

Short communication

An ultrastructural investigation of the surface microbiota present on the leaves and reproductive structures of the resurrection plant *Myrothamnus flabellifolia*

J.P. Moore ^{a,*}, M. Waldron ^b, G.G. Lindsey ^c, J.M. Farrant ^c, W.F. Brandt ^c^a Institute for Wine Biotechnology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa^b Electron Microscope Unit, University of Cape Town, Private Bag, Rondebosch 7701, South Africa^c Department of Molecular and Cellular Biology, University of Cape Town, Private Bag, Rondebosch 7701, South Africa

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Abstract

The leaves, flower and stems of the southern African angiosperm resurrection plant *Myrothamnus flabellifolia* were investigated at the ultrastructural level to determine the source of previously reported fungal contamination. Fungal mycelia and hyphae of the genera *Aspergillus* and *Penicillium* were found localized to the hydathodes of the leaves and stigmatic surfaces of the female flowers in both desiccated and hydrated specimens. A waxy bacterium of the genus *Bacillus* was found to colonise the waxy epidermal surfaces of the leaves and flowers which was also where fungal cells were found to be absent. It is suggested that the wax like deposits within the leaves and stems as well as over the epidermal surface prevent the growth of the fungal organisms. These fungi opportunistically invade moist surfaces, such as the floral stigmas, during periods of moisture availability and may thus negatively impact plant development.

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1. Introduction

Resurrection plants are a unique group of plants found predominantly in the arid and semi-arid areas of the world and so southern Africa possesses a rich diversity of these plants (Gaff, 1971). A remarkable feature of these plants is their ability to survive extensive dehydration (desiccation) of their vegetative organs (e.g. leaves) without suffering permanent injury (Gaff, 1971; Farrant, 2000). *Myrothamnus flabellifolia* Welw. is considered to be the largest resurrection plant and is unique as being the only member possessing a woody stem (Moore et al., 2007a). It has a widespread distribution across southern and eastern Africa where it is located on rock inselbergs composed of granite, shale and quartz (Moore et al., 2005, 2007a). The plant possesses fan-like leaves which fold along the stem exposing the pigmented and waxy abaxial surface to the environment

(Moore et al., 2007b). These fan-like leaves unfold and change colour from dull-brown to green during the summer rainy season when the plants absorb water run-off from the rocky slopes via their roots (Moore et al., 2007b). The plants exist singly or in colonies and are dioecious (Child, 1960). Numerous attempts have been made to cultivate transported plants in greenhouse conditions in order to study the mechanisms of desiccation tolerance in more detail (Goldsworthy, 1992; Glen et al., 1999). However, it was discovered that plants transported out of their native habitats were susceptible to many problems such as disease (e.g. red spider mite infection), seasonal variation effects, soil composition, temperature effects and sub-optimal light intensities (personal observations, J.P. Moore and K. Cooper). There have, however, been successes with cultivating *M. flabellifolia* in a humid atmosphere which appeared to improve survival conditions (Glen et al., 1999). A common observation of rehydrating twigs from *M. flabellifolia* is that prolonged presence in a humid atmosphere promotes extensive fungal growth over the leaves (Child, 1960; personal observation, J.P. Moore). This was previously reported by Child

* Corresponding author. Fax: +27 21 808 3771.

E-mail address: moorej@sun.ac.za (J.P. Moore).

(1960) who stated that no obvious source of fungal infection could be found even after a detailed examination of the leaves and branches. To clarify the source and identity of this fungal contamination we undertook an ultrastructural study of the leaves, stem and reproductive structures of *M. flabellifolia* with the further aim of ascertaining the role these microbes may play during plant revival and growth. This is of particular importance considering that *M. flabellifolia* is endangered due to excessive collection for traditional medicine formulations (Moore et al., 2007a; Van Wyk et al., 1997).

2. Materials and methods

2.1. Plant material

M. flabellifolia plants, collected from the Buffelskloof Nature Reserve, Mpumalanga Province, South Africa, and Outjo, Namibia were maintained in a glasshouse in the Botany Department, University of Cape Town. Excised twigs/leaves from plants kept in a separate section of the glasshouse, were either incubated for 24–48 h in a humid atmosphere at room temperature to induce fungal growth or impregnated on LB agar plates and incubated at 25 and 37 °C for 24–48 h.

2.2. Light and scanning electron microscopy

The material for ultrastructural analysis was prepared as previously described (Moore et al., 2006, 2007b). Photographs were taken with a Wild Photomakroskop equipped with an AxioCam digital camera. Scanning electron microscopy was performed using a Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo transfer system. Certain specimen-containing stubs were coated with palladium–gold prior to viewing.

3. Results

The unusual fan-like appearance of *M. flabellifolia* leaves has been reviewed and discussed previously (Moore et al., 2007a). The concertina-like structure of the leaves, supported by sclerenchymous ribs, is believed to permit controlled folding and tissue compaction upon rehydration whilst a rapid return to the full turgor state at rehydration (Moore et al., 2007b). This can be seen in hydrated (Fig. 1A) and desiccated (Fig. 1E) leaves. Similarly the reproductive structures have been discussed previously with respect to understanding the pollination and seed dispersal mechanisms of the plant in relation to its unique desiccation-associated ecology (Moore et al., 2007a). The male flowers are arranged in catkin-like inflorescences which bear anthers on short lateral filaments (Fig. 1A, B). The purplish-red anthers dehisce longitudinally releasing large amounts of yellow pollen grains (Fig. 1B). This pollen is believed to be carried to the female flowers possibly via a combination of both insect and wind driven mechanisms (Moore et al., 2007a). The female flowers are composed of three carpels with either pink (Fig. 1C) or red-purple (Moore et al., 2007a) stigmatic surfaces which possess a feathery appearance (Fig. 1D). Floral maturation, pollination and seed dispersal are naturally believed to

occur during the summer rainy season when the plants are fully hydrated. Desiccated flowers present on the branches of desiccated plants are found in a state of post-anthesis for male plants and post-pollination or post-seed-dispersal for female ones. Desiccated female flowers appear dark brown, shriveled and the stigmatic surfaces are devoid of colour as well as the previously observed feathery appearance (Fig. 1E, F). Similarly, male florescences are light brown with dried out anthers and little to no pollen present (Fig. 1G). Desiccated fruits are occasionally also found composed of brown bulbous carpels containing numerous minute ‘dust-like’ seeds (Fig. 1H). Visual inspection as well as scrutiny of the material under the dissecting microscope revealed no obvious source of the fungal contamination observed during prolonged exposure to high humidity. Further analysis under a higher magnification using scanning electron microscopy was therefore performed.

Detailed ultrastructural analysis of both desiccated and hydrated leaves of *M. flabellifolia* has been performed (see Moore et al., 2006, 2007b). An interesting feature of *M. flabellifolia* leaves, apart from the fan-like corrugations at the leaf surface and sclerenchymous ribs, is the presence of hydathode-like structures (which appear to be formed from modified stomata) at leaf apices (Fig. 2A; Moore et al., 2007b). The presence of desiccated salt-like deposits at the apex of desiccated leaves (Fig. 2B) provides strong support to the suggestion that these hydathode structures may function to regulate the salt and solute content of the leaves particularly during desiccation and rehydration (Moore et al., 2007b). Higher magnification examination under electron microscopy of the apices of desiccated leaves revealed clear evidence of microbial presence (Fig. 2C). The desiccated droplet of salts present near the hydathodes of desiccated leaves appeared to be covered with rod shaped microbes and fungal hyphae (Fig. 2C). Furthermore, the fungal hyphae were shown to emerge from fold crevices present at the desiccated leaf surface (Fig. 2D) suggesting that the fungi are protected within the folds of the plants leaves. Incubation of leaf segments either in a humid atmosphere or on nutrient agar Petri plates (see Material and Methods) confirmed the emergence of significant fungal growth from the apices and to a lesser extent the leaf folds (Fig. 2E). The fungal mycelium appeared to rapidly spread across the leaf surface producing aerial fruiting bodies (Fig. 2E). To ascertain if the fungus was parasitic or saprophytic on the plant, closer examination of the fungal infected material was undertaken (Fig. 2F). No evidence of penetration of the fungal hyphae into the leaf surface was observed. Copious fruiting bodies emerged from the fungal hyphae growing on the leaf surface (Fig. 2G) and these in turn were composed of numerous fungal spores. Higher magnification of the fruiting bodies revealed spores which bore ‘wheel’ shaped structures that possessed clear ridges spanning their circumference at set intervals (Fig. 2H). The fungal organisms present on the leaf surface were identified as those belonging to the genus *Aspergillus* and *Penicillium* (personal communication, S. Coertze, Department of Plant Pathology, Stellenbosch University). Confirmation of fungal presence on the leaves of *M. flabellifolia* suggested that further analysis be undertaken on the reproductive organs to ascertain if fungi were associated with these structures also.

Scanning electron microscopy of hydrated female flowers revealed similar surface morphology to that observed previously

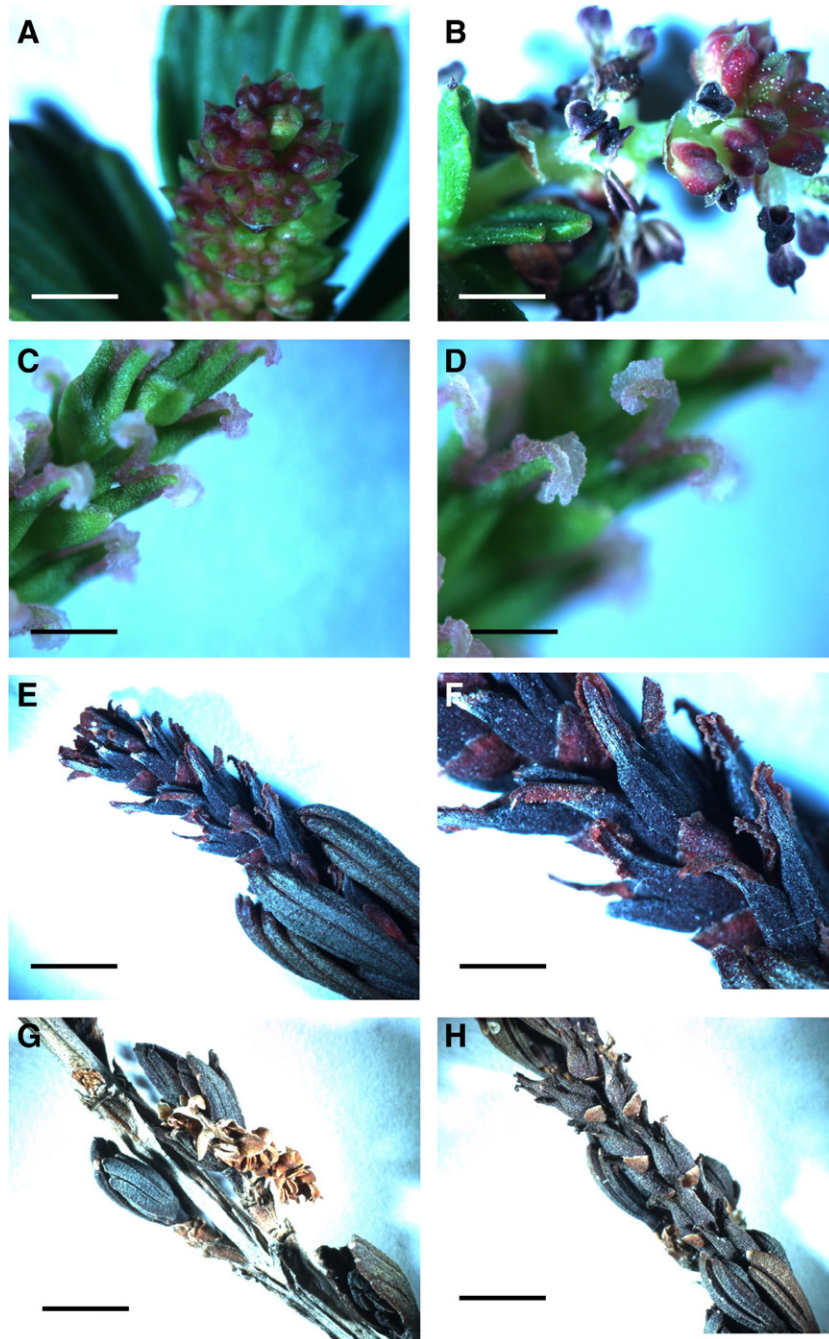


Fig. 1. High magnification photographs of male (A, B, and G) and female (C, D, E, and F) flowers of the resurrection plant, *Myrothamnus flabellifolia*. Fig. 1A–D is hydrated whereas Fig. 1E–H is in the desiccated state. Fig. 1A and E shows leaves in addition to flowers. Fig. 1H represents desiccated seed-containing capsules. Scale bars: a, b=2 mm, c=1 mm, d=0.5 mm, e=3 mm, f=1 mm, g, h=10 mm.

with hydrated leaves (Moore et al., 2007b). The abaxial epidermis consists of gland cells, ordinary epidermal cells, stomata and a wax coating (Fig. 3A). The stigmatic surface previously observed as a pinkish feathery structure was found to be comprised of numerous papillae (Fig. 3B). Each papilla consisted of a bulbous basal part and a tubular projection that presumably acted as an attachment site for pollen grains (Fig. 3B). Observation of the stigmatic surface of desiccated flowers revealed no gross morphological differences other than tissue compaction due to water loss (Fig. 3C). Closer examination however revealed that the bulbous basal parts of the papillae had collapsed due to water

loss resulting in a flattening of the tubular projections (Fig. 3D). Examination of a number of desiccated female flowers revealed that some of the specimens showed similar fungal contamination to that found for the leaves (Fig. 3E). Specifically the desiccated stigmatic surface appeared covered with these fungi (Fig. 3F). Higher magnification of the infected stigmatic surface of desiccated flowers (not shown) revealed similar rod shaped microbes and fungal hyphae as that observed previously at the apices of desiccated leaves (Fig. 2C). By contrast, no fungal contamination was observed on the male desiccated and hydrated male flowers studied (not shown). This suggested that contamination appeared to

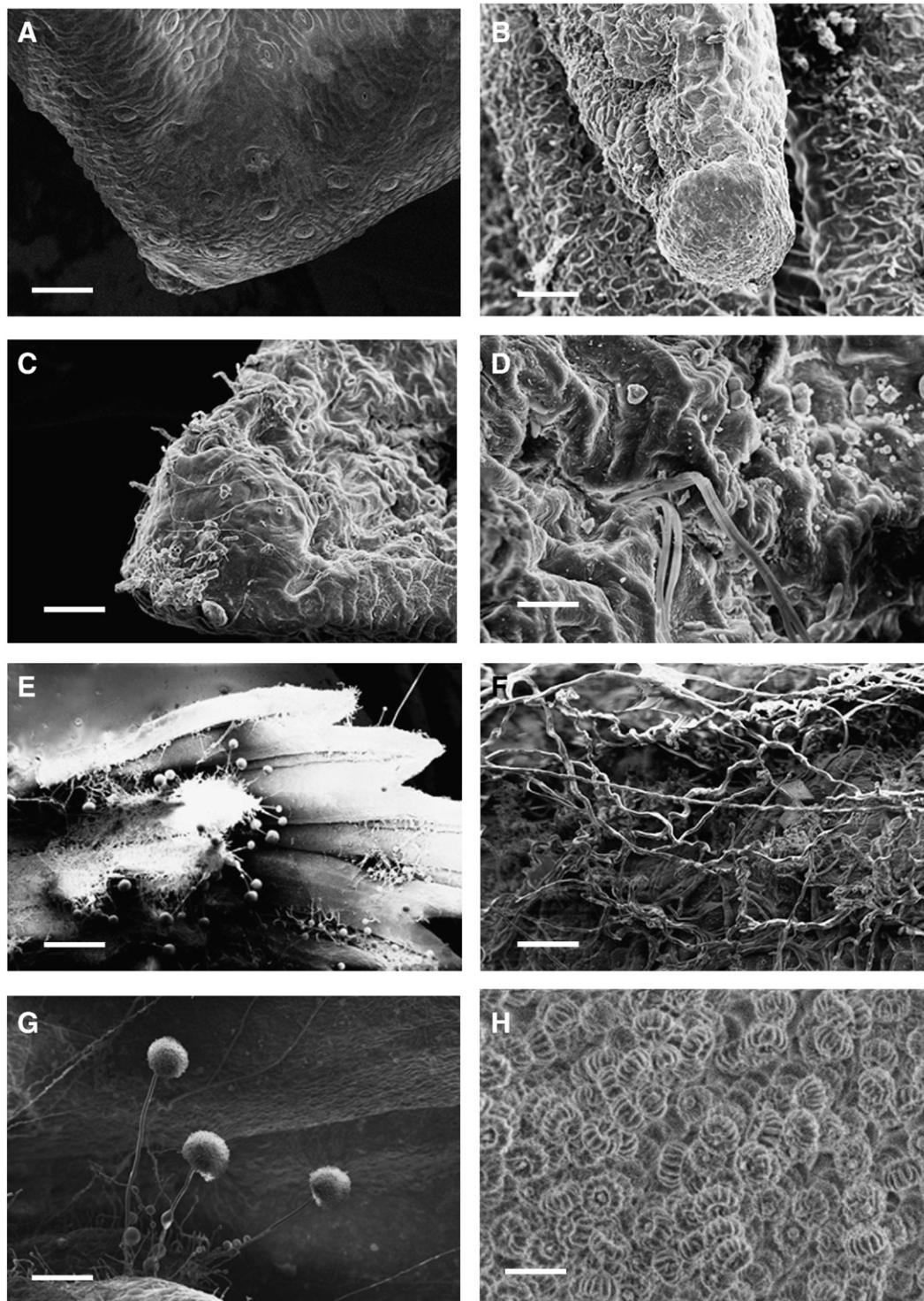


Fig. 2. Scanning electron micrographs of the hydathodes (present on the structures in micrographs A–D) and demonstrating fungal growth at the leaf apices (C–E) and leaf surfaces (F–H) of the resurrection plant *Myrothamnus flabellifolia*. Fig. 2H represents fungal spores. Arrow in Fig. 2B indicates a salt deposit. Scale bars: a=40 μ m, b=80 μ m, c=40 μ m, d=10 μ m, e=300 μ m, f=40 μ m, g=100 μ m, and h=6 μ m.

be present at specific locations on the leaf and female flower surfaces.

During the incubation of leaf and flower segments on nutrient agar it was noticed in addition to fungal growth previously characterized that their also repeatedly occurred growth of a waxy like bacterial film on the agar surface emanating from the

specimen. Scanning electron microscopy of the bacterial culture revealed a multicellular mycelium composed of a waxy sheen (Fig. 4A, individual cells not visible). Individual bacteria, resembling those found in the bacterial culture, were observed on the leaf surface particularly associated with wax-like structures such as leaf ribs or stomata wax-like lips (Fig. 4B; Moore et al.,

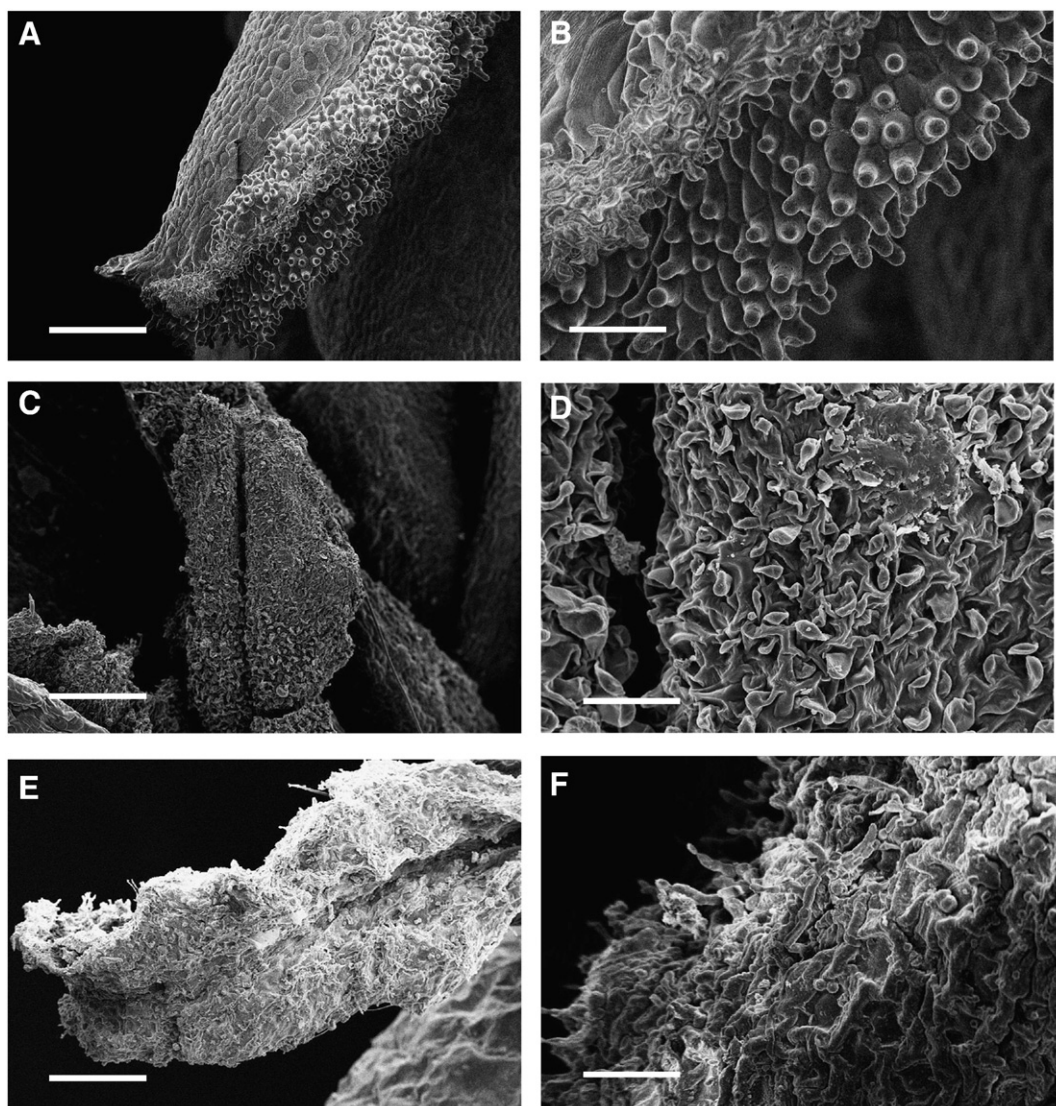


Fig. 3. Scanning electron micrographs of hydrated (A, B), desiccated (C, D) and fungi (E, F) infected stigmatic surfaces of female flowers in the resurrection plant *Myrothamnus flabellifolia*. Scale bars: a=90 μm , b=30 μm , c=100 μm , d=30 μm , e=90 μm , and f=20 μm .

2007b). Isolated bacterial cultures were identified as belonging to the genus *Bacillus* (personal communication, M. Le Roes, Department of Molecular and Cell Biology, University of Cape Town). Wax appears to be deposited liberally around stomata (Fig. 4C) and gland cells (Fig. 4D) present on or near the leaf ribs. Plant wax has been documented to occur in a variety of morphological forms, from plates and globules to crystals and rods (see Cutler et al., 1981). Plant wax has even been characterized as filaments (Juniper and Jeffree, 1983) and occurs within the leaf structure between cells (see Cutler et al., 1981) as documented in this paper. These wax-coated ribs become exposed to the environment during desiccation and have been suggested to function to reflect light away from the leaf surface as well as facilitate water run-off during the rainy season (Moore et al., 2007b). Wax-like depositions present on the leaf surface may be similarly associated with the ‘essential oil’ secreting gland cells (Moore et al., 2007b) as well as the lipid linings present in the vascular tissue of the stem (Moore et al., 2007a). The main composition of waxes is highly variable but usually includes fatty

acids, alkyl esters, primary and secondary lipid alcohols, alkanes, and ketones, and it is noteworthy that many of these components occur in essential oil secretions (Ernst and Samuels, 2009; Juniper and Jeffree, 1983; Kunst and Samuels, 2003). Scanning electron microscopy of cross-sections of woody stem segments of *M. flabellifolia* revealed wax-like deposits particularly located towards the centre of the organ (Fig. 4E). Higher magnification views of the stem cross-section revealed lipid-like globules emanating from what appears to be the pith (parenchyma) of the stem (Fig. 4F). Finally analysis of cross-sections of hydrated and desiccated leaf segments was undertaken to determine whether bacterial or fungal contamination was present within these structures. Cross-sections of hydrated leaf segments revealed wax-like filaments and wax-like globules present on the internal surface of both palisade (Fig. 4G) and spongy mesophyll (Fig. 4H) cells as described previously (Moore et al., 2007b). Similar wax-like arrangements were observed for desiccated leaf tissue (not shown). No microbial contamination was detected within the hydrated or desiccated leaves analysed.

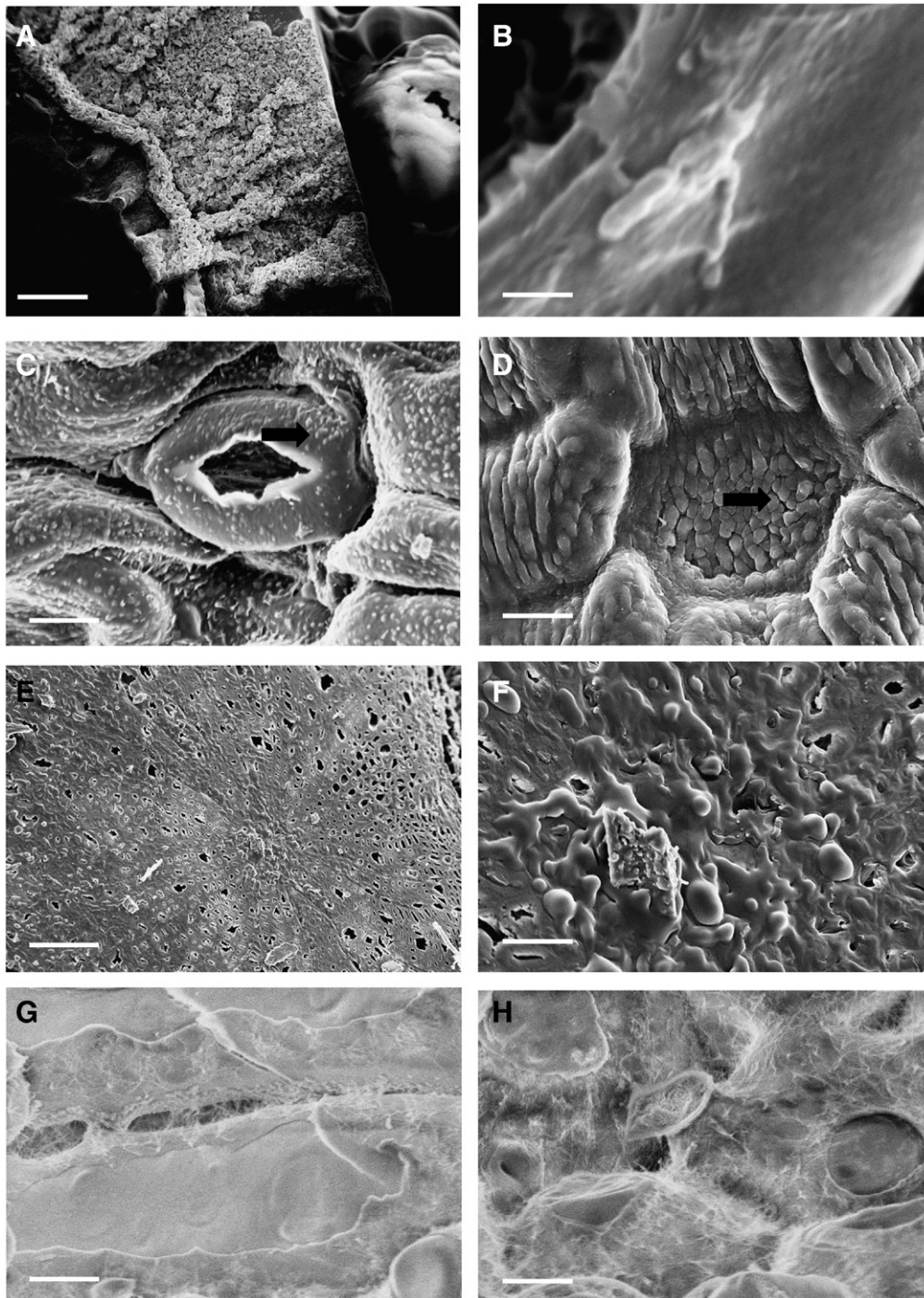


Fig. 4. Scanning electron micrographs of bacteria (wax adhering) (A, B) and wax structures (C, D) present on leaves of the resurrection plant *Myrothamnus flabellifolia*. Arrows in Fig. 4C and D represent wax deposits. Lipid-like globules emanating from the pith (E, F) and wax-like structures (G, H) present within the leaves of *Myrothamnus flabellifolia*. Arrow in Fig. 4D indicates a wax particle. Scale bars: a=400 μm , b=0.4 μm , c=5 μm , d=10 μm , e=30 μm , f=10 μm , g, h=5 μm .

4. Discussion

Fungal contamination is a serious concern of seed scientists where desiccated embryos are vulnerable to parasitic and saprophytic infection significantly reducing seed lot viability. Similarly, in xerophytic plants, such as was recently discovered for the Namib Desert plant *Welwitschia mirabilis*, fungi (*Aspergillus* species) were found associated with the reproductive organs thus possibly threatening the survival of this already threatened species

(Whitaker et al., 2008). In the case of *M. flabellifolia* it appears that the fungi (of the genera *Aspergillus* and *Penicillium*) appear to be associated with the hydathode structures on the leaves (Drennan et al., 1993; Moore et al., 2007b) and at the stigmatic surfaces of the female flowers. These structures are likely to produce aqueous solutions consisting of nutrient rich salts (at the hydathode apices) and sugary exudate (on the stigmatic surface) which facilitates fungal growth at these surfaces. There is no indication that the fungi are desiccation tolerant and so they most probably produce

desiccation-tolerant spores after growth. They appear opportunistic and most likely grow and reproduce during the summer rains when humidity is high and thereafter remain quiescent during the dry winter months in parallel with the plant life-cycle (Farrant and Kruger, 2001). There is no indication whether these fungi interfere with the reproductive process but this may be of concern regarding the slow growing nature of this species. With regards to the waxy bacteria, these appear to be *Bacillus* species which attach to the waxy deposits on the plant epidermis where they similarly may grow only when sufficient humidity is available. They may utilize these waxy layers as a source of nutrients such as reduced carbon for growth. Interestingly, no fungi appeared to be associated with these waxy surfaces where the bacteria were found. Growth of both fungi and bacteria on nutrient agar showed a similar separation of growth and it appeared that the bacteria may be producing an anti-fungal agent secreted into the medium (personal observation, J.P. Moore). Thus the bacteria may limit fungal growth on the leaf surface to areas such as the hydathodes (Drennan et al., 1992) or stigmatic surfaces where wax is not present. Similarly, the lipid lining of the stem as well as the wax filaments and lipid globules coating the internal ultrastructure of the leaves show no fungal contamination. These structures have been previously suggested to facilitate water transport during the dehydration and rehydration phases of the *M. flabellifolia* life-cycle (Moore et al., 2007b; Schneider et al., 2003; Wilson and Drennan, 1992). A further role can now be envisaged where these lipid structures function to limit invasive fungal growth into the plants leaves, stems and flowers during the period that the plant is dehydrating and/or desiccated. This gains support from the observation that essential oils from this species possess marked antimicrobial activity against a range of microbial strains (Chagonda et al., 1999; Da Cunha and De Lurdes Rodriguez Roque, 1974; Viljoen et al., 2002). It has been previously suggested that fungal association may be responsible for the observation of trehalose presence in the leaves of *M. flabellifolia* which has been reported to increase upon desiccation (Bianchi et al., 1993; Drennan et al., 1993; Moore et al., 2007b). This, however, is unlikely as the amount of fungal contamination on both desiccated and hydrated leaves would be insufficient in relation to the mass of leaves analysed to make any significant contribution, particularly as the trehalose measured was of a similar concentration to sucrose which is a known desiccation-induced osmoticum in resurrection plants (Farrant, 2000; Moore et al., 2007a). Only by artificially inducing significant fungal growth (as shown previously) by incubating plants in a humid atmosphere could sufficient biomass be generated. Hence, the role these fungi and bacteria may play in relation to the desiccation-associated biology of this plant remain unclear. However, understanding the relationship between *M. flabellifolia* and its associated microflora is of importance in elucidating the ecology of this remarkable southern African resurrection plant.

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