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Identification, pathogenicity and distribution of *Penicillium* spp. isolated from garlic in two regions in Argentina

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A total of 147 samples of garlic (*Allium sativum*) bulbs affected by blue mould were obtained from a variety of agroclimatic districts between December 1999 and February 2000. *Penicillium* species were identified using both morphological and chemotaxonomic characteristics. *Penicillium allii* was the predominant species isolated (81·8%) in this survey and the only species proven to be pathogenic on garlic. Other species were isolated much less frequently: *P. chrysogenum* (13·7%), *P. brevicompactum* (2·8%), *P. phoeniceum* (0·9%), *P. aurantiogriseum* (0·6%) and *P. flavigenum* (0·2%). Colonies of *P. allii* could be classified into four morphotypes and their distribution seemed to be influenced by seed trade and agricultural practices. *Penicillium allii* isolates were grouped into three aggressiveness phenotypes (low, medium and high) based on their ability to cause disease during field trials on susceptible (Fuego INTA) and less susceptible (Castaño INTA) garlic cultivars. The number of surviving plants at 191 days after planting and postharvest bulb weight contributed the most towards aggressiveness modelling.

Keywords: *Allium sativum*, blue mould of garlic, chemotaxonomy, *Penicillium allii*, seed bulbils

Introduction

Argentina is the world's second largest garlic (*Allium sativum*) exporter (FAO, 2004). The main area of production (14 000 ha, 86%) is located in the provinces of Mendoza and San Juan, distributed across approximately 2400 small farms. Garlic farming in Argentina has both significant social and economic importance. Blue mould rot is responsible for annual crop losses, affecting both plant fitness and survival, resulting in diminished bulb size and/or commercially non-viable bulbs. Characteristic disease symptoms are stunted and chlorotic plants with withered leaves, and infected bulbs are often covered with blue/green conidial masses. Blue mould rot also occurs in storage environments, and is reported as being one of the primary causes of decay in stored garlic (Smalley & Hansen, 1962). Currently in Argentina a loss of 15% of the total harvest yield of red type garlic caused by pathogenic decay in the field is assigned to *Penicillium* spp. (J. L. Burba, INTA La Consulta, personal communication).

Consensus over the causal agent(s) of blue mould rot of garlic has yet to be reached, although many *Penicillium* spp. have been reported as pathogens. Smalley & Hansen (1962) first reported the causal agents of the disease as *P. corymbiferum* and *P. cyclopium*. However, since then *P. corymbiferum* has been subdivided into several different species contained within the *Penicillium* series *Corymbifera* (with synonymy of the original *P. corymbiferum* characterization aligned with that of *P. hirsutum*). In Argentina, *P. viridicatum* (Gatica & Oriolani, 1984), *P. hirsutum* (Cavagnaro *et al*., 2005) and *P. allii* (Valdez *et al*., 2006) have all been reported as garlic pathogens. *Penicillium viridicatum* has also been reported from garlic in Japan (Saito & Tsuruta, 1984) and Poland (Machowicz-Stefaniak *et al*., 1998), although in the Polish study pathogenicity of the isolates was not confirmed. Recently, doubt has been raised regarding the correct taxonomic identification of the reported *P. viridicatum* causal agents, as the *P. viridicatum* strains ATCC 9635 (Smalley & Hansen, 1962), IFO 7736 = CBS 390·48 ex-type (Saito & Tsuruta, 1984), IBT 15053 = CBS 101034 and IBT 16639 (Valdez *et al*., 2006) failed to establish a colony or to sporulate on infected garlic bulbils in damp chambers. As *P. allii* and *P. viridicatum* are macro- and micromorphologically quite similar, it is possible that the *P. viridicatum* isolates previously reported as causal agents were in fact *P. allii*. *Penicillium allii* was originally described from garlic (Vincent & Pitt, 1989), 5 years after the first report of *P. viridicatum* as a garlic

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pathogen, and later taxonomically placed within ser. *Corymbifera* based on shared micromorphological attributes, secondary metabolite production and ecological habitat (Frisvad *et al*., 2000). Other species have also been reported as isolated from mouldy garlic: *P. expansum*, *P. glandicola* (Mazur, 1990) and *P. chrysogenum* (Abdel Sater & Eraky, 2002), but pathogenicity was not confirmed in either of these studies.

The objective of the following study was to identify which *Penicillium* species are responsible for blue mould rot of garlic in Argentina, to determine their distribution and to characterize their pathogenicity. As there are potential crops that can act as reservoirs of the pathogen(s) in specific areas (apples in the central west area of Mendoza; nuts in the south of San Juan and north of Mendoza; maize and grapes in the whole region) isolation of *Penicillium* species associated with these crops from these areas was expected [namely *P. cyclopium* and *P. viridicatum* from cereals (Samson *et al*., 2000), *P. expansum* from apples, pears and grapes (La Guerche *et al*., 2004), *P. glandicola* from nuts (Overy *et al*., 2003), *P. allii* from onions and garlic and *P. hirsutum* from onions and various flowering bulbs (Overy *et al*., 2005a)].

Materials and methods

Sampling and isolation

Between December 1999 and February 2000, 147 samples (one sample for each 100 ha) of 100 randomly selected bulbs were taken from commercially harvested garlic lots in the provinces of Mendoza and San Juan (Argentina).

A total of 40 bulbils per sample were placed in damp chambers under cold fluorescent light (16-h photoperiod) at 25°C. After 7 days, fungal colonies were observed and four colonies were obtained from each sample, transferred to potato dextrose agar (PDA; Difco) and incubated in the dark for 7 days at 25°C. A portion of each pure culture was removed into 200 μL of sterile 1% Tween 20 solution. To create monospore cultures, 10 μL suspension were placed in the centre of a glass Petri dish into which 3 mL monosporization medium [0·25 g K_2HPO_2, 4 g agar and 2·5 mL Czapek concentrate L^{-1} (Pitt, 1973)] was poured in a circular motion from the edge to the centre of the dish. Petri dishes were then incubated in the dark at 25°C for 48 h, after which individual germinating conidia were picked up and placed into a slant tube containing 5 mL PDA. After 1 week, conidia from each monospore culture were removed and suspended in 0·5 mL of 10% glycerol and preserved at –20°C. From the 147 field samples, a total of 538 monosporic cultures of *Penicillium* were obtained. The acronym LJC (Laboratorio José Crnko) was assigned to each accession.

Media preparation and species identification

Two media were used for morphological identification: CYA (Czapek yeast agar) and GSA (garlic sucrose agar). CYA is one of several media commonly used for the identification and description of *Penicillium* spp. (Ramirez, 1982; Pitt & Hocking, 1997; Samson *et al*., 2000). CYA was prepared according to Pitt (1973). The use of media amended with fresh garlic juice was shown to be fungistatic or fungitoxic to *Penicillium* spp. not isolated from garlic (Tansey & Appleton, 1975; Muhsin *et al*., 2000; Obagwu & Korsten, 2003). In preliminary work, the addition of an aqueous garlic powder extract to PDA affected colony diameters of *Penicillium* spp. isolated from garlic (JGV, unpublished data). To prepare GSA, commercial garlic powder was dissolved into distilled water (1/10 w/v) and stored at 5°C for 18–26 h. The suspension was then shaken for 3 min, centrifuged for 20 min at 8512 ***g*** and the supernatant sterilized by vacuum filtration through serial filter membranes (0·8 and 0·2 μm, Sartorius 16510). Sucrose agar was prepared by combining 13·5 g agar and 20 g sucrose L^{-1}, then autoclaving at 121°C for 15 min. The garlic extracts were aseptically added to the warm media (50°C) at a concentration of 60 mL L^{-1} v/v (garlic extract/media), shaken and poured into 90-mm-diameter Petri dishes (10 mL per dish).

Each one of the 538 fungal isolates were three-point-seeded onto both media and incubated in the dark for 7 days at 25°C. Colonies on CYA were measured for diameter, type (velvety, lanose, funiculose, fasciculate), margin (regular or irregular), size of the submerged margin, presence or absence of exudates, colour of exudates, colour of soluble pigments, colour of the colony in the conidial area, and colour of the colony on the reverse. Colony diameter was measured on GSA medium and the ratio (diameter CYA/diameter GSA) was also used in the morphological characterization of the colonies.

Statistical analysis for taxonomical grouping

Cluster analyses were performed to sort the isolates into distinct groups. Quantitative variables were standardized and transformed following Escofier (1979): (1 – stv)/2 and (1 + stv)/2, where 'stv' is the standardized variable. A similarity matrix using a simple matching coefficient was calculated. The unweighted pair group method using an arithmetic average (UPGMA) was used to perform cluster analysis with the software NTSYS v 2·1 (Exeter Software). As a result, 32 morphological groups were obtained, based on 28 macromorphological features.

A representative set of isolates from each group was selected for micromorphological identification and secondary metabolite profiling, and identified isolates were compared with species ex-type strains from the IBT culture collection (CMB, BioCentrum-DTU, Denmark). Culture extracts were prepared according to Smedsgaard (1997) and profiled by RP-HPLC according to Frisvad & Thrane (1987) as modified by Nielsen & Smedsgaard (2003). The software COWTOOL v1·1 (IBT, DTU, Lyngby, Denmark) was used to align the chromatograms (Nielsen *et al*., 1998). The absorbance trace (collected at 280 nm) for each chromatographic run was used to construct a matrix of 32 operational taxonomic units (OTUs) × 1800 files. The Euclidian distance was used to obtain a distance

Table 1 Representative isolates of *Penicillium* spp. used in pathogenicity trials (strains in bold were also used in secondary metabolite profiling). All isolates were obtained from garlic with the exception of *P. tulipae* (isolated from a rotten tulip) and *P. implicatum* (isolated from poultry food). All garlic isolates were obtained from garlic bulbs inocubated in damp chambers with the exception of isolates LJC 542 and 543, which were obtained from blue-mould-affected garlic plants

Species	Morphotype	Culture collection
P. allii	1	**LJC[a] 087 = IBT[b] 26453**, LJC 097, LJC 119, LJC 138, LJC 160, **LJC 197 = IBT 26456**, **LJC 201 = IBT 26457**, LJC 219, LJC 295, **LJC 302 = IBT 26458**, LJC 366, LJC 385, LJC 402, LJC 447, **LJC 482 = IBT 26464**, **LJC 498 = IBT 26465**, LJC 521, **LJC 530 = IBT 26467 = FFR[c] 5534**, LJC 533, **LJC 543 = IBT 26512**.
	2	LJC 029, LJC 079, LJC 137, LJC 157, **LJC 159 = IBT 26454**, LJC 165, **LJC 178 = IBT 26455**, LJC 228, LJC 252, LJC 280, **LJC 313 = IBT 26459**, **LJC 346 = IBT 26461**, LJC 360, **LJC 368 = IBT 26462**, LJC 419, LJC 452, **LJC 517 = IBT 26466 = FFR 5533**, **LJC 542 = IBT 26511**.
	3	**LJC 059 = IBT 26452**, **LJC 319 = IBT 26460**.
	4	**LJC 466 = IBT 26463**.
P. aurantiogriseum	1	**LJC 064 = IBT 26509**.
P. brevicompactum	1	LJC 089.
	2	**LJC 188 = IBT 26501**, LJC 339, **LJC 494 = IBT 26502**.
P. chrysogenum	1	**LJC 005 = IBT 26517**, **LJC 044 = IBT 26506**, **LJC 128 = IBT 26518, LJC 206 = IBT 26507**, **LJC 317 = IBT 26516**, **LJC 394 = IBT 26515**, **LJC 481 = IBT 26514**.
	2	**LJC 215 = IBT 26505**, **LJC 384 = IBT 26504**.
P. flavigenum	1	**LJC 321 = IBT 26508**.
P. implicatum	–	LJC 541.
P. phoeniceum	1	**LJC 537 = IBT 26513**.
P. tulipae	1	**LJC 560 = IBT 26510**.

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[c]FFR: CSIRO, New South Wales, Australia.

matrix to which a cluster analysis (UPGMA) was applied. Representative cultures were deposited into the IBT culture collection (Table 1).

Distribution

The area of study was situated between 65 and 71°S latitude and 28 and 38°W longitude. A cell size of 30×30 feet was chosen to divide the area into 12 columns and 20 rows, establishing 19 grids.

To estimate the relative inoculum of *P. allii* per grid ($R_{P.\ allii\ (g)}$), the sample within the grid (*i*), the number of bulbils affected in the sample *i* (*ba*), the total number of bulbils incubated per sample (normally 40, *bt*) and the area of the field where the sample was obtained (A), were related in the following formula:

$$R_{P.\ allii\ (g)} = \frac{\sum_{i=1} \frac{ba_i}{bt_i} A_i \times 0{\cdot}82}{\sum_{i=1} A_i}$$

The constant 0·82 represented the frequency of *P. allii* in the sample (see Results). The relative inoculum, therefore, was defined as the relative biomass of potential inoculum produced in the grid, taking into consideration the incidence of the pathogen on the sample and the size of the field where the sample was obtained. The number of species per grid (richness) and the relative inoculum of *P. allii* per grid were represented (Fig. 1a,b) using the software DIVA GIS v 4·2 (CIP, Lima, Peru).

Preparation of inoculum

In vitro and pathogenicity trials were carried out with 56 strains representing the morphological diversity obtained from the 538 isolates originally sampled and the additional isolates LJC 541 *P. implicatum*, LJC 542 *P. allii* and LJC 543 *P. allii* incorporated as negative (LJC 541) and positive (LJC 542 and 543) controls. LJC 541 was used as the negative control as it is a monoverticillate *Penicillium* and not reported as a plant pathogen. LJC 542 and LJC 543 were obtained from blue-mould-affected field plants of cvs Castaño INTA (low susceptibility) and Fuego INTA (high susceptibility), respectively (Cavagnaro *et al*., 2005), growing at La Consulta experimental station (33°45′S, 69°02′W), in August 2000. An additional strain of *P. tulipae* (LJC 560), a species related to *P. allii* and *P. hirsutum* within ser. *Corymbifera* (Overy & Frisvad, 2003), isolated from a rotten field tulip bulb in Mendoza, was also included. Spore suspensions were prepared for each isolate growing on CYA by removing conidia from the margin of a 7-day-old pure culture into 500 μL autoclaved 1% Tween 20. Suspensions were adjusted to a final concentration of 5×10^6 conidia mL^{-1} with a haemocytometer to carry 25 000 conidia in a volume of 5 μL.

In vitro trial

A total of 1410 μL of each spore suspension (5×10^6 conidia mL^{-1}) were mixed into 7 mL melted water agar (50°C) in individual Petri dishes (9 cm) and incubated in

Figure 1 (a) Number of *Penicillium* species per grid (species richness) and (b) relative inoculum (relative biomass of potential inoculum produced in the grid) of *P. allii* per grid in San Juan (north) and Mendoza (south) provinces of Argentina. White dots indicate farms where *P. allii* was present. The agroclimatical districts of each province are presented.

the dark for 24 h at 25°C. Plugs 5 mm in diameter (approximately 25 000 conidia per plug) were removed to seed CYA and GSA media (three plugs per dish). A completely randomized design was established with 60 isolates and three replicates. After incubation in the dark for 7 days at 25°C, colonies were morphologically characterized and diameters were recorded.

Pathogenicity trials

Damp-chamber experiments were performed in 2002 and field experiments in 2003 and repeated in 2004. Inoculum was prepared the day before the inoculation and preserved at –20°C following inoculation for control purposes. Bulbils were inoculated once their dormancy was broken as estimated at a visual index of dormancy (VID) of 75% (Burba *et al*., 1983). The VID was calculated as the length of sprouting leaf/length of storage leaf × 100. Healthy bulbils between 6 and 8 g were peeled, surface-sterilized by immersion in ethanol (70%, 5 min) and sodium hypochlorite (1%, 20 min) and left to dry on sterilized paper. To confirm Koch's postulates, morphological comparisons were performed using the original inoculum, compared with re-isolated cultures from damp-chamber trials and obtained from bulbs postharvest. Petri plates containing CYA and GSA were three-point-seeded and incubated in the dark at 25°C for 7 days prior to comparison.

Damp-chamber experiments

Replicate inoculations ($n = 10$) were performed in a randomized design for each of the selected 60 isolates for both cvs Fuego INTA and Castaño INTA. Sterilized peeled bulbils were wounded (1·5 mm deep) with a sterile

needle (0·7 mm diameter), inoculated with 5 μL spore suspension and placed into sterile plastic boxes (20 bulbils per box) containing saturated, sterile filter paper and incubated in the dark for 12 days at 25 ± 2°C. The diameter of the sporulating area was recorded after incubation. To obtain the total sporulation of each bulbil, the affected tissue was cut, put into tubes containing 1 mL autoclaved 1% Tween 20 and vortexed. Mycelia were removed by sterile forceps and 100 μL were diluted in 5 mL of water. Transmittance of the suspension at 340 nm was measured on a Spectronic 20D spectrophotometer (Milton Roy) and suspension concentration was estimated as $y = -86\,497\ T + 9{\cdot}3 \times 10^6$ (Valdez & Piccolo, 2006) where y was the concentration (conidia mL^{-1}) and T the transmittance. Tubes were stored at –20°C prior to morphological re-identification to confirm Koch's postulates.

Field experiment

A total of 1200 healthy bulbils of cvs Fuego INTA and Castaño INTA selected for field trials were peeled, surface-sterilized and wounded (4 mm deep) with a sterile needle (1·5 mm diameter), with 5 μL spore suspension inoculated into the wound. Bulbils were then incubated in dark damp conditions for 24 h at 20°C prior to planting at La Consulta Station in an experimental field plot not cultivated with garlic the previous year. A completely randomized design of 60 treatments and four replications (five in the first year) of five plants per plot was used. Fuego INTA was planted on 21 April 2003 and 28 April 2004 and cv. Castaño INTA was planted on 5 May 2003 and 7 May 2004. Emerging/surviving plants (weekly), stalk diameter (20 days before harvest) and bulb weight (60 days after harvest) were recorded. Bulbs from plants with symptoms of the extra replication of the first year, demonstrating visible sporulation, were released from the field on 14 July 2003 and incubated for 3 days in the dark at 25°C. Emerging fungi were monosporized and kept for comparison purposes.

Fuego INTA was harvested on 2 December 2003 and 1 December 2004 and Castaño INTA on 15 December 2003 and 14 December 2004. Harvesting was performed by hand pulling, using a fork to loosen the soil and facilitate lifting. After curing, the plants were trimmed. Both the tops and the root were removed by hand. The weight of each bulb was recorded and the cured bulbs were stored for 2 months. Six months after harvesting, selected bulbs were placed in damp chambers and the resulting colonies were monosporized and kept for morphological comparison.

Statistical analysis of pathogenicity trials

Statistical differences between results obtained from the field experiments were studied by procrustes analysis (Gower, 1975). The procrustes analysis can centre, rotate and scale one multivariate configuration of points so that they best match another configuration (each configuration of each year in this case). No differences were observed.

Principle component analysis (PCA) was performed with the average field, damp-chamber and *in vitro* variables. A Euclidean distance matrix was calculated with the coordinates of the first two eigenvectors. OTUs were clustered from this matrix using the UPGMA procedure. Clusters were compared by analysis of variance (ANOVA) using the software STATISTICA v6·1 (StatSoft) and homogeneity between them was tested by means of the Tukey test ($\alpha = 0{\cdot}05$, d.f. = 56).

Results

Identification

The species isolated most frequently was *P. allii* (81·8%, 440 isolates). Other *Penicillium* species were also isolated: *P. chrysogenum* (13·7%, 74 isolates), *P. brevicompactum* (2·8%, 15 isolates), *P. phoeniceum* (0·9%, five isolates), *P. flavigenum* (0·6 %, three isolates) and *P. aurantiogriseum* (0·2%, one isolate). Four morphotypes of *P. allii* (Fig. 2), two of *P. chrysogenum* and two of *P. brevicompactum* were observed on CYA while *P. phoeniceum* and *P. flavigenum* presented only a single morphotype. A total of 239 isolates of *P. allii* morphotype one were characterized as being granular in appearance with an umbonate centre, producing small drops of yellow exudate and diffusing yellow pigment (Fig. 2a). Morphotype 2 (179 isolates) differed by producing a velvety/floccose colony texture rather than granular, sulcate, often with a slightly floccose center and not diffusing yellow pigment, while morphotype 3 (21 isolates) exuded a conspicuous ring of medium sized, yellow droplets (Fig. 2b,c). Morphotype 4 was represented by one isolate characterized by the production of an orange brown reverse and yellow exudate (Fig. 2d). On GSA this isolate produced a yellow reverse as a result of the production of the antibiotic TAN-1612 (J.G. Valdez, unpublished data).

Secondary metabolite profile comparison of the representative strains yielded six clusters at a similitude of 45%, each corresponding to the six species obtained. No correlation was observed between *P. allii* morphotypes 1–4 and the *P. allii* subclusters ($R^2 = 0{\cdot}31$) created from the secondary metabolite profile analysis.

Distribution

The area located in the north of Mendoza was the richest in both phenotypic (not shown) and species diversity (Fig. 1a). Representation of the relative inoculum of *P. allii* per grid showed that in Mendoza there was less inoculum than in San Juan province (Fig. 1b). Correspondence analysis used to establish associations between *P. allii* morphotypes and agroclimatical districts based on environmental variables (De Fina, 1978) showed no relationship ($P = 0{\cdot}0057$). Therefore, factors other than the environment, such as human influence, could be involved in the pathogen dispersion.

In vitro trial

The average diameters (mm) reached on GSA were 28 ± 3 (*P. allii*); 32 ± 2 (*P. tulipae*); 24 ± 1 (*P. aurantiogriseum*),

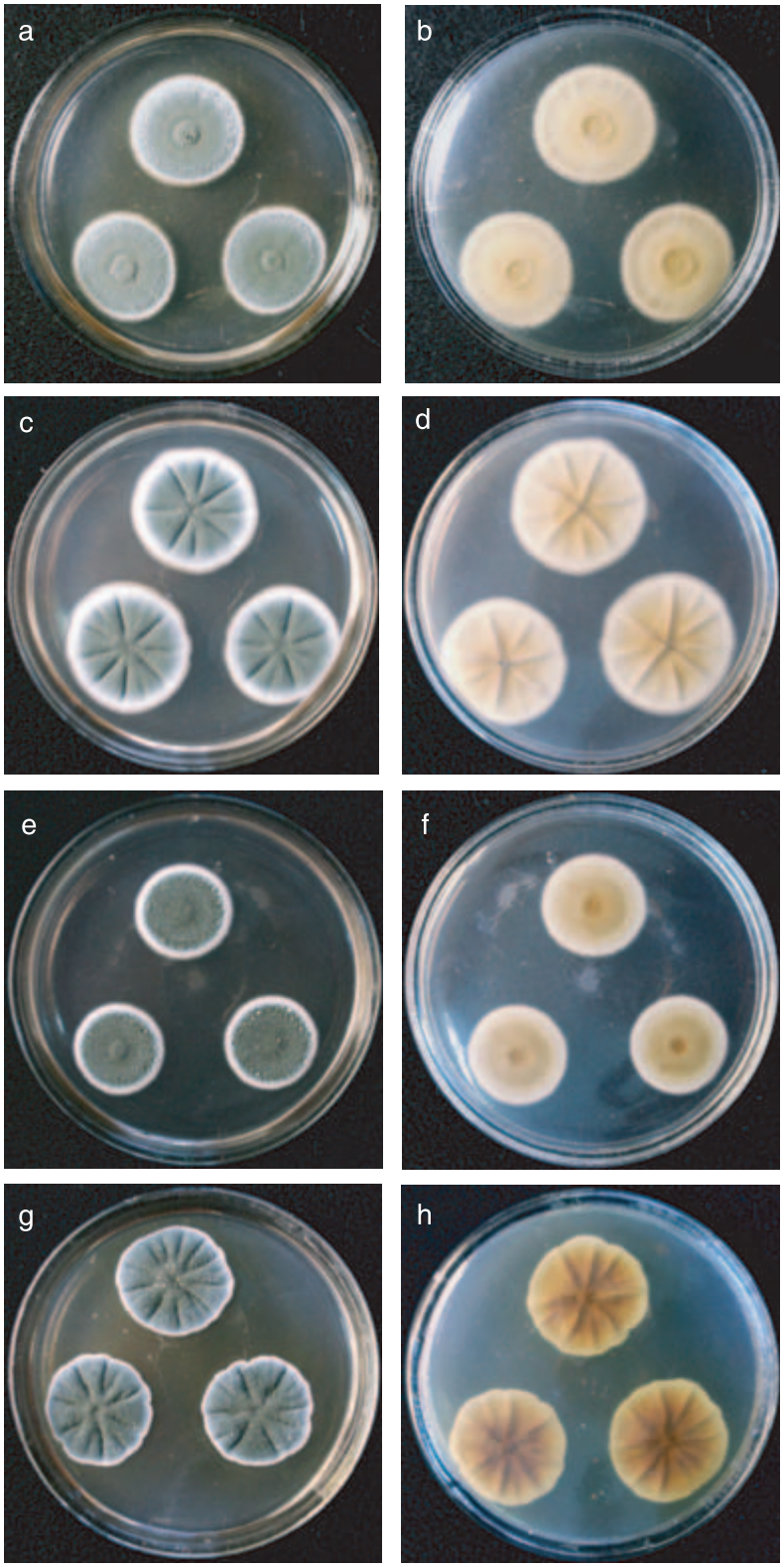

Figure 2 Morphotypes (front and reverse) of *Penicillium allii* isolates from garlic in Mendoza and San Juan provinces, Argentina, after 7 days at 25°C on Czapek yeast agar; (a) and (b) LJC 530, morphotype 1; (c) and (d) LJC 517, morphotype 2; (e) and (f) LJC 319, morphotype 3; (g) and (h) LJC 466, morphotype 4.

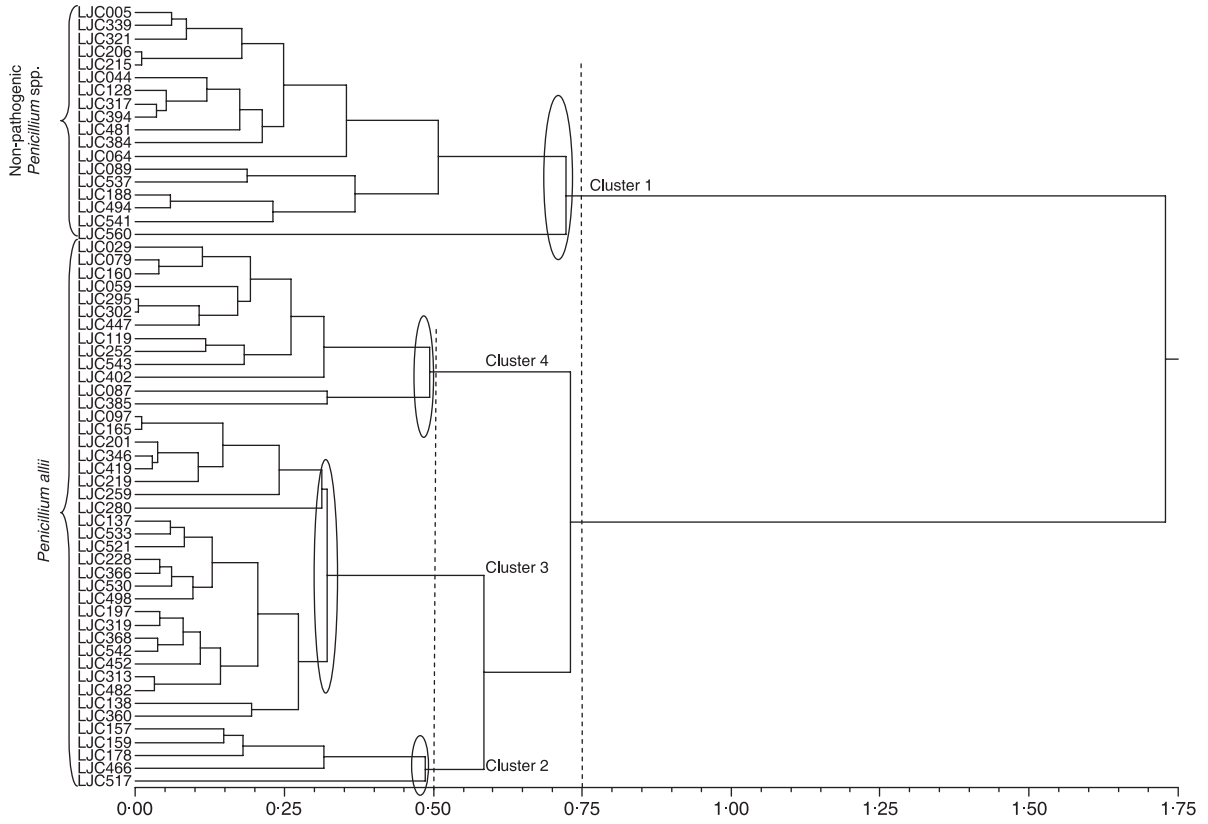

Figure 3 Cluster analysis (UPGMA) performed from a distance matrix obtained with the Euclidean distance of OTUs (operational taxonomic units) projected on the first two axes of component analysis. Dotted line at 0·75 defines cluster 1 (non-pathogenic isolates), segregating it from *Penicillium allii* isolates. Dotted line at 0·50 defines cluster 2 (low aggressiveness phenotype), cluster 3 (medium aggressiveness phenotype) and cluster 4 (high aggressiveness phenotype).

22 ± 2 (*P. chrysogenum*), 13 ± 3 (*P. brevicompactum*) and 7 ± 1 (*P. phoeniceum*). *Penicillium flavigenum* normally did not grow and, when it did grow, sporulation was poor, with colonies reaching only 7–8 mm in diameter. *Penicillium implicatum* failed to sporulate on GSA.

Pathogenicity trials

Penicillium aurantiogriseum (2·50 mm average diameter of sporulating area) and *P. chrysogenum* (0·40 mm average diameter) were able to sporulate on bulbils of cv. Fuego INTA, but not on those of Castaño INTA. However, the average diameter reached by those species was not comparable with the diameter reached by *P. allii* isolates (7·80 mm on Fuego INTA and 4·00 mm on Castaño INTA). *Penicillium brevicompactum* was able to sporulate on both cultivars, with an average diameter of 0·15 and 0·25 mm on cvs Fuego INTA and Castaño INTA, respectively. A corky tissue surrounded the lesion produced by the non-pathogenic species, whereas the lesions produced by *P. allii* isolates were soft, indicating that the enzymatic digestion of cell wall polymers had occurred.

The two first eigenvectors of PCA gave a total variability of 76·06% with a notable predomination of the first over the second (68·87% PC1 and 7·18% PC2, respectively). Four groups could be distinguished from cluster analysis with the two first eigenvectors, one non-pathogenic and three consisting of the *P. allii* isolates (Fig. 3). The non-pathogenic group (cluster 1) was separated from the *P. allii* group at a distance of 0·75. A second line traced at a distance of 0·50 defined the three *P. allii* isolate clusters.

All the *P. allii* isolates were recovered from blue-mould-affected field plants, from both damp-chamber garlic bulbil infections and harvested damp-chamber bulbs, with the exception of isolates LJC 178 and LJC 517 from bulbs of cv. Castaño INTA. In all cases, the recovered isolates showed the same morphology as the original ones when they were seeded on CYA.

In the infection trials in damp chambers, lesion diameter was correlated with sporulation for both Fuego INTA ($R^2 = 0{\cdot}96$) and Castaño INTA ($R^2 = 0{\cdot}91$). For Castaño INTA only, sporulation and lesion diameter on bulbils demonstrated differences in aggressiveness between isolates of clusters 2 and 4 (Table 2).

The three aggressive phenoypes of *P. allii* could be differentiated by observed significant differences (ANOVA, $P < 0{\cdot}01$) in the number of surviving plants at 191 days after planting (and at 65 days after planting for Castaño INTA) and diameter of stalk and weight of bulbs in both cultivars (Table 2). The more aggressive the phenotype,

Table 2 Average value of variables from *in vitro* (CYA, GSA, GSA/CYA culture media[a]) trials and *in vivo* controlled inoculations of 60 *Penicillium* isolates on garlic cvs Castaño INTA (CI) and Fuego INTA (FI). Cluster 1: non-pathogenic species; cluster 2, 3 and 4: low, medium and high aggressiveness phenotypes of *P. allii*. Different letters in the same row indicate significant differences (Tukey test; $\alpha = 0{\cdot}05$. d.f. = 56)

		C1	C2	C3	C4
Variables	GSA (mm)	17·22a	24·28b	29·10b	28·82b
	CYA (mm)	27·93a	31·13ab	33·92b	31·90ab
	GSA/CYA	0·62a	0·78ab	0·86b	0·90b
Sporulation on bulbils[b]	FI	11·10a	225·2b	275·6b	273·0b
	CI	1·00a	80·7b	98·8bc	124·9c
Diameter of lesion (mm)	FI	0·45a	7·14b	7·95b	7·75b
	CI	0·13a	3·40b	3·97bc	4·35c
Surviving plants	FI at 65 days[c]	4·97a	4·95a	4·97a	4·69b
	FI at 191 days	4·93a	3·98b	3·19c	1·67d
	CI at 65 days	4·85a	4·58ab	4·48b	4·12c
	CI at 191 days	4·88a	4·43a	3·69b	2·63c
Diameter of the stalk (mm)	FI	13·42a	11·25b	9·46c	8·30d
	CI	14·66a	12·72b	11·29c	9·32d
Weight of the postharvest bulb (g)	FI	28·53a	20·58b	15·75c	12·50d
	CI	33·13a	27·68b	21·72c	14·30d
Height of the plant (cm)	FI	28·47a	16·00b	12·29c	10·77c
	CI	19·58a	14·50b	11·35c	10·58c

[a]Czapek yeast agar; GSA, garlic sucrose agar.
[b]Conidia ($\times 10^6$).
[c]Days after planting.

the more reduced in size the surviving plants became, resulting in smaller, lighter, harvested bulbs. Fuego INTA was more susceptible to all three pathogenic phenotypes of *P. allii* than Castaño INTA. Postharvest bulb weight was found to be positively correlated with the diameter of the plant pseudostalk ($R^2 = 0{\cdot}96$ Castaño INTA, $R^2 = 0{\cdot}98$ Fuego INTA), variables that decreased as a result of infection by *P. allii*.

Discussion

Penicillium allii was the only pathogenic species isolated from the geographic area under study. The species *P. flavigenum* and *P. chrysogenum* are associated with desert soils and dry habitats, respectively (Frisvad & Samson, 2004). *Penicillium phoeniceum* (teleomorph = *Eupenicillium cinnamopurpureum*) has been isolated worldwide from rice, beans and dry food (Pitt & Hocking, 1997), whilst *P. brevicompactum* is of widespread occurrence, especially because of its xerophilic nature on dried foods (Pitt & Hocking, 1997). The latter has been cited as a weak pathogen on grapes, apples and ginger (Overy & Frisvad, 2005), while *P. aurantiogriseum* has been mostly related to cereals (Pitt & Hocking, 1997).

Two other species previously reported as pathogens of garlic (*P. viridicatum* and *P. hirsutum*) were not identified among the samples. The overall acreage of garlic-cultivated farmland sampled totalled 1235·25 ha, i.e. *c.* 10% of the area usually cultivated with garlic in Argentina. In the original report of *P. viridicatum* as the causal agent of blue mould rot in Argentina (Gatica & Oriolani, 1984) the collected strains produced yellowish, but not brown, exudates. Both *P. allii* and *P. viridicatum* exhibit terverticillate conidiophores, rough stipes and similarities in colony appearance on standard media. Pale yellow exudates are also characteristic of *P. allii* (Vincent & Pitt, 1989). *Penicillium allii* morphotype 3 is similar in appearance to the characterization of *P. viridicatum*. Additionally, the presence of morphotype 3 in the area most densely cultivated with garlic [the area previously sampled by Gatica & Oriolani (1984)] and the lack of isolation of *P. viridicatum* in this survey and failure of strains to produce an infection in wounded garlic bulbils (Valdez *et al*., 2006) suggests that the isolates identified as *P. viridicatum* in 1984 were in fact *P. allii*. Similarly *P. hirsutum* was reported by Cavagnaro *et al*. (2005) as pathogenic to garlic in Argentina, but closer examination confirmed that these isolates corresponded to the species *P. allii*, accessioned as LJC 545 (*P. hirsutum* IMI 386756) and LJC 550 (*P. hirsutum* IMI 386757). Previous pathogenicity trials conducted in damp chambers at 25°C demonstrated that *P. hirsutum* produced only minor lesions of discoloration around the point of inoculation on garlic bulbils, while *P. allii*, in comparison, was an aggressive pathogen (Overy *et al*., 2005a). Additional studies investigating extracellular enzyme production at various temperatures indicate that *P. allii* is a more aggressive species in field conditions whereas *P. hirsutum* is enzymatically more active and more likely to dominate in low temperature storage (Overy *et al*., 2005b).

Penicillium chrysogenum, previously reported from garlic (Abdel Sater & Eraky, 2002) was non-pathogenic in the current study. Commonalities in morphological features exist between *P. chrysogenum* and *P. allii* that can lead to potential misidentification (terverticillate conidiophores and yellow drops on CYA), although these species can be separated using the secondary metabolite profiles.

The distribution of the pathogen across the surveyed area showed considerable anthropogenic influence. Of the sampled farms, 27% were planted with bulbils acquired from different areas. On the other hand, the same morphotype of *P. allii* was recovered from garlic bulbs which had escaped the disease, even when they were stored in outdoor conditions, allowing for infection with other *Penicillium* strains. This would suggest that *P. allii* is a pathogen transmitted by seed bulbs, remaining viable following desiccation on garlic tissues. If garlic bulbils used as seed are moved within and across various cultivated regions at the beginning of each garlic season, it will be more difficult to obtain interpretable ecological relationships between the presence of *P. allii* and environmental influences from the region.

It was not possible to determine the aggressiveness of *P. allii* phenotypes from *in vitro* assays using GSA. The species *P. implicatum* and *P. viridicatum* did not grow on garlic extract media; *P. flavigenum*, *P. brevicompactum*, *P. phoeniceum* and *P. aurantiogriseum* only developed slightly on garlic extract, whilst colony growth of the non-pathogenic species *P. chrysogenum, P. tulipae* and *P. hirsutum* was similar to that of *P. allii*.

In the pathogenicity trial *P. aurantiogriseum*, *P. chrysogenum*, *P. phoeniceum* and *P. tulipae* were able to sporulate on injured garlic bulbils in damp-chamber trials, but they did not produce typical blue mould symptoms in the field and could not be recovered after harvesting. The reported susceptibility of cv. Fuego INTA, in comparison to Castaño INTA (Cavagnaro *et al*., 2005), was supported here as the former was found to be more severely affected by all three aggressive *P. allii* phenotypes used in the damp-chamber trials and also suffered a greater mortality rate in the field trials.

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