CHAPTER 3

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY OF POWDERY MILDEW ON CASHEW

ABSTRACT

Anamorphs belonging to the genus *Oidium* are often morphologically indistinguishable from each other. Cashew powdery mildew caused by *O. anacardii* was originally described in 1898 and later revised by Castellani and Casulii in 1981. In this study, the infection process of the pathogen was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Conidia from naturally infected cashew leaves were collected and inoculated on young healthy leaf surfaces of three different clones: one tolerant (H1), one moderately tolerant (Veloso 32) and one susceptible (Corane) to the pathogen. After inoculation, samples were collected two-hourly and were prepared for SEM and TEM viewing according to standard procedures. Septa and outer wall ornaments of the conidia, sequence of the germination process and conidial formation on the cashew leaf surface were observed. The results indicated that conidia occurred in depressions on the leaf surfaces. Single germ-tubes emerged from individual conidia within 4 hours post inoculation (hpi). The infection process took place 14 hpi with emission of a penetration peg. Later, the penetration peg differentiated into a globose haustorium. At this stage, cytoplasm aggregates were observed and their relative consistency was higher in the disease tolerant clone as compared to the other clones. The morphology of the fungus including conidiophore and conidia, was observed and analysed using Cook's key. Based on the morphological observations we supported the taxonomic positioning of *O. anacardii* as a member of the subgenus *Pseudoidium*. The mechanism of infection was observed and no obvious host structural differentiation as response to the pathogen could be detected.

INTRODUCTION

Various factors have been ascribed to the decline in cashew (*Anacardium occidentale* L.) production in Mozambique (Uaciquete, 1997; Neto & Caligari, 1998). However, a critical analysis revealed that the lack of varieties and hybrids capable of producing more significant and durable levels of resistance to biotic stresses is one of the major causes of decline in production (Prasad, 1998).

The occurrence of apparent partial resistance in local populations and collections of cashew germplasm has been reported (Waller *et al.*, 1992). This is sometimes termed slow mildewing and is thought to be polygenically inherited and independent of known major race-specific genes for resistance (Aist & Bushnell, 1991). So far there is no evidence that major genes exist that give desirable levels of powdery mildew tolerance or resistance in cashew (Caligari, 1997).

Three approaches (Sijaona & Mansfield, 1998) have been used to study variations in cashew susceptibility or resistance due to mildew infection, covering both laboratory and field observations. Organs such as detached leaves, flowers and panicles including entire seedlings have been used in screening for tolerance (Sijaona & Mansfield, 1998). However, none of the previous studies illustrated the mechanism of interaction involved in host- pathogen relationships.

Anamorphs belonging to the genus *Oidium* Link are often morphologically indistinguishable from each other (Ialongo, 1993). Therefore, the taxonomy and identification of powdery mildews are based largely on the characteristics of the teleomorph (Cook *et al.*, 1997). However, the habit of using the name of the teleomorph to indicate the anamorphs resulted in limited studies of the latter (Ialongo, 1993). Identification based on teleomorphs are also a problem when the powdery mildew pathogen widens its host range or increases its ecological area of occurrence (Cook *et al.*, 1997). This is because the teleomorphic stage may not be found for some time or may not be produced at all. Furthermore, accurate identification is essential in plant quarantine and in locating the origin or source of inoculum (Cook *et al.*, 1997).

Determining the source of inoculum, as an element of disease dynamics, is of importance in developing a management strategy (Maddison *et al.*, 1998). In trying to do so, Waller *et al.* (1992), described the possibility that the powdery mildew of mango extended its host range to include cashew. Maddison *et al.* (1997) suggested that the cashew powdery mildew anamorph may also attack a wild fabaceae plant, *Julbernardia globiflora* (Benth.) Troupin, in Tanzania. However, no further clarification was provided on pathogen specificity and the potential of this miombo vegetation as an active reservoir or source of inoculum. Thus, a clear understanding of the pathogen's distinctiveness is required. The present study was designed to 1) provide further information on the characteristics of the cashew powdery mildew anamorph using scanning electron microscopy (SEM) to address the issue of identity and; 2) to describe the mechanism of infection on different cashew clones known to express different levels of susceptibility or tolerance to the disease using transmission electron microscopy (TEM).

MATERIALS AND METHODS

Plant inoculation

Vegetatively propagated and rain fed cashew trees over 30 years old grown at Ricatla cashew research station, Maputo, were used in this experiment. These trees were not used for infection studies due to a lack of appropriate facilities in Mozambique for an in-house study. In addition, South African quarantine authorities did not grant a permit for cashew plants to be brought into South Africa for experimental purposes. Since mature tissues are not susceptible to the disease (Sijaona *et al.*, 2001), only symptomless emerging twigs were used. One day prior to inoculation, conidia were shaken from infected cashew leaves to encourage new production before collection of inoculum. Young leaves from individual twigs were randomly selected and surface sterilised by dipping them up to the fifth leaf into 70% v/v ethanol for 2 min. The twigs with leaves were then rinsed twice in sterile distilled water and air dried before being inoculated with prepared inoculum. A dry brush was used to gently harvest spores and transfer them onto the new target young leaves.

Targeted leaves were inoculated on the upper surface over the whole lamina just after sunrise (06h00). Inoculated leaves were immediately covered in plastic bags previously moistened with sterile distilled-water. Three compatible combinations of cashew and powdery mildew inoculum were tested on clone H1 representing tolerant material, Veloso 32 as intermediate and Corane 2 as susceptible. Naturally infected and non-infected controls were included.

Samples consisting of 0.5 x 0.5 cm pieces cut from any site on the inoculated leaf lamina, were collected two-hourly until late at night (22h00). At each sampling time, individual samples were fixed in 2.5% glutaraldehyde, buffered with 0.075 M phosphate buffer. Samples were maintained at 6°C until transported to the laboratory for processing.

Scanning electron microscopy

After rinsing three times in 0.075 M phosphate buffer (pH = 7.4) for 15 min, samples were post-fixed in 1% unbuffered osmium tetroxide for 30 min at room temperature (Glauert, 1975). All material was successively washed three times in distilled water and then dehydrated in an ascending ethanol series (50%, 70%, 90% and three times 100%). Samples were critical point-dried in a Bio-rad E3000 critical point drier (Polaron Equipment Ltd, Hertfordshire, England) and mounted on SEM stubs. Specimens were coated with gold in a Polaron E5200 Sputter Coater (Polaron Equipment Ltd, Waterford, England) before examination with a JEOL JSM840 (JEOL LTD, Tokyo) SEM, operated at 5 kV.

Transmission electron microscopy

Samples were prepared for TEM as described up to the 100% ethanol dehydration step and were then infiltrated with quetol resin at 33% for 1 h, 66% for 1 h and twice in 100% for 18 h and 3 h respectively (Van der Merwe & Coetzee, 1992). Samples were polymerised at 60°C for 24 h. Gold sections were cut on an ultramicrotome (Ultracut E, Reichert-Jung, C Reichert, Vienna). Sections were stained with uranyl acetate for 30 min followed by lead citrate for 3 min. Sections were then viewed in a Philips 301 TEM (Philips, Eindhoven) operated at 60 kV.

RESULTS

Conidial germination and appressorium development

Conidia were generally clustered in depressions on the leaf surface (Fig. 3.1a). In certain cases, conidia were observed slightly embedded in the waxy layer of the upper leaf surface (Fig. 3.1b).

A single-germ tube emerged from individual conidia within 4 h after inoculation (Fig. 3.1c) and elongated (Fig. 3.1d). When it reached about 40 nm long, it swelled, especially at the apex, producing a long (10 nm), lobed structure (Fig. 3.1e), which is the primary

appressorium formed within 8 hpi. At 10 hpi, the primary appressorium differentiated dichotomously on the host epidermis in all material studied (Fig. 3.1f). No obvious differences between clones could be detected up to this stage.

Infection process

The infection process took place within 14 hpi. A penetration peg emerged and penetrated perpendicularly through the epidermal cell wall of the host (Fig. 3.2a). The host cell wall appeared visually thicker than that of adjacent uninfected cells and the cytoplasm beneath the penetration site aggregated (Fig. 3.2a). The penetration peg appeared narrowed at the penetration site and then expanded into a globular structure once inside the cytoplasm (Fig. 3.2b). The cytoplasm aggregates as well as the thickening of the cell wall were universal phenomena in all three clones. However, cytoplasm density was comparatively higher in clone H1 (as revealed by the concentration of stains) than other clones (Fig. 3.2b as compared to Fig. 3.2a). Otherwise, no obvious differences between clones could be observed.

Conidiophore and conidial development

A mature colony consisted of a matrix of abundant filamentous, septate and whitish hyphae. Conidiophore initiation was observed at 72 hpi as small globose swellings on the hyphae of mature colonies on infected leaves (Fig. 3.3a). The swellings differentiated into a typical conidiophore with a straight basal cell (Fig. 3.3b) and mature conidium with a distinct septum at the apex (Fig.3.3c). This in turn developed into two to three cells separated by septa (Fig. 3.3d). The distal cell matured and was released from the chain at the apical septum (Fig. 3.3d).

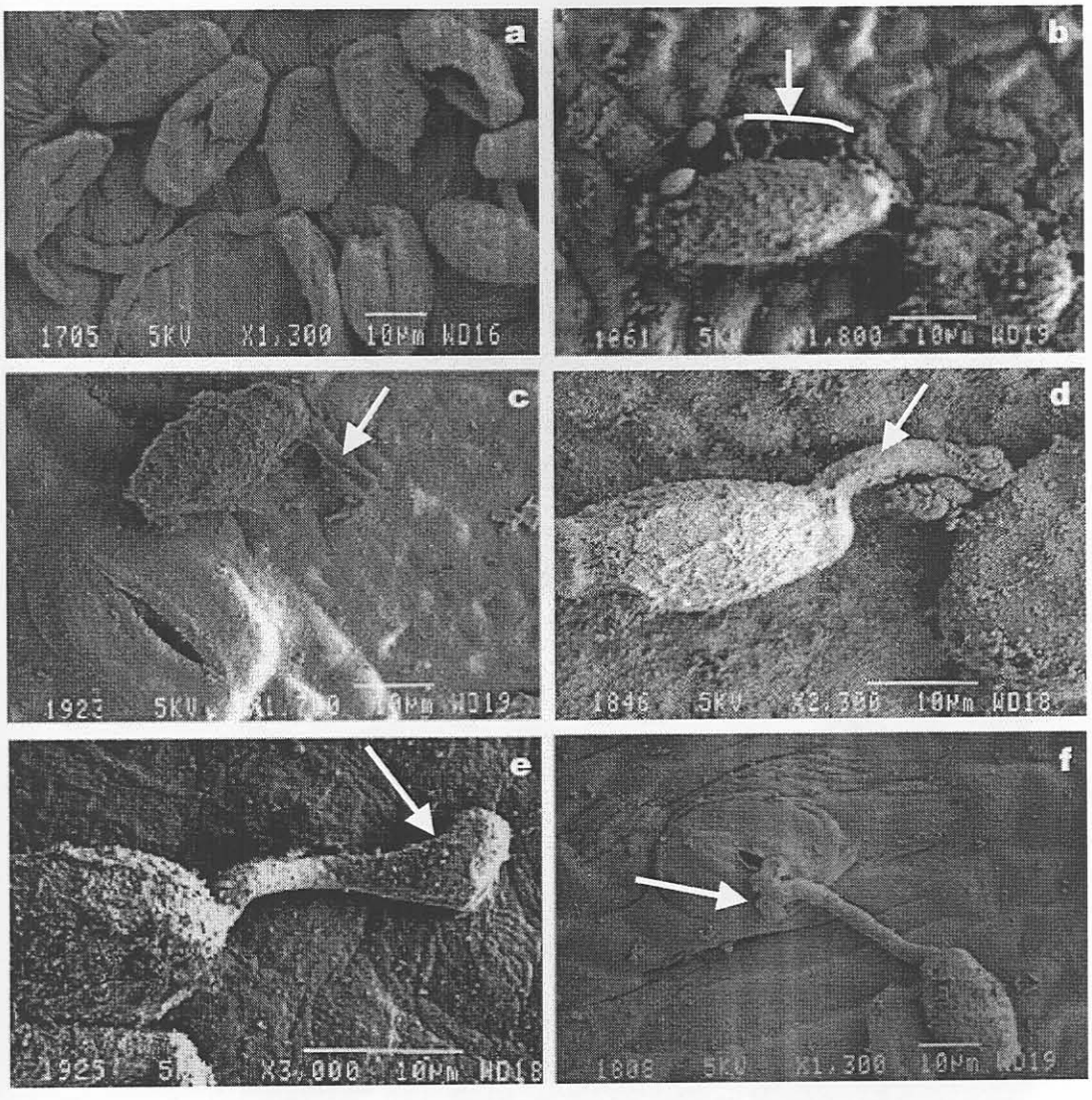
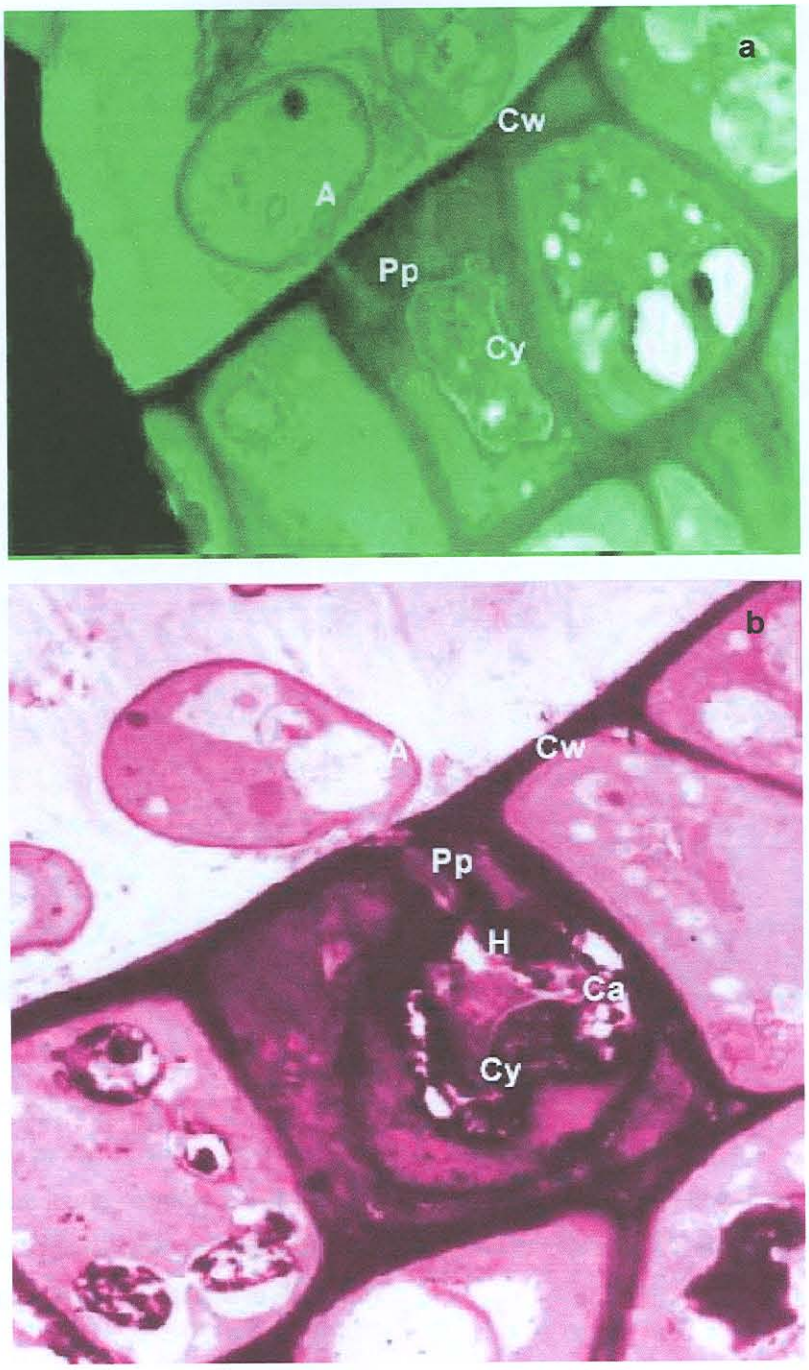


Figure 3.1 Scanning electron micrographs of germinating conidia of *Oidium anacardii* on young cashew leaves. a) Clusters of conidia on the upper leaf surface; b) Wax layer degradation by conidia; c) Elongation of primary germ tube; d) Elongated primary germ tube; e) Differentiation into an appressorium and f) Mature, expanded appressorium. The arrows indicate degraded area b) or fungal structures c) to f).



A = apressorium; Ca = cytoplasm aggregates; Cw = Thickened cell wall;
Cy = cytoplasm; Pp = penetration peg; H = haustorium

Figure 3.2 Transmission electron micrographs of *Oidium anacardii*, infection process 18 hpi, showing a) Penetration of haustorium into epidermal cells of clone Corane 2 and cytoplasm aggregation beneath haustorium. Mag. 3780x. b) Advanced infection with dense cytoplasm aggregation, clone H1. Mag. 4515x.

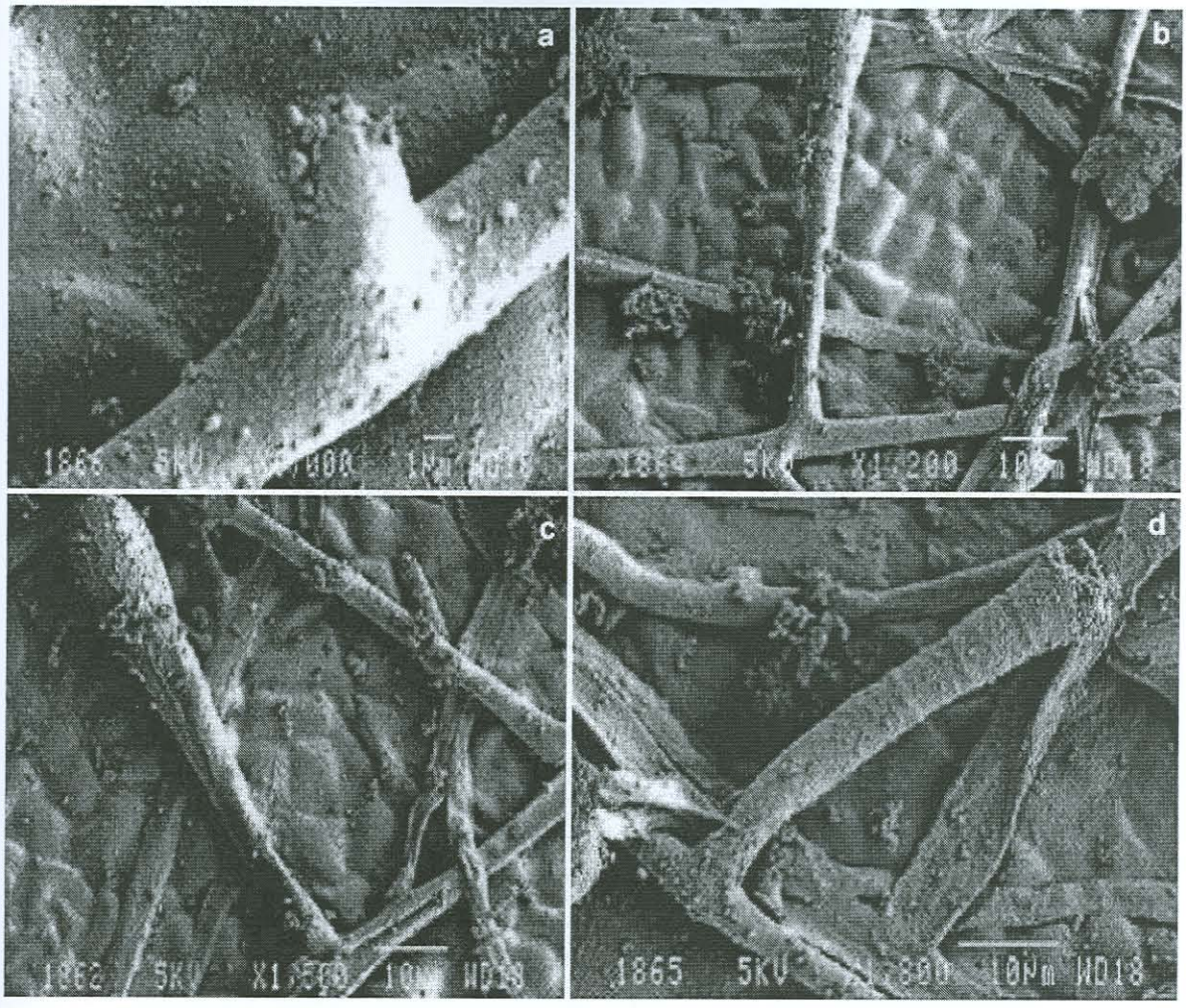


Figure 3.3 Scanning electron micrographs of *Oidium anacardii* condiophore and conidium development 72 hpi.on leaves of clone H1 a) Conidiophore initiation; b) Differentiating conidium with a straight basal cell; c) Conidium maturation; d) Conidiophore with conspicuous septa after conidium release. The arrows indicate fungal structures referred to in a) to d).

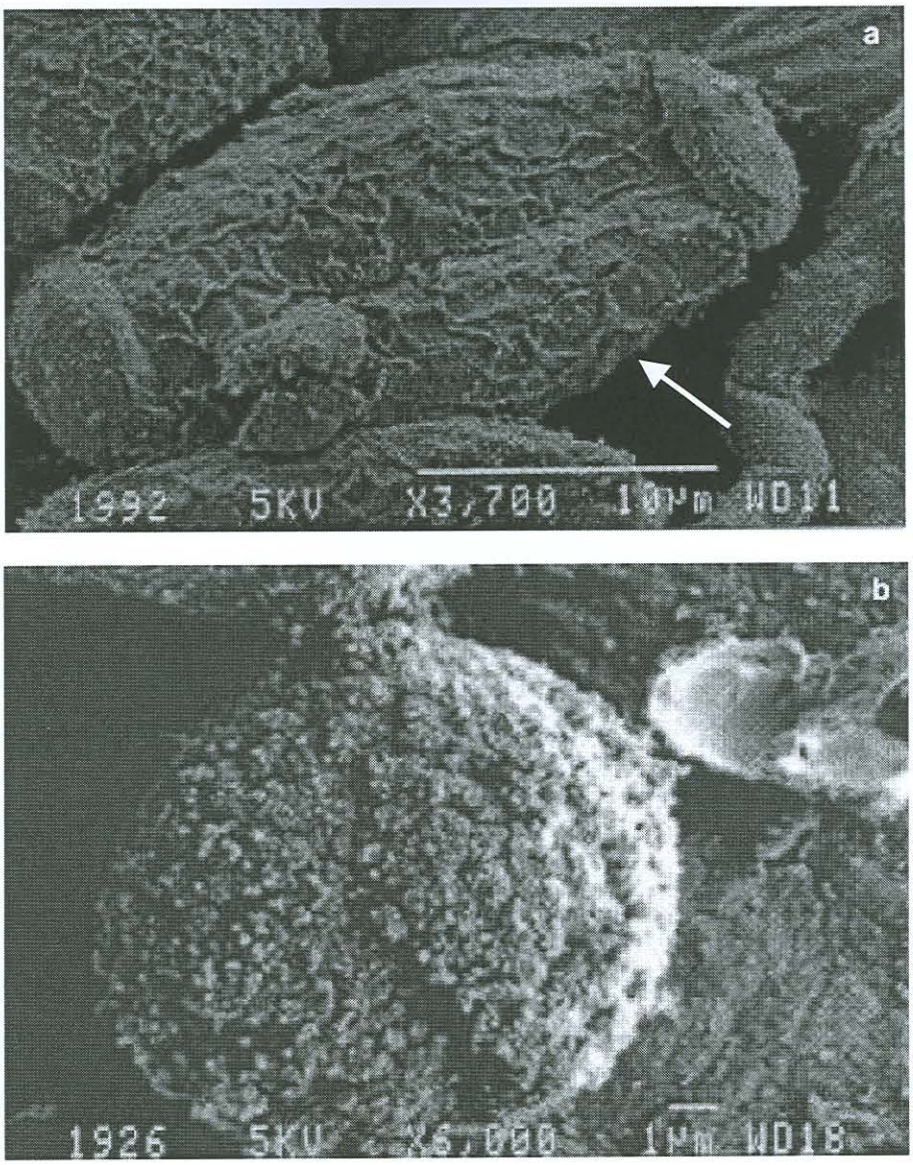


Figure 3.4 Scanning electron micrographs of *Oidium anacardii* conidial structures on leaves of clone H1 a) Outer wall wrinkles on collapsed conidium; b) Conidial septum. Arrows indicate the wrinkles a) and septum b).

Conidial structures

Conidia are hyaline and elliptical in shape. The outer wall on collapsed conidia consisted of a dense honey-combed wrinkling (Fig. 3.4a) and the pattern observed on a turgid conidial septum was characterised as smooth (Fig. 3.4b), i.e. neither verrucose nor particularly protuberant following Cook's SEM description of conidia.

DISCUSSION

The species *O. anacardii* was originally described by Noack in 1898 (Castellani & Casulii, 1981; Waller *et al.*, 1992; Shomari, 1996). Based on light microscopy, Castellani and Casulii (1981) provided a preliminary review on the morphology of this pathogen: The mycelium is superficial and abundant. The hyphae are whitish to light-grey in colour, septate with a diameter of about 3.5 µm. Conidiophores are short, measuring up to 45 µm in length with one or two basal cells. In this study, SEM and TEM were employed to further describe the fungus' morphology and its development on the leaf surface of three different cashew clones. Our observations on the external characteristics of the fungus were similar to those of previous researchers.

In the present study, conidia were found to have commenced germination 4 hpi, regardless of the clone. This confirms prior observations made *in vitro* on the same pathogen, where conidia germinated 4 hpi under environmental conditions of 15-35°C and 20 to 100% relative humidity (Shomari & Kennedy, 1998; Shomari & Kennedy, 1999). Similarly, Leinhos *et al.* (1997), working with another powdery mildew (*Uncinula necator* Schwein.) on grapevine leaves, found that the pathogen produced a germ tube within 4 hpi. Earlier germination 1-2 hpi has been reported for other powdery mildews (Plotnikova *et al.*, 1998).

To our knowledge, none of the earlier studies on *O. anacardii* has attempted to examine, at microscopic level, the relationship between the process of conidial germination and hosts of different resistance levels. Reported comparative studies on the relationship between different varieties and the pathogen have been based on superficial host tissue coverage by actively sporulating mycelia (Nathaniels, 1996; Sijaona *et al.*, 2001). In some cases, a reduced rate of germination is associated with the host's partial resistance to infection (Aist & Bushnell, 1991). None of the tested clones expressed any morphological or temporal differences upon conidial germination on the leaf surface which could be related to resistance

or tolerance to the disease. Therefore, our results suggest that germination of individual conidia on the host surface does not necessarily determine the severity of the disease. Disease severity is possibly determined once the pathogen has penetrated the leaf and organic interactions between the pathogen and the host are established.

Partial resistance to infection by powdery mildews can be determined by the failure to produce haustoria or having reduction in their size and development of papillae. More commonly, slower colony development without a hypersensitive reaction in the host, has been attributed to partial resistance (Aist & Bushnell, 1991). It has been hypothesized that tolerance to cashew powdery mildew is characterised by a slower development of infection and consequently slower growth of mycelium (Martin *et al.*, 1997). The hypothesis was supported by a higher level of cytoplasmic reaction by the tolerant clone H1 compared to the others. Possibly a more dense cytoplasm restricts the availability and absorption of nutrients through haustoria and consequently reduced development of the pathogen.

Our study illustrates the mechanism of infection. No obvious clonal differentiation could be made. However, more dense cytoplasm on clone H1 may be associated with a higher concentration of tannins (Coetzee, 2000, personal communication) which in turn may be involved in a biochemical response. Kanter *et al.* (1996) found that tannins conferred chemical and physical properties which protected *Vicia faba* L. germinating seeds against fungal infection. Spiers *et al.* (1998) also found association of these compounds with seasonal host tolerances of various plant species to fungal diseases. Finally, cashew varieties are known to possess different levels of tannins (Ferrão, 1995). This could also explain the resistance observed in mature tissues of all clones in the field. Nevertheless, the potential role of tannin production as defense strategy against *O. anacardii* infection requires further investigation.

Previous observations by Castellani and Casulii (1981) and Shomari (1996) revealed that *O. anacardii* possesses a short conidiophore with one or two basal cells. In our observations, the structure of the conidiophore coincided with the description given above, except that only one straight basal cell was observed. This type of conidiophore has also been reported for other powdery mildew fungi (Celio & Hausbeck, 1997). *Oidium anacardii* conidia are ellipsoidal, unicellular, hyaline, thin walled (Shomari, 1996) and produced in short chains (Waller *et al.*,

1992) of 4-8 spores (Ponte, 1984). Our findings support this description. Shomari (1996) further described that the conidia mature singly.

Under high humidity, two or three conidia may mature simultaneously on one conidiophore (Castellani & Casulii, 1981). The conidia produce a short germ-tube which terminates in a lobate appressorium measuring 8-10 µm (Shomari, 1996). Our observations agree with the above description. However, in our work the germ-tube appeared to be quite long. In addition, the SEM observations revealed for the first time that the conidial outer wall pattern is honeycomb ornamented with whorled or smooth septa according to the technique adopted in this study. The findings also confirm that separation of anamorphs with whorled septa from those with fibrillar septa is the most fundamental difference visible by SEM (Cook *et al*., 1997).

On the basis of their observations, Castellani and Casulii (1981) concluded that *O. anacardii* belongs to the *Pseudoidium* and fitted well with group A of the classification made by Zaracovitis (1965) cited by Shomari, 1996. Based on the pattern seen on turgid conidia on septa and on outer conidial walls, our investigation revealed that *O. anacardii* fits in well with the new classification proposed by Cook *et al*. (1997) as a member of *Oidium* subgenus Pseudoidium (Y.S. Paul & J.N. Kapoor) comb. Et. stat. Nov. (Holomorph *Erysiphe* Sect. *Erysiphe* U.Braun).

REFERENCES

Aist, J.R. & Bushnell, W.R. 1991. Invasion of plants by powdery mildew fungi and cellular mechanisms of resistance. Pages 321-340 *In*: The Fungal Spore and Disease Initiation in Plants and Animals. Cole, T.G. & Hoch, H.C. (Eds). Plenum Press, USA.

Caligari, P.D.S. 1997. Breeding and improvement of cashew in Mozambique. Report on visit to Mozambique. Consultancy for USAID/Agribusiness and Marketing strategies (AMIS II), Mozambique cashew subsection. Rehabilitation study, production and propagation via the Postharvest Institute for Perishables, University of Idaho, USA.

Castellani, E. & Casulii, F. 1981. Osservazioni preliminari su *Oidium anacardii* Noack agente del mal bianco dell'anacardio. *Revista di Agricultura Subtropicale e Tropicale* LXXV: 211-222.

Celio, G.J. & Hausbeck, M.K. 1997. Conidial germination, infection structure formation, and early colony development of powdery mildew on Poinsettia. *Phytopathology* 88: 105-113.

Cook, R.T.A., Inman, A.J. & Billings, C. 1997. Identification and classification of powdery mildew anamorphs using light and scanning electron microscopy and host range data. *Mycological Research* 101: 975-1002.

Ferrão, J.E.M. 1995. O cajueiro (*Anacardium occidentale* L.). Instituto de Investigacao Cientifica Tropical, Lisboa.

Glauert, A.M. 1975. Fixation, dehydration and embedding of biological specimens. Practicals in Electron Microscopy. North Holland Publishing Company. Holland.

Ialongo, M.T. 1993. Biostatistical characterization of the genus *Oidium*. *Mycotaxon* XLVII: 193-199.

Kanter, F., Hebblethwaite, P.D. & Pibeam, C.J. 1996. Factors influencing disease resistance in high ad low tannin *Vicia faba*. *Journal of Agricultural Science* 127: 83-88.

Leinhos, G.M.E., Gold, R.E., Duggelin, M. & Guggenheim, R. 1997. Development and morphology of *Uncinula necator* following treatment with the fungicides kresoxim-methyl and penconazole. *Mycological Research* 101: 1033-1046.

Maddison, A., Boma, F., Topper, C. & Shomari, S. 1997. Sanitation in the management of cashew powdery mildew disease in Tanzania. Pages 236-240 *In*: Proceedings of the International Cashew and Coconut Conference. Dar es Salaam, Tanzania, 17-22 February, 1997. Topper, C.P., Caligari, P.S.D., Kullaya, A.K., Shomari, S.H., Kasuga, L.J., Masawe, P.A.L. & Mpunami, A.A. (Eds). BioHybrids International Ltd, Reading.

Maddison, A., Shomari, S., Sijaona, M. & Topper, C. 1998. Disease dynamics in the cashew powdery mildew pathosystem in Tanzania: a review. Pages 266-269 *In*: Proceedings of the International Cashew and Coconut Conference. Dar es Salaam, Tanzania, 17-22 February, 1997. Topper, C.P., Caligari, P.S.D., Kullaya, A.K., Shomari, S.H., Kasuga, L.J., Masawe, P.A.L. & Mpunami, A.A. (Eds). BioHybrids International Ltd, Reading.

Martin, P.J. Topper, C.P., Bashiru, R.A., Boma, F., De Waal, D., Harries, H.C., Kasuga, L.J., Katanila, N., Kikoka, L.P., Lamboll, R., Maddison, A.C., Majule, A.E., Masawe, P.A., Millanzi, K.J., Nathaniels, N.Q., Shomari, S.H., Sijaona, M.E. & Stathers, T. 1997. Cashew nut production in Tanzania: Constraints and progress through integrated crop management. *Crop Protection* 16: 5-14.

Nathaniels, N.Q.R. 1996. Short communication. Methods, including visual keys for the assessment of cashew powdery mildew (*Oidium anacardii* Noack) severity. *International Journal of Pest Management* 42: 99-205.

Neto, V. & Caligari, P.D.S. 1998. The effect of variability on cashew yield trials. Pages 74-75 *In*: Proceedings of the International Cashew and Coconut Conference. Dar es Salaam, Tanzania, 17-22 February, 1997. Topper, C.P., Caligari, P.S.D., Kullaya, A.K., Shomari, S.H., Kasuga, L.J. Masawe, P.A.L. & Mpunami, A.A. (Eds). BioHybrids International Ltd, Reading.

Plotnikova, J.M., Reuber, T.L., Ausubel, F.M. & Pfister, D.H. 1998. Powdery mildew pathogenesis of *Arabidopsis thaliana*. *Mycologia* 90: 2009-2016.

Ponte, J.J. 1984. Doencas do cajueiro no nordeste Brasileiro. Embrapa, Brasilia.

Prasad, M.V.R. 1998. Final report on cashew applied research (1995-1998). Cashew rehabilitation project. Louis Berger International Inc., Nampula.

Shomari, S.H. 1996. Studies on the biology and epidemiology of *Oidium anacardii* (Noack), the powdery mildew pathogen of cashew. PhD thesis. University of Birmingham.

Shomari, S.H. & Kennedy, R. 1998. Field and laboratory investigations on the development of *Oidium anacardii* in relation to environmental factors. Pages 260 – 265 *In*: Proceedings of the International Cashew and Coconut Conference. Dar es Salaam, Tanzania, 17-22 February, 1997. Topper, C.P., Caligari, P.S.D., Kullaya, A.K., Shomari, S.H., Kasuga, L.J., Masawe, P.A.L. & Mpunami, A.A. (Eds). BioHybrids International Ltd, Reading.

Shomari, S.H. & Kennedy, R. 1999. Survival of *Oidium anacardii* on cashew (*Anacardium occidentale*) in Southern Tanzania. *Plant Pathology* 48: 505-513.

Sijaona, M.E.R. & Mansfield, J.W. 1998. Studies on cashew resistance to powdery mildew (*Oidium anacardii* Noack). Pages 241-248 *In*: Proceedings of the International Cashew and Coconut Conference. Dar es Salaam, Tanzania, 17-22 February, 1997. Topper, C.P., Caligari, P.S.D., Kullaya, A.K., Shomari, S.H., Kasuga, L.J., Masawe, P.A.L. & Mpunami, A.A. (Eds). BioHybrids International Ltd, Reading.

Sijaona, M.E.R., Clewer, A., Maddison, A. & Mansfield, J.W. 2001. Comparative analysis of powdery mildew development on leaves, seedlings and flower panicles of different genotypes of cashew. *Plant Pathology* 50: 234-243.

Spiers, A.G., Brewster, D.T., Bus, V.G. & Hopcroft, D.H. 1998. Seasonal variation in susceptibility to xylem tissue of *Malus, Pyrus, Prunus*, and *Salix* species of *Chondrostereum purpureum* in New Zealand. *Mycological Research* 102: 881-890.

Uaciquete, A. 1997. Contribuicao para o entendimento da epidemiologia do Oidio do cajueiro na provincia de Gaza. INIA. Serie Investigacao no. 32, Maputo.

Van der Merwe, C.F. & Coetzee, J. 1992. Quetol 651 for general use. A revised formulation. *Electron Microscopy Society of Southern Africa* 22: 1-7.

Waller, J., Nathaniels, N., Sijaona, M.E.R. & Shomari, S.H. 1992. Cashew powdery mildew (*Oidium anacardii* Noack) in Tanzania. *Tropical Pest Management* 32: 160-163.

Zaracovitis C. 1965. Attempts to identify powdery mildew fungi by conidial characters. *Trans. Br. Mycological Society. 48:553-558.* Cited by Shomari, 1996.