Archivos de Zootecnia
Journal website: https://www.uco.es/ucopress/az/index.php/az/

Studies of *Ascosphaera apis* strains isolated from commercial pollens of different Spanish provinces and their enzymatic production capacity

Tejerina, M.R.[1], Cabana, M.J.[1], Flores, J.M.[2] and Benitez Ahrendts, M.R.[1@]

[1]National University of Jujuy. San Salvador de Jujuy. Argentina.
[2]Universidad de Córdoba. Córdoba. Spain.

SUMMARY

Ascosphaera apis is a pathogenic fungus of *Apis mellifera* bees larvae that causes the disease-chalkbrood, which causes serious damage to hives, causing serious economic losses. The disease is usually recurrent during the rainy season and low temperatures, but there are also other factors that predispose the colony to become ill, such as lack of protein reserves, poor ventilation inside the hive and weak hives, especially in those where there is not an adequate use of antimicrobials., In this work six strains of the commercial pollen fungus were isolated from four Spanish provinces, which were conserved for 48 months, they were identified genetically using the ITS (internal transcription space) regions coding for rRNA 5.8S. In order to verify its viability, it was performed an in vitro enzymatic screening of the secretion capacity of products and proteases in conventional media, all strains were found to be viable at 30°C and under microaerophilic conditions, it was also shown that the strains are related phylogenetically, far from other countries, which would be showing that the spores are transported among Spanish provinces. This work brings an approximation to the viability of *A. apis* fungus spores in commercial pollens, to extend sanitary measures and to control the pollen used in the feeding of beehives when it is scarce.

ADDITIONAL KEYWORDS
Ascosphaera apis.
Phylogenetic.
Pollen.
Chitinases.
Proteases.

Estudios de cepas de *Ascosphaera apis* aisladas de pólenes comerciales de diferentes provincias españolas y su capacidad de producción enzimática

RESUMEN

Ascosphaera apis es un hongo patógeno de larvas de abejas *Apis mellifera* que provoca la enfermedad cría yesificada, la cual ocasiona serios daños a las colmenas, y llega a producir graves pérdidas económicas. La enfermedad suele ser recurrente durante el periodo de lluvias y temperaturas bajas, pero también existen otros factores que predisponen a la colonia a enfermarse, como la falta de reservas proteicas, la mala ventilación dentro de la colmena y colmenas débiles, especialmente en aquellas donde no hay un uso adecuado de antimicrobianos. En este trabajo se aislaron seis cepas del hongo de pólenes comerciales proveniente de cuatro provincias españolas, que fueron conservados durante 48 meses, las mismas fueron identificadas genéticamente utilizando las regiones ITS (internal transcription espace) que codifican para ARNr 5.8S, para comprobar su viabilidad se procedió a realizar un screening enzimático in vitro de la capacidad de secreción de quitinasas y proteasas en medios convencionales, se comprobó que todas las cepas fueron viables a 30°C y en condiciones de microaerofilia, además se demostró que las cepa están relacionadas filogenéticamente, alejadas de otros países, lo que estaría evidenciando que las esporas son transportadas entre provincias españolas. Este trabajo trae una aproximación a la viabilidad de las esporas del hongo *A. apis* en pólenes comerciales, para ampliar las medidas sanitarias y controlar el polen usado en la alimentación de las colmenas cuando este es escaso.

PALABRAS CLAVE ADICIONALES
Ascosphaera apis.
Filogenética.
Polen.
Quitinasa.
Proteasa.

INFORMATION
Cronología del artículo.
Recibido/Received: 05.07.2017
Aceptado/Accepted: 02.07.2019
On-line: 15.07.2019
Correspondencia a los autores/Contact e-mail:
mrba71@yahoo.com.ar

INTRODUCTION

Bees play an important ecological role worldwide since they pollinate numerous crops as well as wild plants, making them of great economic importance in agriculture around the world, nowadays the demand for pollination has increased (Koh *et al.*, 2016, p. 140). In the beehive, bees face a continuous exposure to different pathogenic microorganisms, either due to the alteration of the population, low temperatures and the increase of humidity that make factors predisposing to diseases by parasites such as *Varroa* sp.,

Bacterial diseases), Fungal diseases (Chalkbrood), or viral diseases among others (Flores *et al.* 1996, p. 188, Shin *et al.*, 2016, p. 1).

The genus *Ascosphaera* (Ascomycota: Eurotiomycetes: Ascosphaerales), is mainly associated with the larvae of bees and products of the hive, among them pollen. The *Ascosphaera apis* species causes the disease known as chalkbrood, which affect the developing larvae. The spores of this fungus remain viable for years, when the larvae ingest them together with the food supplied by the nurses, germinate in the intestine and pass through the intestinal wall, spreading throughout the body, appearing on the body surface when the larva reaches the pre-pupae phase (Maxfield-Taylor *et al.*, 2015, p. 7). On the body surface the fruiting bodies (Sporeballs) are developed and they will produce a new generation of spores (Jensen *et al.*, 2013, p. 4). The mechanism by which spores go through the intestine is due to the coordinated production of extracellular enzymes secreted into the medium, together with the mechanical pressure of the hyphae in the exoskeleton and/or peritrophic membrane of the intestine. The genes expressed in the virulence correspond to hydrolytic enzymes, such as chitinases, proteases and esterases (Alonso *et al.*, 1993, p. 385; Wang 2000, p. 135; Teerayut 2008, p. 71; Cornman *et al.*, 2012, p. 6).

A. apis can also infect adult bumble bees, increasing the potential to infect native bees through the spread of pathogens (Maxfield-Taylor *et al.,* 2015, p. 7*).* With the exception of natural essential oils, no other compound has been shown to inhibit *A. apis* in its growth (Ansari *et al.*, 2017, p. 1003).

The viability of the spores in pollen transported to the hives is unknown, so the objective of the present work was to isolate strains of the fungus *A. apis* from different provinces of Spain from conserved pollen samples and determine their phylogenetic relationship. As well as, how to evaluate the viability of the spores, from hydrolytic enzymes.

MATERIAL AND METHODS

Isolation of *Ascosphaera* spp.

The commercial pollen was purchased from herbalists of Spanish provinces, Madrid, Aragón Zaragoza, Castilla-La Mancha, Ciudad Real, Castilla-La Mancha, Guadalajara, Castilla-La Mancha, Cuenca, Catalonia-Barcelona; it was preserved at 20°C in the dark for 48 months after its acquisition in 2012. 1g of pollen was seeded in selective medium malt extract and yeast agar with 20% dextrose MY20 (Takatori 1982, p. 91) and incubated under microaerophilic conditions at 30±2°C for 4 to 10 days. They were identified based on their morphology, six strains of *Ascosphaera apis* were isolated from the Spanish provinces: strain P1, Madrid, strain P3: Aragón Zaragoza, strain P4: Castilla-La Mancha, Ciudad Real, strain P5: Castilla-La Mancha, Guadalajara, strain P6: Castilla-La Mancha, Cuenca, strain P8: Catalonia-Barcelona.

Analysis and identification of *Ascosphaera* spp.

Obtaining genomic DNA from collected samples

The DNA was isolated from mycelium grown in MY20 liquid medium. The mycelium was washed twice with 1mL of 0.1M TRIS-HCl solution, 0.02M EDTA in cold and centrifuged at 12000 rpm for 3 min. Subsequently the mycelium was ground with a sterile glass rod in the presence of 1mL of extraction buffer (100mM Tris-HCl pH 8, 1.5M NaCl, 50mM EDTA pH 8, Proteinase K, 0.1 mg/ml, 10mM β-mercaptoethanol , SDS 2%) and digested at 60°C for 1 vortexing every 10 min. The DNA was purified from the supernatant of this digestion using chloroform: isoamyl alcohol (24:1), twice, and 3M potassium acetate for one time. Subsequently the DNA was precipitated with 100% Isopropanol, washed with 70% cold Ethanol, centrifuged at 12000 rpm for 5 min, dried at room temperature and resuspended in nucleated free distilled water and stored at -20°C until use.

Amplification and analysis of ITS sequences

In order to perform the characterization of the requested fungal isolates the ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primers described by White et al (1990), ITS1 5'-TCCGTAGGT-GAACCTGCGG and ITS4 5'-TCCTCCGCTTATTGA-TATGC. PCR was carried out in 20 µl final volume containing 1X Buffer, 2.5 mM Magnesium Chloride, 200 µM dNTPs, 10 µM of each primer, 0.5 U Taq polymerase, using a cyclin composed of an initial denaturation 4 min at 94°C and 35 cycles of: 40s 94°C, 40s 52°C and 40s 72°C, with a final extension of 10 min at 72°C.

PCR products were screened on 2% agarose gels, sequenced using the Macrogen Korea service and analyzed with the MEGA6 program which allows visualization of the chromatograms, facilitates their control and the conformation of a consensus sequence for each sequence obtained. The sequences were compared against the Fungal barcoding database (http://www.fungalbarcoding.org/), using the Pairwise sequence alignment tool and NCBI database (http://www.ncbi.nlm.nih.gov/) using the BLASTn tool. The sequences were deposited in the GenBank database under the accession number KX622164; KX622165; KX622166; KX622167; KX622168; KX622169.

Determination of phylogenetic relationships of strains

All sequences were analyzed using MEGA6, BLASn, BioEdit and CLUSTAL W, before the tree construction. Phylogenetic analysis was carried out using the TNT program. The gaps (indels) were treated as a fifth state as they represent insertion-deletion events. The analysis includes 18 sequences including *Ascosphaera aggregata* (U68323), which was used as outgroups. The set of data was reduced, in the heuristic search, 1000RAS was implemented, saving a tree by TBR. In order to evaluate the support in the identification of groups, a bootstrap and a parsimony analysis of Jack-knifing was performed. Both bootstrap and Jack-knife analysis include 1000 resampled matrices. For each, 100 cycles of RAS + TBR were performed.

Detection of enzymatic activity proteases and chitinases

Solid media

A primary culture was performed on solid media on 90 mm diameter petri dishes containing MY20 medium.

The mycelia were inoculated with ansa needle, puncture a pad of approximately 36-mm² (mycelial agar kept at 4°C) and allowed to grow for 5 to 7 days under microaerophilic conditions at 28±1°C. At the end of this time period a colony of sufficient size could be obtained for the generation of mycelial blocks intended for repechage and for the seeding of the chitinase and protease detection means. The fungi were picked in the same medium every 30 days and stored at 4°C.

For inoculation of liquid media, mycelial agar blocks were extracted from the edge of the growin colony with the aid of a 36 mm² glass punch.

Detection of chitinases

An identification medium was made for chitinases with modifications, described by Carrillo (1998, p.74). To obtain colloidal chitin as a substrate, the protocol described by Agrawal (2012, p. 24) was followed by weighing 1 g of chitin (SIGMA), adding 10 ml of concentrated HCl and leaving it standing 6 hours. 250mL of 70% cold ethanol was added under continuous stirring for about 10 to 20 min, the mixture was expected to stabilize, the pH was adjusted to 7.0. It was centrifuged at 8000 rpm for 30 min at 4°C. The pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4°C until the alcohol odor was completely removed. The colloidal chitin obtained had a soft, pasty consistency and was stored at 4°C until its further use.

Qualitative determination of chitinase activity

On the other hand, the strains were seeded in colloidal chitin agar composed of: 5 g colloidal chitin, 1 g NH4Cl, 1 g K2HPO4 and 1mL trace elements (1% FeSO4-7H2O solution, MnCl2-4H2O and ZnSO4-7H2O), agar 15g And 0.15 g of bromocresol purple, adjusted to pH 7. Subsequently strains *A. apis* were incubated for 7 to 10 days at 30 ° C in microaerophilic conditions. The test was done in triplicate, leaving them incubating until the formation of a yellow zone around the colonies in the purple medium.

Detection of extracellular proteases

The agar-milk medium was used for the selection of protease-producing microorganisms. 20g of skim milk was dissolved in 100mL of distilled water and on the other hand a suspension of 13g of agar in 900mL of meat peptone and 15g of agar was prepared. They were sterilized in separate containers and let them cool down to about 60 ° C and the media were mixed, dispensed into sterile 90mm Petri dishes (Sarath *et al.*, 1989, p. 30; Lumi *et al.*, 2015, p. 15335). Blocks of the mycelium of the *A. apis* strains were inoculated. The tests were performed in triplicate. As the milk agar is opaque, the enzymatic activity by the presence of a clear halo around colonies was evaluated.

RESULTS

Macroscopic characteristics and phylogenetic study of the studied strains This work was focused on six strains of the *Ascosphaera* genus of the A. apis species isolated from commercial pollen from the Spanish provinces **(Table I)** preserved for 48 months. All strains grew in MY20 between 4 and 10 days, but showed differences in sporulation, strains P1, P3, P8, sporulated at 5 days and the mycelial surface was more scarce, whereas P5, P4 and P6 made them in On day 7, but there is a uniform cottony mycelial growth developing above the ascas **(Figure 1)**. In the phylogenetic analysis the nucleotide sequence of the ITS1-5.8-ITS2-28S region of the strains P1 (585), P3 (491), P4 (483), P5 (481), P6 (391) and P8 (453bp), the blast search showed that the sequences exhibited an identity greater than 89% with other *A. apis* sequence from the NCBI database, based

Table I. Reference number in database (*) https://www.ncbi.nlm.nih.gov/ (Número de referencia en la base de datos (*) https://www.ncbi.nlm.nih.gov/)

Ascosphaera sp.	Strains	Isolation	Origin	Referece (*)
A. apis	P1	Pollen	Madrid	KX622164
A. apis	P3	Pollen	Aragón Zaragoza	KX622165
A. apis	P4	Pollen	Castilla-La Mancha, Ciudad Real	KX622166
A. apis	P5	Pollen	Castilla-La Mancha, Guadalajara	KX622167
A. apis	P6	Pollen	Castilla-La Mancha, Cuenca	KX622168
A. apis	P8	Pollen	Cataluña, Barcelona	KX622169
A. apis	ST-OR11-A1	Bumble Bee Adults	USA	KJ158165
A. apis	4081-2	*Apis mellifera*	Rusia	KM242592
A. apis	4080-2	*Apis mellifera*	Rusia	KM242591
A. apis	4079-2	*Apis mellifera*	Rusia	KM242590
A. apis	4078	*Apis mellifera*	Rusia	KM242589
A. apis	13785	*Apis mellifera*	USA	GQ867764
A. apis	ATCC MYA-4450	*Apis mellifera*	USA	FJ172292
A. apis	NAP	*Apis mellifera*	USA	GQ867766
A. apis	ARSEF 5142	*Apis mellifera*	Australia	U68313
A. aggregata	ARSEF 689	*Apis mellifera*	Australia	U68323
A. atra	CBS 524.75	*Apis mellifera*	Australia	U68314

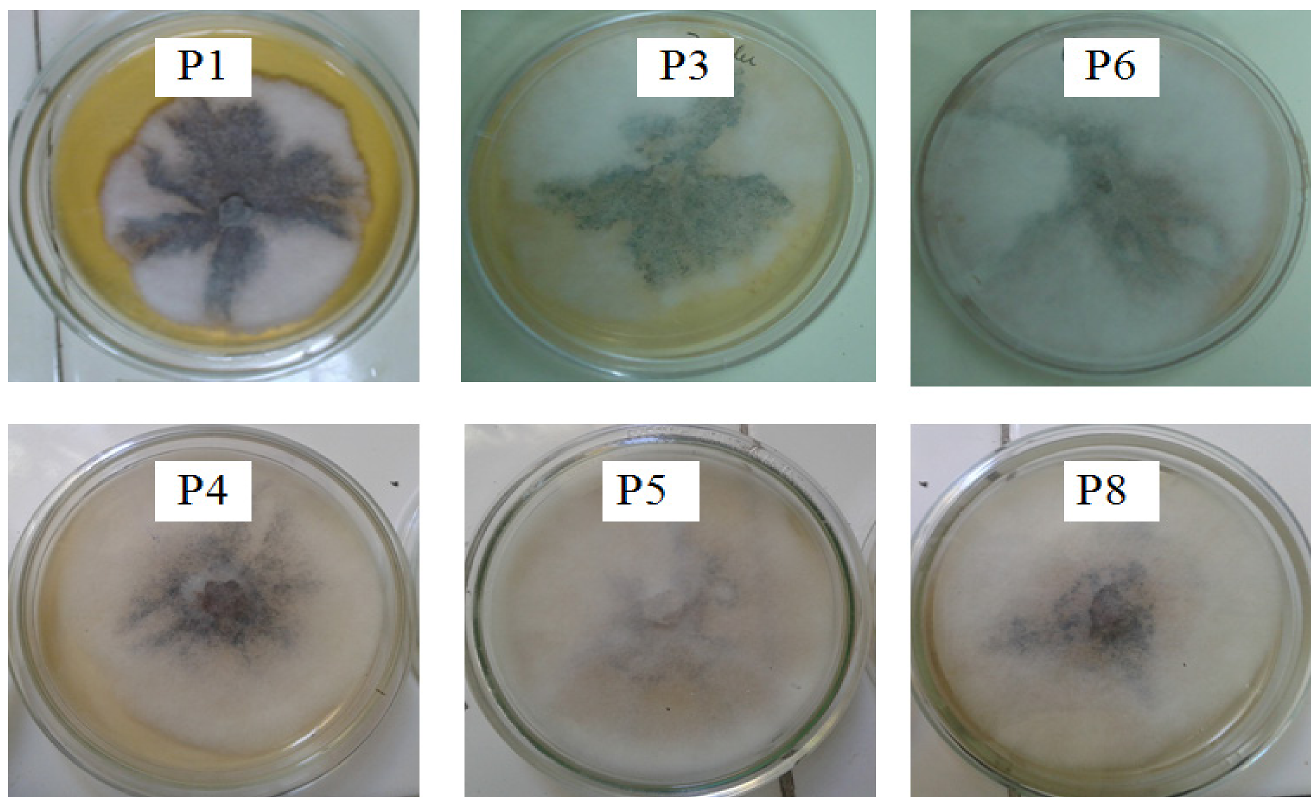

Figure 1. Characteristics in *Ascosphaera apis* culture, isolated from commercial pollen in MY20. The letters indicate P (pollen) and the code number of the strain (Características en cultivos de Ascosphaera apis, aisladas a partir del polen comercial en MY20. Las letras indican P (polen) y el número de código de la cepa).

on the blast search results, for subsequent phylogenetic analysis species of *A. apis* from three countries, the United States, Australia and Russia, which are registered at NCBI (https://www.ncbi.nlm.nih.gov/). The informative sites were defined by not being in ambiguous regions, not presenting gap and not presenting consensus sequence in each site. The sequences were aligned with 658 characters, with 285 informative sites parsimoniously. The analysis of parsimony resulted in 5 more parsimonious trees of 1077 steps. Both the bootstrap and jack-knife processes established the same topology, and their support values were very similar **(Figure 2)**.

The resulting trees showed that all the strains studied in this work formed a monophyletic clade that was closely related to *A. apis*. With a high bootstrap support of 98% and a support of Jacknifing also of 91%, the strains of P3 and P8 were established in a monophyletic clade, with a high bootstrap support of 100% and a Jacknifing support also of 100%, As did the strains P4 and P5 which exhibited the same supports **(Figure 2).**

Enzymatic analysis

The detection of extracellular chitinases with the Carrillo et al. (1998) method was positive at 7 days of inoculation of strain P1, P3, P6 and P8, while P4 and P5 secreted chitinases at 10 days ofinoculation **(Table II, Figure 3)**. On the other hand, strains P1, P3, P6 and P8 secrete proteases at 48 hours, and strains P4 and P5 secrete proteases at seven days **(Table II and Figure 3)**.

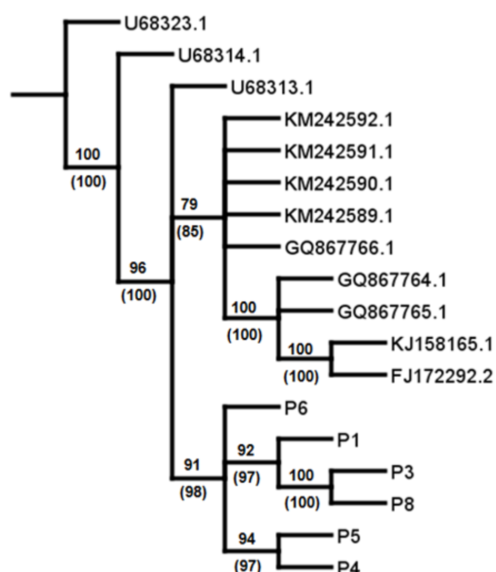

Figure 2. Phylogenetic relationship between strains of ITS region of 5.8S rDNA, The support group was evaluated using 1000 replications, bootstrapping and parsimony of Jack-kniffing. The numbers in parentheses correspond to the Jack-knife holder. The bootstrap support is found without parentheses. P1: Madrid, P3: Aragon-Zaragoza, P4: Castilla-La Mancha, Ciudad Real, P5: Castilla- La Mancha, Guadalajara, P6: Castilla-La Mancha, Cuenca, P8: Catalonia-Barcelona (Relación filogenética entre cepas de la región ITS de 5.8S rDNA, el grupo de apoyo fue evaluado usando 1000 replicaciones, bootstrapping y parsimonia de Jack-kniffing. Los números entre paréntesis corresponden al soporte Jack-knife. El soporte de arranque se encuentra sin Paréntesis. P1: Madrid, P3: Aragón-Zaragoza, P4: Castilla-La Mancha, Ciudad Real, P5: Castilla- La Mancha, Guadalajara, P6: Castilla-La Mancha, Cuenca, P8: Cataluña-Barcelona.).

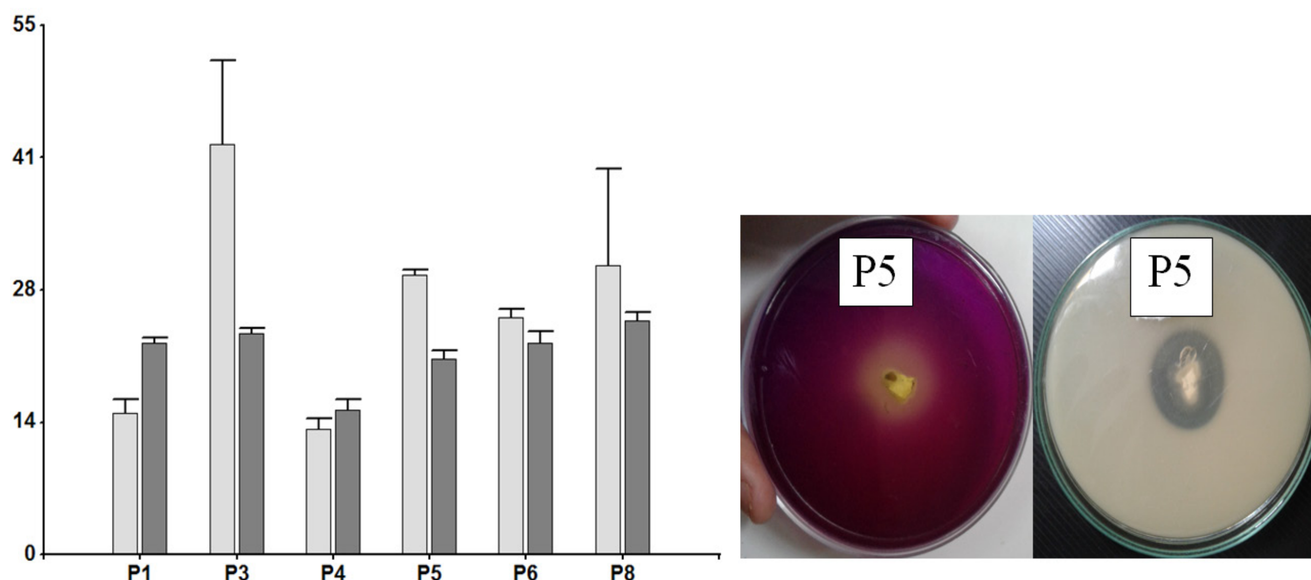

Figure 3. A) Extracellular enzymes secretion average of six strains of *A. apis*, B) Left; Degradation of colloidal chitin (strain P5). Right action of extracellular proteases in skimmed milk (strain P5) (A) Secreción media de enzimas extracelulares de seis cepas de *A. apis*, B) Izquierda; Degradación de quitina coloidal (cepa P5). Derecha, acción de las proteasas extracelulares en la leche descremada (cepa P5).

DISCUSSION

A. apis is a fungus that is spread by bees when they extract pollen and nectar from the plants they visit, transporting the spores to the hives infecting the larvae when the nurses feed them. In this work we have examined commercial pollen from different Spanish provinces which were acquired in the year 2012, being kept under laboratory conditions at 20ºC. After four years of pollen conservation six strains of *A. apis* were isolated, which would indicate the presence of the fungus after 48 months of conservation, which allowed to establish the duration of the spores. *Palacio et al*. (2007) reports that in brown rice the spore conservation decreases after 360 days of maintenance, unlike in MY20 that is conserved until a period of 77 days, the isolation of the strains of *A. apis* after 48 months resulted In viable sporulation after 5 days of planting in MY20. The six isolated strains were identified genetically and their phylogenetic relationship allowed to establish that there are differences with strains of USA, Russia and Australia, the Spanish strains forming a monophyletic group, forming six intergenic polymorphic loci that can differentiate the haplotypes of *A. apis*. Jensen *et al*. (2012, p. 5) describes twelve polymorphic variants of *A. apis* from Denmark and USA, using ITS regions which had high levels of intraspecific variation. Zervakis *et al*., (2004, p. 76) using the ITS regions, inferred the phylogenetic relationships of closely related species and evaluated the variability present within a population; establishing evolutionary paths, between geographically distant isolates (ecotypes). In this work phylogenetic relationships revealed that isolated Spanish strains are far from the *A. apis* branch of the above countries. In our case, all the samples studied were genetically different depending on the Spanish zone, those that are grouped in a narrow monophyletic clade (100% bootstrap support) are found in remote areas Zaragoza (P3) and Cataluña (P8) which would be caused by the transhumance of hives between provinces, while the strain that comes from Madrid would be related he same, establishing their own evolutionary path. On the other hand the strains of Castile (P4, P5 and P6) have genetically diverged, pollen strain from Cuenca has moved away from the other regions (genetic divergence). This study demonstrated a geographical subdivision pattern in the genetic structure of *A. apis* in Spain. The distance of the Asian, American and Oceanian clades proves that there would be no transhumance between continents, allowing the spores to remain in geographic regions close to Spain, which would be related to the works of Jensen et al. American strains are far from those in Denmark.

On the other hand, the analysis of the enzymes involved in the infection turned out to be different according to the strains studied as also showed Teerayut *et al.* (2008, p. 70), determined the production of extracellular enzymes chitinases and proteases using seven culture media to determine proteases, only two produced results at 7 days those containing yeast extract and glucose. The strains that secreted protease enzymes at 48 hours in the medium, based on skim milk were P1, P8, P3 and P6, they were cultivated under the same conditions of growth of *A. apis* (30ºC and in microaerophilia), P4 and P5 secreted proteases at seven days. Teerayut *et al*. (2008, p. 70) concluded that serine protease and metalloprotease are the proteolytic enzymes involved in the pathogenesis of bee larvae, which would be correlated with the proteases secreted by Spanish strains, since the skim milk has sites of action for these enzymes on the other hand, confirming hypotheses predicted (Alonso *et al.*, 1993, p. 386) the studies of genes coding for hydrolytic enzymes, elucidated the mechanism of coordinated penetration in the peritrophic membrane of *Apis mellifera* larvae as well as the mechanical pressure exerted by hyphae

Table II. Summary of average secretion of chitinases and proteases from six Spanish strains (Resumen de la secreción media de quitinasas y proteasas de seis cepas españolas).

Strains	chitinese (mm)	protease (mm)
P1	14,6±2,5	22±1
P3	42,6 ±15,1	23±1
P4	13 ±2	15 ±2
P5	29±1	20,3±1,5
P6	24,6±1,5	22±2
P8	30±17,4	24,3±1,5

In the exoskeleton, where 42 proteases would be involved in the pathogenesis process. Several genes encoding cutinase transcription factor homologues Ctf1 have also been identified, indicating the ability of this *A. apis* to use different substrates as nutrients, including plant cutin, a variety of lipids, as well as triacylglycerols and synthetic esters (Cornman *et al*., 2012, p. 6).

On the other hand, degradation of colloidal chitin by extracellular chitinases from *A. apis* was evaluated. The P1, P3, P8 and P6 strain was determined to secrete chitinases at 30 ° C and in microareophilic conditions at pH 5 for seven days, whereas that, strains P4 and P5 secretion chitinases in on day 10. Teerayut *et al*. (2008, p. 74) determined that the molecular weight of β-N-acetylglucosaminidase was 55.042Da on SDS-PAGE showing that the enzyme expressed in a monomeric form, the optimum pH was about 5.5. On the other hand, like the extracellular proteases

Cornman *et al*. (2012) identified the genes coding for three extracellular chitinases, in addition to a glucoamylase, 16 amidases, 30 esterases, 24 lipases among others.

Gilliam (1993, p. 21) and Chorbinski (2003, p. 21) identified enzymes present in *A. apis*, among them, valine aminopeptidase was produced only by unpaired strains and was proposed for the identification of mycelial stage of the fungus (anomorph). Thus β-galactosidase and a-mannosidase enzymes were also suggested for the identification of both mated and unpaired strains since few other fungi associated with honey bees produce these enzymes.

CONCLUSION

This study determined the conservation of the fungus *Ascophaera apis* in commercial pollen and its viability after forty-eight months. The phylogenetic relationship of six strains was determined by demonstrating that they retain the ability to secrete chitinases and proteases, important in the pathogenesis of *Apis mellifera*, over a prolonged period of time.

ACKOWLEGMENTS

This work has the support of the Secretary of Science and Technology of the National University of Jujuy.

BIBLIOGRAPHY

Agrawal, T & Kotasthane, AS 2012, `Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India´, *SpringerPlus*, vol. *1*, no. 73, pp. 1-10. http://doi.org/10.1186/2193-1801-1-73

Alonso, JM, Rey, J, Puerta, F, Hermoso de Mendoza, J, Hermoso de Mendoza, M, Flores, JM 1993, `Enzymatic equipmet of Ascosphaera apis and the development of infection by this fungus in *Apis mellifera*´, *Apidologie, vol.* 24, no. 4, pp. 383-390. https://doi.org/10.1051/apido:19930404

Ansari, MJ, Al-Ghamdi, A, Usmani, S, Khan, KA, Alqarni, AS, Kaur, M, & Al-Waili, N 2017, `*In vitro* evaluation of the effects of some plant essential oils on *Ascosphaera apis*, the causative agent of Chalkbrood disease´, *Saudi Journal of Biological Sciences*, vol. *24*, no. 5, pp. 1001–1006. http://doi.org/10.1016/j.sjbs.2016.04.016

Aronstein, KA & Murray, KD 2010, `Chalkbrood disease in honey bees´, *Journal of Invertebrate Pathology, Elsevier*, vol. 103 Supplement, pp. S20–S29. https://doi.org/10.1016/j.jip.2009.06.018

Carrillo, L & Gómez Molina, SE 1998, `Producción de quitinasa por una cepa de streptomyces griseoruber aislada de la rizósfera de caña de azúcar´, *Revista Argentina de Microbiología, vol.* 30, no. 2, pp. 73-78.

Chorbinski, P 2003, `Enzymatic activity of strains of *Ascosphaera apis*´, *Medycyna Weterynaryjna*, vol. 59, no. 11, pp. 1019–1022.

Cornman, RS, Bennett, AK, Murray, KD, Evans, JD, Elsik, CG, Aronstein, K 2012, `Transcriptome analysis of the honey bee fungal pathogen, *Ascosphaera apis:* implications for host pathogenesis´, *BMC Genomics*, vol. *13*, no. 285, pp. 1-13. http://doi.org/10.1186/1471-2164-13-285

Flores, JM, Ruiz, JA, Ruz, JM, Puerta, F, Bustos, M, Padilla, F, Campano, F 1996. `Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions´, *Apidologie, vol.* 27, no. 4, pp. 185–192. https://doi.org/10.1051/apido:19960401. Gilliam, M, Lorenz, BJ 1993,`Enzymatic activity of strains of *Ascosphaera apis*, an entomopathogenic fungus of the honey bee, *Apis mellifera*´, *Apidologie,* vol. 24,no. 1, pp. 19–23. https://doi.org/10.1051/apido:19930102

Jensen, AB, Welker, DL, Kryger, P, James, RR 2012, `Polymorphic DNA sequences of the fungal honey bee pathogen *Ascosphaera apis*´, FEMS Microbiology Letters, vol. 330, no. 1, pp. 17–22. http://dx.doi.org/10.1111/j.1574-6968.2012.02515.x

Jensen, AB, Aronstein, K, Flores, JM, Vojvodic, S, Palacio, MA, Spivak, M 2013, `Standard methods for fungal brood disease research´, *Journal of Apicultural Research*, vol. *52*, no. 1, pp. 1-20, http://doi.org/10.3896/IBRA.1.52.1.13

Koh, I, Lonsdorf, EV, Williams, NM, Brittain, C, Isaacs, R, Gibbs, J, Ricketts, TH 2016, `Modeling the status, trends, and impacts of wild bee abundance in the United States´, *Proceedings of the National Academy of Sciences of the United States of America*, vol. *113*, no. 1, pp. 140–145. http://doi.org/10.1073/pnas.1517685113.

Lumi-Abe, CA, Bertechini-Faria, C, Fernandes-Castro, F, De Souza, SR, Dos Santos, FC, Novais da Silva, C, Barbosa-Tessmann, IP 2015, `Fungi Isolated from Maize (*Zea mays* L.) Grains and Production of Associated Enzyme Activities´, *International Journal of Molecular Sciences*, vol. 16, no. 7, pp. 15328–15346. http://doi.org/10.3390/ijms160715328

Maxfield-Taylor, SA, Mujic, AB, Rao, S 2015, `First Detection of the Larval Chalkbrood Disease Pathogen Ascosphaera

apis (Ascomycota: Eurotiomycetes: Ascosphaerales) in Adult Bumble Bees´, PLoS ONE, vol. 10, no. 4, pp. 1-11. http://doi.org/10.1371/journal.pone.0124868.

Palacio, MA, Clemente, G, Ruffinengo, S, Escande, AR, Peña, N 2007, `Viability and pathogenicity of "Ascosphaera apis" preserved in integral rice cultures´, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), vol. 5, no.4, pp. 481-486

Sarath, G, De la Motte, R, Wagner, F W 1989, `Protease assay methods´, in Proteolytic Enzymes: A Practical Approach, eds Beynon R. J., Bond J. S., editors. (Oxford: IRL Press), pp. 25–56.

Shin, YK & Kim, KY 2016,`Macelignan inhibits bee pathogenic fungi *Ascophaera apis* growth through HOG1 pathway´, *Brazilian Journal of Medical and Biological Research,* vol. 49, no. 7, pp. 1-9. http://doi.org/10.1590/1414-431X20165313

Takatori, K, Tanaka, I 1982, `Ascosphaera apis isolated from chalkbrood in honey bees´, *Japan Journal of Zootechnics*, vol. 53, no. 2, pp. 89-92.

Teerayut, T & Panuwan, C 2008, `Protease and β-N-acetylglucosaminidase of honey bee chalkbrood pathogen Ascosphaera apis´, *Journal of Apicultural Research*, vol. 47, no. 1, pp. 68-76. http://dx.doi.org/10.1080/00218839.2008.11101426

Wang, P & Granados, RR 2000,`Calcofluor disrupts the midgut defense system in insects´, Insect. Biochem´. *Molecullar Biology*, vol. 30, no. 2, pp. 135–143. https://doi.org/10.1016/S0965-1748(99)00108-3

Zervakis, GI, Moncalvo, JM, Vilgalys, R 2004. `Molecular phylogeny, biogeography and speciation of mushroom species *Pleurotus cystidiosus* and allied taxa´, *Microbiology*, vol. 150, no. 3, pp. 715–726. http://dx.doi.org/10.1099/mic.0.26673-0