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Ultrastructure of the Initial Interaction of *Puccinia arachidis* and *Cercosporidium personatum* with Leaves of *Arachis hypogaea* and *Arachis stenosperma*

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Abstract

Cultivated peanut, *Arachis hypogaea* L., is an economically important species. It is very susceptible to different stresses to which wild species are mostly resistant. Foliar diseases, such as late leaf spot (LLS) caused by the fungus *Cercosporidium personatum*, and rust caused by the fungus *Puccinia arachidis*, are responsible for decrease in plant growth and productivity. The peanut wild relative *Arachis stenosperma* accession V10309 was identified as resistant to a number of pests and diseases, including LLS and rust. Aiming to better understand the mechanisms of resistance of *A. stenosperma* to *C. personatum* and *P. arachidis*, determine initial key steps of the plant–pathogen interaction and to contribute for studies on genes involved in this interaction, ultrastructural analysis was performed on leaves of *A. stenosperma* V10309 (wild, resistant) and *A. hypogaea* cv. IAC-Tatu (cultivated, susceptible) inoculated with *C. personatum* or *P. arachidis*. For both fungal species, adhesion, germination of spores and hyphal proliferation occurred in both species but was more limited and later in *A. stenosperma* than in *A. hypogaea*, and no successful penetration was observed in the former. These data suggest that in *A. stenosperma*, infection is hampered at the stage of penetration. This is the first morphological description of the first hours of the interaction of plant pathogenic fungi and the resistant wild species *A. stenosperma*.

Introduction

The genus *Arachis* is native to South America (Krapovickas and Gregory 1994). The most economically important member of this genus is peanut, *Arachis hypogaea* L. Peanut is an allotetraploid which probably originated via hybridization of two diploid wild species (*Arachis duranensis* and *Arachis ipaensis*) followed by a spontaneous duplication of chromosomes (Kochert et al. 1996; Seijo et al. 2004; Robledo et al. 2009). This hybrid was isolated reproductively from its wild relatives, leading to low diversity for some traits of agricultural interest. In contrast, wild diploid species of *Arachis* are more diverse genetically and have evolved by selection to diverse factors including various environments and stresses, providing a rich source of variation in agronomically important characters.

Peanut yields can be reduced dramatically by biotic stresses such as fungal diseases. *Cercospora arachidicola* (S. Hori) (causing early leaf spot, ELS), *Phoma arachidicola* Marasas, Pauer, and Boerema) (causing web blotch), *C. personatum* (Berk. and M.A. Curtis) Deighton (causing late leaf spot, LLS) and *Puccinia arachidis* (Speg.) (causing rust) are important fungi occurring worldwide. The latter two are considered to be the most severe foliar diseases in peanut (Shokes and Culbreath 1997; Subrahmanyam 1997).

LLS lesions begin on the leaf surface as small necrotic flecks that enlarge to form coalescing, blackish-brown spots, up to *c.* 8 mm in diameter. Mature spots occasionally develop a yellow halo. Conidial sporulation occurs on almost all lesions, on the abaxial surface, from a conspicuous dark stroma. Conidia are pigmented (olivaceous), more or less cylindrical, straight or slightly curved, relatively short and wide (20–70 × 4–9 um) and mostly with 3–4 septa. Conidia are the main initial inoculum source, but ascospores, chlamydospores and mycelial fragments can also be infective. Conidia germinate on the leaf surface during periods of high humidity, penetrate either through stomata or directly through epidermal cells and produce intercellular haustoria. Symptoms develop within 10–14 days at temperatures above 21°C (Chupp 1954; Shokes and Culbreath 1997).

Rust appears as minute pustules that are visible from both sides of the leaf. As the number of infections increases and pustules become older, leaves become a 'rusty' yellow colour. Infections may also develop on stems and leaf petioles. Lesions viewed from the abaxial surface of leaves will exhibit masses of reddish-brown spores (urediniospores) that are easily spread by air movements to other leaves. These spores are capable of causing new infections and increasing the severity of disease. Pustules, that can appear in all aerial parts except flowers, are usually circular and 0.5–1.4 mm in diameter (Subrahmanyam 1997).

To look for sources of disease resistance, a large number of *A. hypogaea* accessions, some interspecific derivatives and some wild accessions, have been challenged with *C. personatum* and *P. arachidis*, and their responses were evaluated by classic phytopathological methods (Abdou et al. 1974; Subrahmanyam et al. 1983, 1985; Mehan et al. 1994; Moraes and Godoy 1995; Pande and Rao 2001; Dwivedi et al. 2003; Fávero et al. 2009). Different levels of response to the infection were reported and among the species tested, and some accessions of *Arachis stenosperma* were identified as highly resistant or immune (Fávero et al. 2009).

In spite of the availability of different studies on plant–pathogen interaction, details of mechanisms of resistance to *C. personatum* and *P. arachidis* in *Arachis* remain largely unknown. This study was carried out to examine the initial stages of the interaction of the *C. personatum* and *P. arachidis* with leaves of *Arachis hypogea* var. Tatu (susceptible) and *A. stenosperma* (resistant) under scanning electron microscopy to increase the knowledge of these two peanut diseases.

Materials and Methods

Cercosporidium personatum spores were collected from infected peanut plants at the Agronomic Institute of Campinas (IAC), São Paulo, Brazil. Fungal cultures were replicated in BDA medium (potato-dextrose-agar), and spores were isolated after multiplication in rice. *Puccinia arachidis* urediniospores were collected from infected *Arachis* plants in the greenhouse in Embrapa Genetic Resources and Biotechnology, Federal District, Brazil.

Plants used were *A. stenosperma* accession V10309, from the Active Germplasm Bank at Embrapa Genetic Resources and Biotechnology. *Arachis hypogaea* cv IAC-Tatu was obtained from IAC. Plants were kept in greenhouse conditions at Embrapa Genetic Resources and Biotechnology. Detached leaves were inoculated as previously described (Moraes and Salgado 1982). The first expanded leaves of the main stem of 10 individuals of each species were inoculated with a suspension of 50 000 spores/ml of Tween 20. Spore suspensions were spread on leaf surfaces with the help of a soft brush. Inoculated leaves were maintained in a growth chamber at 24°C with 12-h light photoperiod. Another ten Petri dishes containing inoculated *A. stenosperma* accession V10309 or *A. hypogaea* cv. IAC-Tatu leaves were kept with either fungus for longer periods to test susceptibility or resistance by observation of macroscopic symptoms and the observation of spores under light microscopy using Zeiss SV 16 Stereomicroscope (Carl Zeiss, Jena, Germany).

Samples were collected at 3, 6, 12, 24, 48, 72 and 96 h after inoculation (HAI). For each stage, samples of 1 cm^2 were collected randomly from leaflets and processed for scanning electron microscopy. Samples were immersed for 2 h in a solution of 0.05 M cacodylate buffer, pH 6.8, containing 2.5% glutaraldehyde, postfixed for 30 min in 1% osmium tetroxide in the same buffer and dehydrated in increasing concentrations of ethanol solutions (30, 50, 70 and 90%) for 30 min in each solution and for 1 h in 100% ethanol. Samples were dried in a critical point drier (Emitech K850, Emitech, Kent, UK) using CO_2 as transition fluid. The dried samples were mounted over copper stubs and coated with approximately 20-nm-diameter gold particles in a sputter coater (Emitech K550). Specimens were observed under a Zeiss DSM 962 Scanning Electron Microscope. At least four randomly chosen samples from each batch were analysed.

Results

In this work, the first steps of the interaction of *C. personatum* and *P. arachidis* with detached leaves of the wild resistant *A. stenosperma* (V10309) accession and cultivated susceptible cultivar *A. hypogaea* cv. IAC-Tatu were ultrastructurally analysed to improve the understanding of the plant–pathogen interaction and resistance mechanisms in *Arachis* species.

All samples of *A. hypogaea* cv. IAC-Tatu had visible symptoms typical of *C. personatum* 35 days after inoculation: necrotic, round, dark brown lesions with spores on the surface of the leaves. Similarly, symptoms of *P. arachidis* infection were detected 15 days after inoculation on peanut leaves, such as masses of reddish-brown spores on the abaxial surface of the leaves. No macroscopical symptoms were observed in *A. stenosperma* after inoculation with either fungal species (not shown).

Samples from both species inoculated with *C. personatum* collected 3 HAI lacked conidia on either leaf surface (not shown), suggesting that the inoculum was washed off during sample preparation due to the fact that no adhesion has occurred by this time. At 6 HAI, fungal spores were observed adhered to the leaf surface (not shown). Germinated spores were observed 12 HAI in both species and each conidium produced one or two germ tubes from terminal or intercalary cells and the germ tubes often branched (Fig. 1a,c). However, at this stage, it was already clear that conidia proliferation was more evident in *A. hypogaea* than in *A. stenosperma*. Regular germination was observed 48 HAI in *A. hypogaea* (Fig. 1d) but not in *A. stenosperma*, where the growth of the germ tubes appeared to have ceased (Fig. 1b). In *A. hypogaea*, there was a well-developed net of germ tubes and the presence of some of them on stomata openings 72 HAI (Fig. 1e)

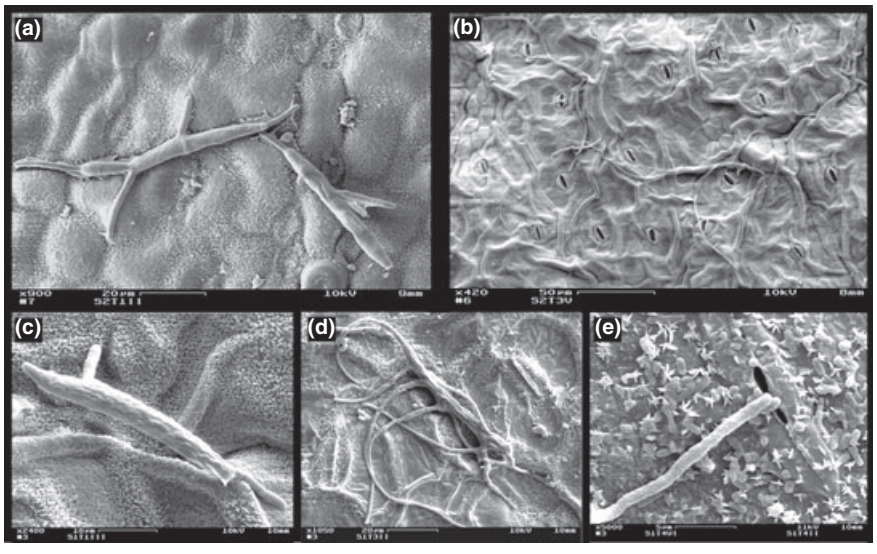

Fig. 1 Interaction of *Cercosporidium personatum* with detached leaves of *Arachis stenosperma* V10309 (a,b) and *Arachis hypogaea* cv. IAC-Tatu (c,d,e). Branched germ tubes originated from terminal or intercalary cells were observed in both species 12 HAI (a,c). Cylindrical, straight, relatively short and wide with 3–4 septa conidia were observed 48 HAI (b,d). Hyphal penetration into stomatal openings was observed only in *A. hypogaea* cv. IAC-Tatu 72 HAI (e). HAI, hours after inoculation

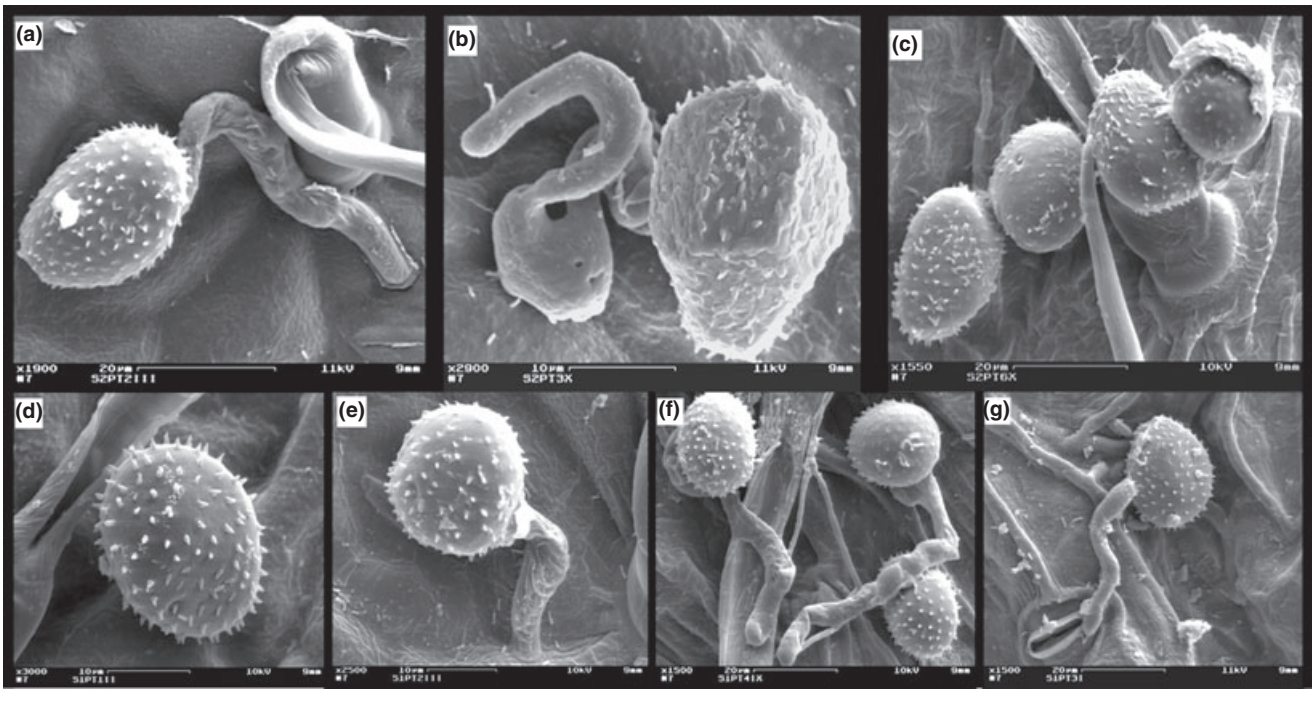

Fig. 2 Interaction of *Puccinia arachidis* with detached leaves of resistant *Arachis stenosperma* V10309 (a,b,c) and susceptible *Arachis hypogaea* cv. IAC-Tatu (d,e,f,g). Presence of adhered spores on the abaxial surface of the leaves of *A. hypogaea* cv. IAC-Tatu collected 3 HAI (d). Germinated spores 6 HAI showing cylindrical and long germ tubes (a,e) and appressoria 12 HAI (b,f) on the leaves of both species. Reduced germ tube proliferation in *A. stenosperma* 24 HAI relative to *A. hypogaea* (c). Presence of germ tubes on the stomata opening in *A. hypogaea* cv. IAC-Tatu 72 HAI (g). HAI, hours after inoculation

with the tip towards the leaf epidermis, suggesting that penetration could be occurring around this time of the infection. At 96 HAI, development of conidia and attempts to penetrate were observed in *A. hypogaea*, and no signal of conidia growth or penetration was observed on the adaxial leaf surface of *A. stenosperma* (not shown).

Samples inoculated with *P. arachidis* showed germ tubes and appressoria on the leaves of *A. stenosperma* and *A. hypogaea* (Fig. 2). Spores on the abaxial surface of the leaves were observed 3 HAI (Fig. 2d), indicating that adhesion occurs earlier than *C. personatum* under these experimental conditions. However, for both species, spore germination was detected only

6 HAI, with developing cylindrical and long germ tubes present (Fig. 2a,e). Formation of appressoria was observed 12 HAI for both species (Fig. 2b,f). At 24 HAI, expansion of germ tubes was evident in *A. hypogaea* but was poor in *A. stenosperma* (Fig. 2c). In *A. hypogaea*, samples collected 72 HAI germ tubes were observed on the stomata opening, probably trying to penetrate the leaf (Fig. 2g), whilst just a reduced net of germ tubes was detected in *A. stenosperma* 72–96 HAI (not shown).

Discussion

This is the first report on the ultrastructural analysis of the early steps of the interaction of *C. personatum* and *P. arachidis* with leaves of the wild species *A. stenosperma* and cultivated peanut (*A. hypogaea* cv. IAC-Tatu). The observations made in this study were in agreement with previous studies, where susceptibility of *A. hypogaea* cv. IAC-Tatu and apparent complete resistance of *A. stenosperma* to both fungal diseases were reported (Subrahmanyam et al. 1985; Fávero et al. 2009).

Observations of *C. personatum* inoculated leaves of both species indicated that adhesion of spores occurs at 3–6 HAI and conidia germination occurs 12 HAI, this is consistent with previous data of Nobile et al. (2008) for *A. hypogaea* cv. 850 (partially resistant) and *A. hypogaea* cv. IAC-Tatu (susceptible). The slight differences in penetration time observed between the studies could be due to differences in experimental conditions, fungal strain or physiological state of the plants.

On *A. stenosperma* leaves inoculated with either pathogen, development of germ tubes and formation of apressoria were delayed and reduced, suggesting that resistance mechanisms act during early steps of the interaction. Additionally, no penetration could be observed. These data suggest that *A. stenosperma* is immune to *C. personatum* and *P. arachidis*.

It is well known that resistance to fungal foliar diseases, here evidenced by the restriction of conidia development and penetration, is under genetic control, and recently quantitative trait loci (QTLs) controlling resistance were mapped in an F_2 population derived from a cross between *A. duranensis* K7988 and *A. stenosperma* V10309 (Moretzsohn et al. 2005; Leal-Bertioli et al. 2009). The proximity of these QTLs to regions rich in resistance genes analogues suggests the involvement of proteins with the *nucleotide binding site* (NBS) domain in the resistance process. The genetics of this immune response is of considerable interest, and the recombinant inbred lines developed from this same population provide a tool that could be used to investigate this further.

The source of resistance of *A. stenosperma* is diploid and cannot be used directly for gene introgression into cultivated peanut by crossing and breeding. Gene isolation through map-based cloning and transformation could be contemplated, or alternatively, the sterility barriers could be overcome by the incorporation of *A. stenosperma* into a synthetic tetraploid. In this latter aspect, promising advances have been made (unpublished results).

In summary, we hope that the knowledge of the early interaction between *A. stenosperma* and the fungi will be useful to guide further work: both experiments aimed at further characterizing the genes associated with the resistance and susceptible responses of the cultivated and wild species of peanut, and the introgression of resistance genes from *A. stenosperma* into the peanut crop.

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