew disease in the studied area.

In all farms monitored, chasmothecia were found from late August until the end of October on senescent leaves of crop plants (Fig. 2a), and mainly in October in the soil under the plants. No asci were found inside chasmothecia during the years 2010 and 2011. This because, at the time of collection, chasmothecia were not completely mature, as they were yellow/light brown when observed under the stereo microscope. For 2010, this is probably due to the summer rainfall pattern, characterized by abundant rain, and to the higher mean RH values than 2011 and 2012 (Table 3). The latter were higher in both provinces especially in August, the period when chasmothecia presumably start to form. The 2011 growing season in both provinces was characterized by higher temperatures during September than 2010 and 2012 (Table 3). Considering that chasmothecia formation is favoured by dry atmosphere and low temperatures (Yarwood, 1935; McGrath, 1994), the climate conditions of both 2010 and 2011 could have delayed ascospore formation inside chasmothecia. However, considering that the only powdery mildew species that infected the crops at that time of the growing season was *P. xanthii*
Table 3. Climate data of temperature (T °C), relative humidity (RH %) and total rainfall (mm) measured in Bologna and Mantova provinces in 2010, 2011 and 2012 during the months when chasmothecia were collected.

<table>
<thead>
<tr>
<th>Climate parameter</th>
<th>Year</th>
<th>Bologna</th>
<th>Mantova</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>August</td>
<td>September</td>
</tr>
<tr>
<td>Mean T</td>
<td>2010</td>
<td>22.19</td>
<td>17.60</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>24.28</td>
<td>21.00</td>
</tr>
<tr>
<td>Mean minimum T</td>
<td>2010</td>
<td>16.19</td>
<td>11.75</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>15.50</td>
<td>14.00</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>16.87</td>
<td>13.30</td>
</tr>
<tr>
<td>Mean maximum T</td>
<td>2010</td>
<td>28.27</td>
<td>24.55</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>31.97</td>
<td>27.83</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>31.87</td>
<td>24.17</td>
</tr>
<tr>
<td>Mean RH</td>
<td>2010</td>
<td>70.38</td>
<td>74.09</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>58.50</td>
<td>68.56</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>58.39</td>
<td>75.29</td>
</tr>
<tr>
<td>Mean minimum RH</td>
<td>2010</td>
<td>44.56</td>
<td>46.43</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>30.97</td>
<td>40.17</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>30.81</td>
<td>47.17</td>
</tr>
<tr>
<td>Mean maximum RH</td>
<td>2010</td>
<td>94.73</td>
<td>95.53</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>90.48</td>
<td>92.33</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>91.94</td>
<td>96.83</td>
</tr>
<tr>
<td>Rainfall</td>
<td>2010</td>
<td>74.70</td>
<td>73.40</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>36.80</td>
<td>14.40</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>0.60</td>
<td>82.40</td>
</tr>
</tbody>
</table>

(316) Table S1), we can also suppose that the chasmothecia belonged to this species. In 2012, mature chasmothecia were collected, as they were already black when observed under the stereo microscope. Only one ascus containing eight ascospores was found inside (Fig. 2b; Fig. 2c), a morphological feature that corresponds to chasmothecia of *P. xanthii*. Furthermore, in spring 2013 chasmothecia were collected from zucchini plants left over from the previous summer in the experimental field after powdery mildew infection. Observation of 300 chasmothecia under the fluorescence microscope showed that 12.7% of them were mature and belonged to *P. xanthii*. A very high percentage of them (94.7%) fluoresced green, indicating the presence of viable ascospores (Fig. 2d).

As chasmothecia of *G. orontii* are considered to be very rare (Braun and Cook, 2012), it was not surprising that they were not recorded in all three years of sampling. Their absence suggests that *G. orontii* overwinters in the form of mycelium on alternative hosts, as already hypothesized by Sharma (1989). Pathogenicity of *G. orontii* to cucurbits was also observed for isolates collected from *Picridium vulgare* and *Senecio vulgaris* (Alvarez and Torés, 1995), *Cichorium pumilum*, *Nicotiana tabacum* and *Lactuca serriola* (Cohen and Eyal, 1988).

Considering that only chasmothecia from *P. xanthii* were found, a multiplex-PCR assay was designed to determine the frequency of mating types of this species and to study the impact of sexual reproduction in the populations of northern Italy. The primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 successfully amplified PCR products of 167 and 228 bp, corresponding to *MAT 1-2-1* (KJ438825) and *MAT 1-2-1* (KJ438826) idiomorphs, respectively (Fig. 3). The difference of 61 bp in the size of the amplified products allowed an easy discrimination of the two idiomorphs.

The multiplex-PCR assay was used to evaluate the *MAT* frequencies among the 147 monoconidial isolates of *P. xanthii* collected in the areas under investigation. Results

![Fig. 3. Molecular detection of *MAT* idiomorphs in *P. xanthii* isolates. DNA was amplified by primers aboxF2/aboxR2 and hmgF2/hmgR2 in a multiplex-PCR reaction that made it possible to detect the corresponding idiomorph. M is the molecular size marker MassRuler Low Range DNA ladder (Thermo Scientific, USA). Isolates SF60 and 2086 were used as positive controls respectively for *MAT 1-2-1* and *MAT 1-1-1*.](image-url)
obtained from all three years of investigation suggested that the MAT ratio could tend to be 1:1. This tendency was verified by testing the MAT frequencies by the χ² test. The results of the test indicated that MAT frequencies were not statistically significant for P < 0.05 as no departures from the 1:1 ratio were observed (Table 2).

As mature chasmothecia were collected, this is probably the overwintering strategy of P. xanthii, like other powdery mildew fungi such as Erysiphe necator (Gadoury et al., 2012). In this case, ascospores are released only after the breaking of these structures and act as primary inoculum for initiation of the disease cycle. Moreover, the observation in April 2013 of a high percentage of chasmothecia with viable ascospores suggests that the optimal conditions for breaking chasmothecia seem to occur in mid spring. Because ascospore infections are presumably slower than those causes by mycelia (Glawe, 2008), this could explain the delay in the appearance of the pathogen on cucurbit crops (June-October).

Chasmothecia of P. xanthii were never or rarely found in many cucurbit production areas of several countries (McGrath, 1994; Álvarez and Torés, 1995; Bardin et al., 1997; Miazzi et al., 2011) and more importantly, even when their differentiation was obtained in laboratory conditions (McGrath, 1994), successful ascospore infections were never achieved. These are the main reasons why the epidemiological role of the sexual cycle of P. xanthii has yet to be determined. Interestingly, the occurrence of chasmothecia with viable ascospores was also supported by an estimated ratio for MAT idiomorphs of 1:1, indicating the existence of populations of the pathogen actively mating and suggesting that P. xanthii could undergo sexual recombination in northern Italy. However, for powdery mildews, the production of huge numbers of spores, which are wind-dispersed from one susceptible host to another, is essential for reproduction and survival because these pathogens are completely dependent on living host tissue for survival (Brown and Hovmøller, 2002). In this sense, long-distance dispersal of cucurbit powdery mildew spores could also and additionally be a source of primary inoculum especially for P. xanthii, which is the predominant species in the Mediterranean basin, where cucurbits are grown the whole year round and the pathogen is always present either on protected crops or open fields (Bardin et al., 1997; Miazzi et al., 2011). In this case, an unbalanced ratio of MAT idiomorphs should have been observed, considering the fact that, for example, in Spain, the most important country for cucurbit production, a preferential selection for MAT 1-2-1 was observed (A. Pirondi, unpublished information).

To better investigate the relevance of sexual (ascospores) and asexual (conidia) infections in powdery mildew epidemics, a systematic analysis of the genetic diversity of P. xanthii populations during the entire growing season should be conducted. Furthermore, a study on the genetic structure of Italian populations of P. xanthii should be carried out to look for evidence of genetic recombination and for investigating the importance and role played by the sexual stage in this powdery mildew species.

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