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Cucurbit Powdery Mildew: First Insights for the Identification of the Causal Agent and Screening for Resistance of Squash Genotypes (*Cucurbita moschata* (Duchesne ex Lam.) Duchesne ex Poir.) in Mendoza, Argentina

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The cucurbit powdery mildew (CPM) caused by different fungal species is a major concern for cucurbit crops around the world. In Argentina CPM constitutes the most common and damaging disease for cucurbits, especially for squash crops (*Cucurbita moschata*). The present study displays initial insights into the knowledge of the disease in western Argentina, including the determination of the prevalent species causing CPM, as well as the evaluation of the resistance of squash cultivars and breeding lines. Fungal colonies were isolated from samples collected in Mendoza province, Argentina. A field trial was also performed to assess the resistance of five squash accessions, including commercial cultivars and breeding lines. The severity of CPM was analyzed and epidemiological models were built based on empirical data. The morphological determinations and analysis with specific molecular markers confirmed *Podosphaera xanthi* as the prevalent causal agent of CPM in Mendoza. The results od the field trial showed differences in the resistance trait among the squash accessions. The advanced breeding line BL717/1 showed promising results as source of CPM resistance for the future development of open pollinated resistant cultivars, a crucial tool for an integrative control of the disease.

Keywords **:** breeding for resistance, disease epidemics, plant pathogens

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The cucurbit powdery mildew (CPM) is a major disease of cucurbits around the world (Lebeda and Sedláková, 2010), both under field and greenhouse conditions. It is also the most common disease in Argentina (Della Gaspera, 2013). As many other powdery mildews, the disease is typically recognized by the signs of the pathogen, which appear as whitish colonies in both sides of the leaves (Fig. 1A and B), comprising mycelium and asexual sporulation structures (conidia). The colonies may coalesce and cover the complete surface of leaves, petioles and stems (Kiehr and Delhey, 2013). Disease symptoms include leaf yellowing, necrosis and premature death of leaves (Robinson and Decker-Walters, 1997). The loss of leaves threatens the crop by lowering the potential yield and affecting the commercial quality of the fruits, mainly due to sun burn and incomplete ripening (Pérez-García et al., 2009).

Worldwide the main causal agents of CPM are *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff (Px, formerly *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll.) and *Golovinomyces cichoracearum* (DC.) V. P. Heluta (Gc, formerly *Erysiphe cichoracearum* (DC.) V. P. Gelyuta). The pathogen most commonly associated to CPM is Px, especially in tropical or subtropical areas and in greenhouse crops, while Gc is more prevalent in temperate regions and field crops (Lebeda and Sedláková, 2010; Silveira Maia, 2012). Px and Gc display a broad range of host cultivars and virulent races, and their populations may also vary

temporally and spatially (Lebeda et al., 2016), which adds complexity to the disease management. The absence of the teleomorphic phase (production of chasmotecia, shape of the asci and ascospores) leads to difficulties distinguishing both species. However, some differences in the size and shape of asexual conidia, and the presence of fibrosin bodies exclusively in Px (Lebeda and Sedláková, 2010) are helpful features to identify the causal agent of CPM. A secondary pathogen that may also appear is *Leveillula taurica* (Lév.) Arnaud (Lt), which is easily recognizable under stereomicroscope for its single pyriform conidia and mycelium developing in the mesophyll, unlike Px and Gc which produce several cylindrical conidia in chains (Fig. 1C) with the mycelium growing over the leaf epidermis. In different regions of Argentina Px has been cited previously as the causal agent of CPM (Delhey et al., 2003), while the occurrence of Gc has been cited on many crops but not on squash (Kiehr and Delhey, 2013; Sistema Nacional Argentino de Vigilancia y Monitoreo de Plagas, 2021). Although both pathogens are present in the country, little is known about their geographical distribution and relative prevalence in the different cucurbits growing areas. Moreover, a study to determine the prevalent species in the region of Mendoza is lacking.

The application of scheduled fungicide sprays is the most common technique to manage CPM in squash in Argentina. However, this procedure may trigger the proliferation of fungal strains that are resistant to different active ingredients, hence leading to less effective control (Lebeda and Sedláková, 2010) and environmental pollution. Therefore, the use of resistant cultivars is the most suitable tool for managing the disease in a cost and ecological effective manner. The development of new resistant cultivars to CPM is a laborious and time consuming process that requires extensive selection of resistant lines and the utilization of conventional breeding approaches (Mandal et al., 2020). In Argentina some hybrid resistant cultivars of squash are known to perform well under powdery mildew conducive conditions, while open pollinated (OP) cultivars with acceptable levels of resistance are not available yet. Field observations suggest that some widespread OP cultivars released by the National Institute of Agricultural Technology (INTA), namely Cokena INTA and Pecas INTA, are susceptible, but formal trials to test their levels of resistance/susceptibility are lacking.

The National Breeding Program of Cucurbits held at the INTA La Consulta Experimental Station (EEA La Consulta) is currently on the way to develop new OP squash cultivars with increased resistance to CPM. Identifying which pathogen species are prevalent is crucial, together with the availability of a screening test to determine the resistance of local varieties and breeding lines. Hereafter the aims of the present work were to isolate and identify the pathogens associated with CPM in different cucurbit crop growing areas in the region of Mendoza, Argentina, and to evaluate the resistance to CPM of different squash genotypes under field conditions.

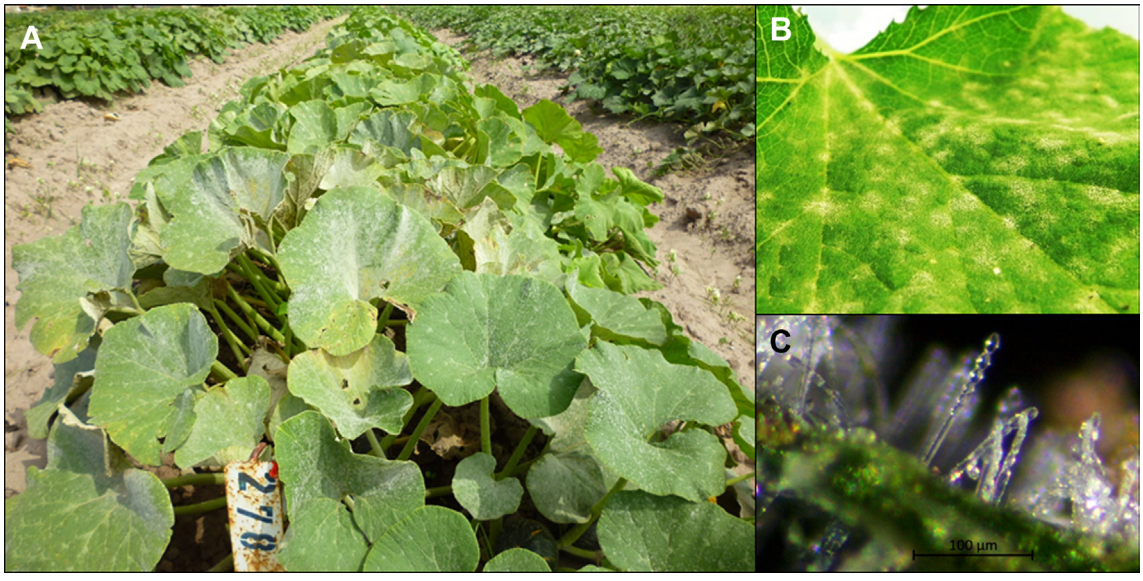

Fig. 1. Powdery mildew of cucurbits. (A) Disease symptoms (leaf yellowing) and pathogen signs (colonies over leaf surface) in the susceptible cultivar Pecas INTA, as it appears under field conditions. (B) Zoom-in to the pathogen colonies present on a leaf (leaf yellowing and white sporulating mycelium). (C) Conidia of *Podosphaera xanthii*, formed in long chains.

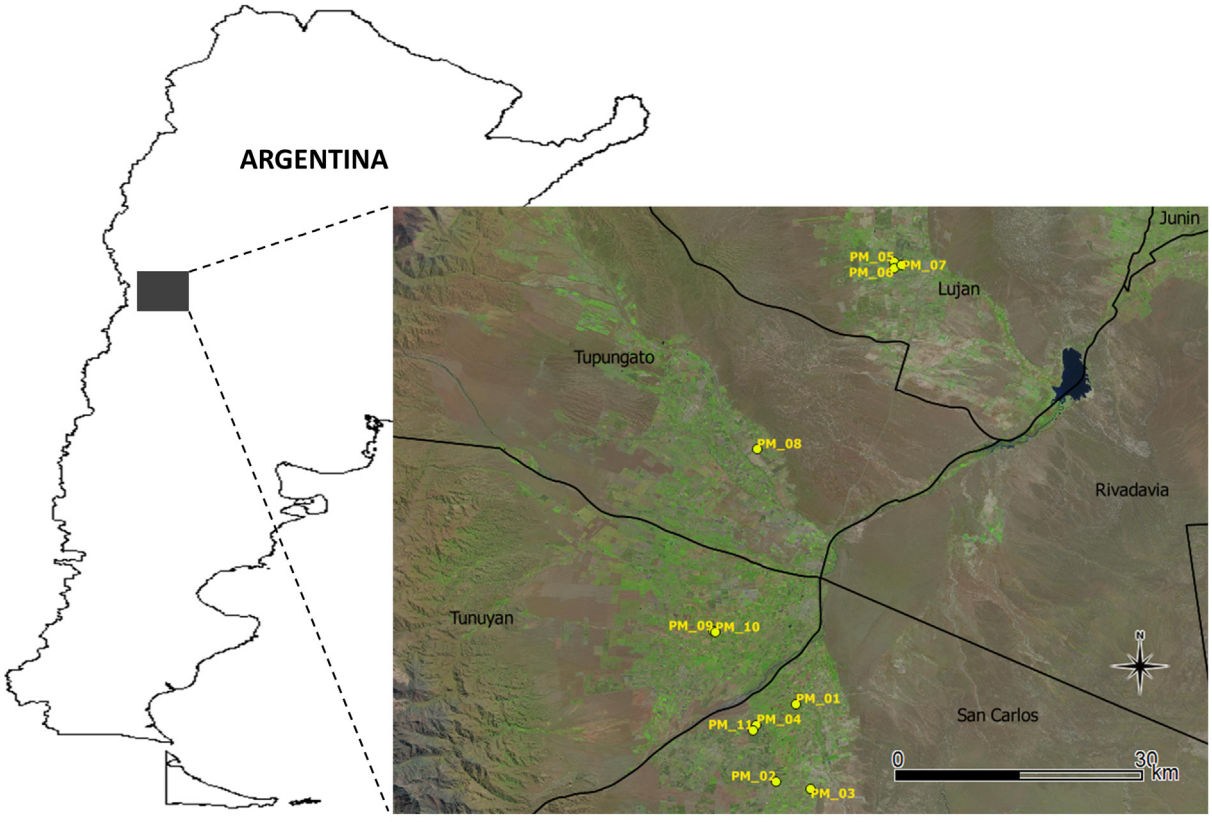

Fig. 2. Geographical distribution of the sampling locations for powdery mildew in cucurbits. Source: U.S. Geological Survey (2022), https://www.usgs.gov/.

Materials and Methods

Sample collection. The samples were collected in 11 locations representing different ecological zones of the province of Mendoza, Argentina (MZ) (Fig. 2). The selected sites are located in traditional cucurbit producing areas. Samples consisted in leaves with signs of the pathogen (=mycelial colonies) that were collected during March 2019 in cucur-

Table 1. General information recorded for the samples collected in different cucurbits production areas of Mendoza, Argentina (MZ)

Code	Location	Host	Cultivar	Latitude	Longitude
PM_01	Capiz	Cucurbita moschata	Male parent of cv. Cokena Argentum INTA	–33,6847	–69,0299
PM_02	San Carlos	*C. maxima* × *C. moschata*	n/a	–33,7693	–69,0514
PM_03	Casas Viejas	*C. maxima*	cv. Zapuco INTA	–33,7780	–69,0138
PM_04	La Consulta	*C. moschata* × *C. moschata*	cv. Cokena Argentum INTA	–33,7074	–69,0729
PM_05	Ugarteche	*Cucurbita moschata*	n/a	–33,2019	–68,9219
PM_06	Ugarteche	*Cucurbita moschata*	n/a	–33,2087	–68,9229
PM_07	Ugarteche	*Cucurbita moschata*	n/a	–33,2053	–68,9141
PM_08	El Zampal	*C. maxima* var. *zapallito*	n/a	–33,4061	–69,0725
PM_09	Colonia Las Rosas	*Cucurbita moschata*	n/a	–33,6050	–69,1198
PM_10	Colonia Las Rosas	*C. maxima* × *C. moschata*	n/a	–33,6064	–69,1181
PM_11	La Consulta	*C. maxima* × *C. moschata*	n/a	–33,7133	–69,0764

n/a, not available.

bit fields, including squash (*C. moschata, C. maxima*), interspecific hybrids and others (Table 1). Geographical data, as well as general crop information was recorded for each sample. The samples were properly conserved and taken to the laboratory, where pieces of leaves containing one single colony were carefully excised with a scalpel, obtaining four isolates for each sample that were later processed separately.

Morphological and molecular identification of the isolates. The shape of conidia and their aggregation form (solitary or in chains) was recorded under stereomicroscope. The presence/absence of fibrosin bodies was observed under microscope, assembling fresh conidia excised from the colonies in KOH 3% (Lebeda and Sedláková, 2010). DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) extracting total DNA from the fungal colonies. Leaves that were free of powdery mildew were included as negative controls. The identity of the causal agents was determined via polymerase chain reaction (PCR) reactions, using a set of primers previously reported to be useful to discriminate the three species that may cause powdery mildew in different crops (Table 2). The PCR reaction mix contained buffer 1×, 0.12 U/µl of polymerase (GoTaq DNA polymerase, Promega), 1.8 µM of dNTPs, 0.60 µM of each primer and 1 µl of DNA template, in a final volume of 15 µl. Sterile distilled water (SDW) and DNA extracted from asymptomatic leaves served as negative controls. The reactions were performed in a Bioneer MyGenie 96 Gradient Thermal Block thermocycler. The conditions for the reaction were set as follows: initial denaturation of five minutes at 94°C, followed by 30 cycles of 40 s at 94°C, 60 s at 57°C and 90 s at 72°C with a final elongation step of five minutes at 72°C. The amplified bands were resolved by electrophoresis (agarose 3% in TBE buffer, 90 V, 60 min) using GelGreen (Biotium Inc., Fremont, CA, USA) as nucleic acid stain and visualized in a Dark Reader DR46B (Clare Chemical Research, Dolores, CO, USA) transilluminator.

Resistance of squash to powdery mildew under field conditions. The field trial was carried out during the summer of 2019/2020 at the INTA EEA La Consulta experimental field (33°42′34.4″S, 69°04′27.9″W) to test the resistance of different squash materials to CPM. A set of five accessions, comprising cultivars and breeding lines was evaluated (Table 3). The trial was designed in complete randomized blocks, with four replications, each replication consisting of five plants. Natural primary inoculum of the causal agent of CPM was present in the field as disease occurred in previous seasons and corresponded to *Podosphaera xanthii*, as shown by the results below. The relative quantity of diseased plants (incidence) and the severity index were recorded twice a week, during six weeks, starting when the first diseased plant was detected (epidemic onset). Each plant was carefully evaluated and rated for incidence with 0 (no disease observed) and 1 (plant with at least one leaf infected with CPM). Following the same procedure, the severity index (S) was rated using a modified scale (Lebeda and Sedláková, 2010), estimating the percentage

Table 2. Species specific primers used to discriminate the isolated pathogens associated to powdery mildew of cucurbits

Primer name	Species	Sequence (5′-3′)
S1	*Podosphaera xanthii*	GGATCATTACTGAGCGCGAGGCCCCG
S2	*Podosphaera xanthii*	CGCCGCCCTGGCGCGAGATACA
G1	*Golovinomyces cichoracearum*	TCCGTAGGTGAACCTGCGGAAGGAT
G2	*Golovinomyces cichoracearum*	CAACACCAAACCACACACACGGCG
L1	*Leveillula taurica*	CCCTCCCACCCGTGTCGACTCGTCTC
L2	*Leveillula taurica*	CTGCGTTTAAGAGCCGCCGCGCCGAA

Source: Chen et al. (2008).

Table 3. Set of squash (*Cucurbita moschata*) accessions evaluated in this study

Name[a]	Genetic background	Origin/Additional information
Carruecano	OP population	INTA Squash Breeding Program
Cokena INTA[a]	OP cultivar	INTA Squash Breeding Program
BL717/1[a]	Breeding line	INTA Squash Breeding Program
Pecas INTA[a]	OP cultivar	INTA Squash Breeding Program
RCvar/2020	F1 hybrid cultivar	Seed Company, Resistant control

[a]Seeds of INTA cultivars and breeding lines are available under request.

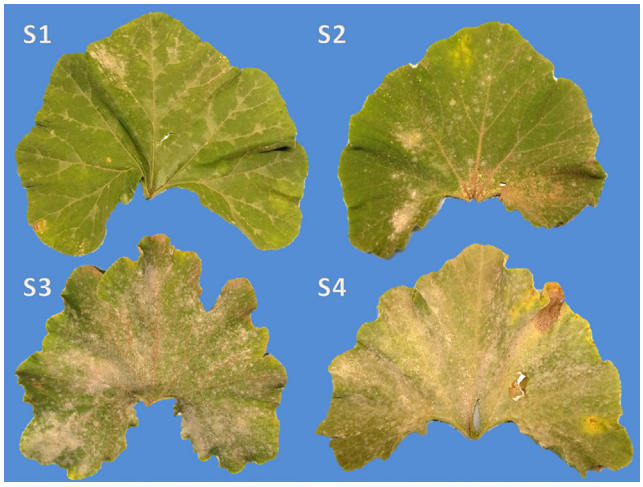

Fig. 3. Schematic figure to guide the indexing of cucurbit powdery mildew severity in leaves (*Cucurbita maxima* cv. Pecas INTA). 0% < S1 < 25% of leaf surface covered by colonies; 25% ≤ S2 < 50% of leaf surface covered by colonies; 50% ≤ S3 < 75% of leaf surface covered by colonies; S4 ≥ 75% of leaf surface covered by colonies, chlorotic or dead leaf.

of leaf covered by powdery mildew colonies as follows: S0 no visible signs/symptoms of the disease; 0% < S1 < 25% of leaf surface covered by colonies; 25% ≤ S2 < 50% of leaf surface covered by colonies; 50% ≤ S3 < 75% of leaf surface covered by colonies; S4 ≥ 75% of leaf surface covered by colonies, chlorotic or dead leaf (Fig. 3). During the complete trial fungicidal sprays were not applied and the epidemic was allowed to develop without intervention.

The first diseased leaf of each plant was identified and the severity evaluation was sequentially assessed in that specific leaf until the end of the assay. The entire plants were visually assessed to confirm the leaf severity indexes. Collected data was analyzed through restricted maximum likelihood estimation for parameters (binomial distribution, logit as link function), using a generalized linear mixed models (GLMM) approach, employing the GLMM package of InfoStat (Di-Rienzo et al., 2020). To write the terms of the model, squash accession, time and their interaction were considered as fixed effects; experimental (plot) error was included as random effect (treatment by block interaction). Linear predicted values (PredLin) for each treatment (squash accession) were statistically compared using least significant difference Fisher ($\alpha = 0.05$). The calculated parameters of the models were used to build non lineal disease progress curves adjusted for each squash accession, using the following transformation for incidence or severity index, as expressions of disease intensity (DI):

$$DI = \frac{EXP(PredLin)}{1 + EXP(PredLin)}$$

Results

Chasmothecia were not found on any of the collected leaf samples. Microscope observations of conidia showed the presence of conspicuous linear structures in all the isolates evaluated. These structures corresponded to fibrosin bodies, a distinctive feature of Px (Lebeda and Sedláková, 2010). Confirming the identity of the isolates, the PCR test led to positive reactions for all the isolates with the primer pair S1/S2 (specific to Px), which yielded a specific amplicon of around 450 bp (Fig. 4). In contrast, only negative reactions were achieved with the primer pairs G1/G2 and L1/L2. The reactions containing SDW or DNA extracted from asymptomatic leaves showed negative results for the three primer pairs tested.

The parameters for severity progression were calculated through GLMMs. The fixed effects 'squash accession' and

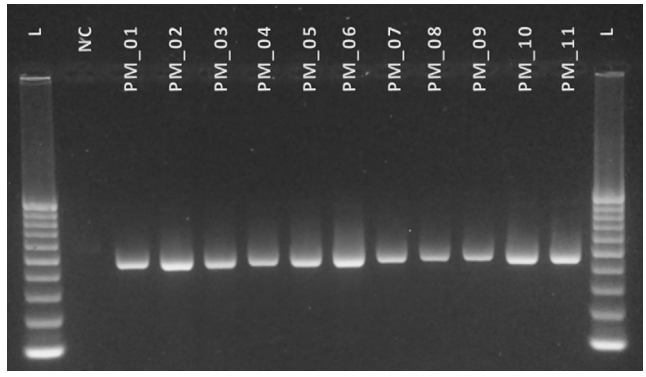

Fig. 4. Polymerase chain reaction (PCR) products amplified by primers S1/S2. L, 100 bp ladder; NC, negative control (sterile distilled water); lines 01-11 powdery mildew isolates. PCR products were separated by electrophoresis in a 2% agarose gel.

Table 4. Lineal predicted parameters and mean severity values calculated by GLMMs for each squash accession

Squash cultivar	PredLin	Mean[a]
Pecas INTA	0.04	0.51 A
Cokena INTA	–1.51	0.20 B
BL717/1	–2.18	0.10 BC
Carruecano	–2.37	0.09 C
RCvar/2020	–3.09	0.04 C

GLMM, generalized linear mixed model; INTA, National Institute of Agricultural Technology.
[a]Mean values with the same letter are not statistically different (least significant difference Fisher, $P > 0.05$).

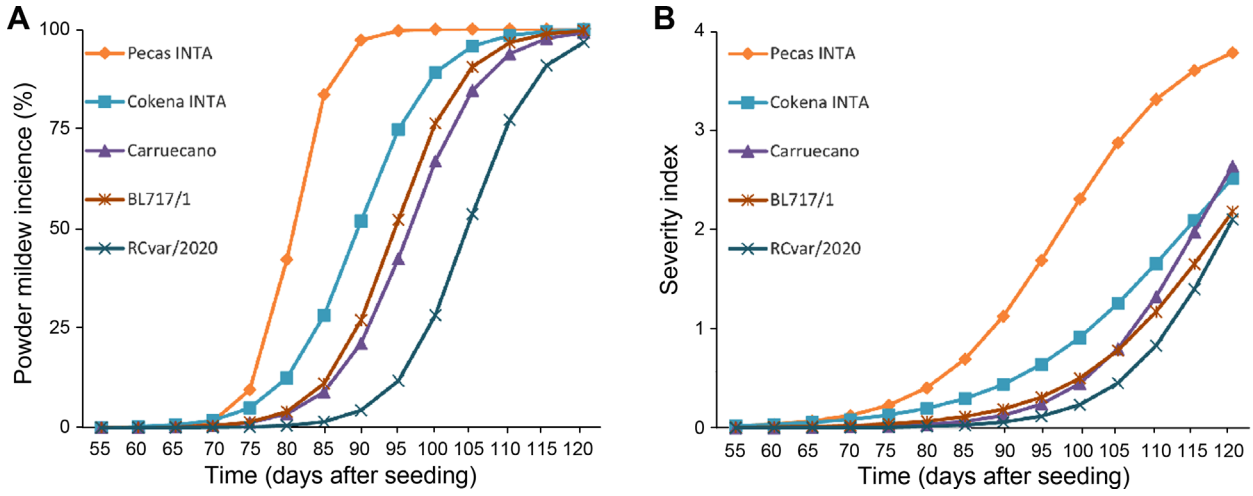

Fig. 5. Simulation of disease progress curves of cucurbit powdery mildew incidence (A) and severity index (B), for each of the tested squash accessions. Parameters calculated through generalized linear mixed models.

'time' showed high significance each in the chi-squared test ($P < 0.0001$) for the severity index. The interaction of the fixed effects was not significant ($P = 0.3417$). The comparison of squash accessions was performed via ANOVA of mean values of severity (Table 4). The results showed significant differences among the tested materials for both parameters. The cultivar Pecas INTA behaved as the most susceptible accession, while the resistant control RCvar/2020 recorded the lowest values of disease intensity. The rest of the tested materials, including the breeding line BL717/1 showed intermediate levels of severity.

The analysis of disease progression in the resistant materials showed a delayed onset of the epidemics and a slower increase of disease incidence. This can be noted in Fig. 5, where 50% of the Pecas INTA plants were diseased at day 81, while the same incidence was reached in RCvar/2020 only 23 days after. The same was found for disease severity in leaves. The susceptible cultivar Pecas INTA showed a continuous increase in disease intensity, while the rest of the materials showed an intermediate behavior.

Discussion

Based on the recorded morphological features, confirmed by simple PCR analysis, the complete set of isolates obtained from the field samples were classified as *Podosphaera xanthii*. The presence of other pathogens like *Golovinoomyces cichoracearum* or *Leveillula taurica* causing CPM was not recorded in the collected samples. Hence Px was identified as the causal agent of CPM in the sampled area. This species has been reported as the prevalent pathogen associated to CPM in temperate regions in many different countries, namely USA (Kousik et al., 2008), Spain (Pérez-García et al., 2009), Australia and New Zealand (Křístková et al., 2009). It is also the main pathogen associated to CPM in warm regions and climates (Lebeda and Sedláková, 2010), which corresponds to the geographical region considered in our study. The detection of Px as the most common and widely spread causal agent of powdery mildew of cucurbits in Mendoza is in accordance with previous citations for other regions of Argentina (Delhey et al., 2003; Kiehr and Delhey, 2013; Mazzanti de Castañon et al., 1987). The present work applied for the first time molecular markers to confirm the identity the pathogens associated to CPM in Argentina. However, more studies are needed in the future to assess the population structure, to detect the occurrence or physiological races and development of sexual structures in Px. The presence of additional species as causal agents of PMC, such as Gc, should also be checked, including samples taken in other regions of the country. The influence of the time when the samples were collected, the geographical location and their interaction could also determine which species are isolated. CPM populations may vary in their composition temporally and spatially (Lebeda et al., 2016), thus further studies are mandatory to reach a comprehensive knowledge of the disease in the region comprised by this survey.

Respect to the evaluation of resistance under field conditions, the results presented above demonstrate that the statistical effects of squash genotype and time significantly influence epidemiological variables, as incidence and severity, indicating that both conditions are critical aspects to explain the disease progression curves. Particularly, a high number of diseased plants which hold effusing sporulating colonies accelerate the epidemics. The simulated models describe a logistic curve, according to a polycyclic disease

as expected to CPM (Madden et al., 2007a). Differences in the resistance trait were found for the evaluated accessions, confirming the cultivar Pecas INTA as susceptible control and RCvar/2020 as resistant. The accession BL717/1, a breeding line originally generated by a double hybrid cross of two quantitative resistant cultivars, followed by recurrent selection under field conditions, showed intermediate levels of resistance and displayed promising results as source of CPM resistance for the breeding program. This finding supports the hypothesis of more than one single gene being involved in its performance and also suggests that several cycles of field selection of good performing lines are a suitable approach to accumulate genes of minor effect involved in the resistance trait. However, genetics assays to confirm this hypothesis are lacking. Interestingly, some materials like Carruecano, BL717/1 and particularly RCvar/2020, despite allowing high levels of infection (=incidence reaching 100%), showed a delay in the rate of the epidemics, as well as low severity indexes at harvest time. Eventually the delayed onset and late increase of the epidemics is a valuable feature for practical purposes and for the development of resistant cultivars. In western Argentina the crop cycle for these cultivars takes around 120 days, comprising hot summer and long sunshine conditions. Thus, the low proportion of diseased leaves in the resistant genotypes, especially at the end of the cycle, in addition to low severity scores, facilitates proper protection of fruits until the date of harvest. The foliar collapse caused by strong infection with CPM in susceptible cultivars and hence the risk of sunburn and quality issues in fruits (Pérez-García et al., 2009) is minimized by the use of partial resistant materials. In our study, the incidence reached 100% in all squash materials, as expected for a polycyclic disease, but differences were evident for severity, which was reduced in the resistant materials, particularly RCvar/2020 and Carruecano. Meanwhile, the susceptible cultivars Pecas INTA and Cokena INTA showed high levels of fruit damage and foliar death, leading to significant yield and quality losses.

The minimized increase of the epidemics suggests the possible activation of effective mechanisms of partial resistance in the resistant accessions. The extended latency period for sporulation and low number of released spores in comparison with the susceptible cultivars (Niks et al., 2011) could explain this behavior, though the components of resistance remain unstudied and should be properly evaluated in future trials. The inheritance of the resistance to CPM in cucurbits is partially understood. Some studies report that the trait is conferred by a single dominant gene in most cases (Pérez-García et al., 2009), but other reports mention the occurrence of recessive genes for resistance, particularly in other members of the cucurbit family, as cucumber (Morishita et al., 2003). The phenotypical expression of the resistance trait also depends on the particular resistant gene and climate conditions, like temperature (Morishita et al., 2003). All these particular features implicate that local validation tests are essential to evaluate resistant materials and adapt cultivars to the particular regional environment. Furthermore, the importance of determining the key factors that influence the onset of the disease and its progression are major concerns to consider in the management of CPM.

To properly analyze the data on infection severity we employed GLMMs, a common approach in many disciplines that may be preferred for non-normally distributed data, over standard analysis of variance (Madden et al., 2007b). In these cases, variables like incidence are characterized by a binomial or beta-binomial distribution, not a normal distribution (an assumption of ANOVA). Then GLMMs can, in principle, deal with the statistical properties of disease incidence and account for multiple random and fixed factors in the analysis (Madden et al., 2007b). Our study showed that the use of GLMMs is and advisable approach to analyze diseases like CPM in cucurbits in field trials, using data collected in different times of the crop cycle.

The employment of simple techniques, such like the observation of distinctive microscopical structures and the PCR with species-specific primers, was rather enough to support the preliminary identification *Podosphaera xanthii* as the causal agent of CPM, at least at the species level. Unfortunately, a set of squash differentials was not available to check the (possible) occurrence of races in the Px populations. Therefore, new complementary studies are needed to assess the genetic variability of Px isolates, including the identification of races within its population (Lebeda and Sedláková, 2010). In this regard, the heterothallic nature of the genus *Podosphaera* reduces the possibility of occurrence of sexual reproduction (Pérez-García et al., 2009), a strong driver for population diversity. Furthermore, chasmothecia have rarely or never been observed in several of the world's most important cucurbit growing areas, including Argentina. The prevalence and epidemiological relevance of the sexual stage of the pathogen remains questioned (Pérez-García et al., 2009), probably not playing a key role to explain genetic population structures or constitute a relevant primary inoculum. New isolations and identifications are needed to check the occurrence of other species causing CPM, such as Gc, which is present in Argentina affecting melon, lettuce and sunflower (Sistema Nacional Argentino de Vigilancia y Monitoreo de Plagas, 2021), crops usually present surrounding squash fields. Remarkably, Px and Gc differ in their host ranges, ecologi-

cal requirements, sensitivity to fungicides and pathogenicity (Křístková et al., 2009; Sistema Nacional Argentino de Vigilancia y Monitoreo de Plagas, 2021). The present results, as well as future advances, will contribute to better understand the disease epidemics, provide new management tools and develop OP squash cultivars with increased resistance to CPM, all of which will provide an integrative, ecological and economic alternative for managing the disease.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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