

THE LIFE CYCLE OF DRAGON FRUIT CANKER CAUSED BY *NEOSCYTALIDIUM DIMIDIATUM* AND IMPLICATIONS FOR CONTROL

R.A. Fullerton¹, P.A. Sutherland¹, R. S. Rebstock¹, Nguyen Thanh Hieu²,
Nguyen Ngoc Anh Thu², Dang Thuy Linh², Ngo Thi Kim Thanh², Nguyen Van Hoa²

¹Plant and Food Research Limited,
Private Mail Bag 92169, Auckland Mail Centre, Auckland 1142

²The Southern Horticultural Research Institute,
Box 203, My Tho, Tien Giang, Vietnam.

ABSTRACT

Dragon fruit canker caused by the fungus *Neoscytalidium dimidiatum* is potentially the most destructive disease of dragon fruit (*Hylocereus* spp.) in Asia. This paper describes the life cycle of *N. dimidiatum* and identifies key features of the life cycle that could be targeted for more efficient control. The fungus infects by the formation of appressoria on the surface followed by direct penetration into an epidermal cell. There is rapid collapse of chlorenchyma cells surrounding the site of infection but minimal colonization of the tissue in the early stages. The host responds to infection by the formation of a cambial layer surrounding the damaged tissue that ‘walls-off’ the damaged tissue which prevents the further development of many infections. Other infections progress to form large spreading lesions with pycnidia embedded at their surfaces. Internally, cells of the hyphae in the dead tissue separate from one another to form phragmospores. Lesion expansion is typically preceded by the formation of conspicuous, large yellow halos in advance of colonization by the fungus. The rapid collapse of tissues surrounding the infection point and extensive halo formation around lesions suggest the production of a diffusible toxin by the pathogen. Chlorosis of cladode tissue following injection with cell free culture filtrates of the fungus provided further evidence of the role of a toxin in the *N. dimidiatum*/dragon fruit pathosystem. Infection studies identified the very young tissues immediately behind the growing point of the cladode as the site most susceptible to infection. The knowledge and life cycle generated by this study has been applied to develop an effective control strategy for the disease.

Key words: Dragon fruit, disease, *Neoscytalidium dimidiatum*, toxin, histology, life cycle, control

INTRODUCTION

Dragon fruit canker, caused by the fungus *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers is potentially the most destructive disease of dragon fruit (*Hylocereus* spp.). Without effective control measures it can destroy the canopy, disfigure fruit and render orchards uneconomic. The pathogen is well known as a human pathogen, for example Khan *et al.* (2009) and Moutran *et al.* (2012), and has been recorded from a range of horticultural tree crops including citrus, mango, and walnut (Polizzi *et al.* 2009; Sakalidis *et al.* 2011; Chen *et al.* 2013; Rolshausen *et al.* 2013; Adesemoye *et al.* 2014; Al-Sadi *et al.* 2014; Chen *et al.* 2014).

N. dimidiatum has been recognized as a pathogen of dragon fruit for a relatively short time. It is only from 2009 onwards that it has been reported to be causing significant damage on dragon fruit throughout South East Asian countries and Israel (Chuang *et al.* 2012; Lan *et al.* 2012; Ezra *et al.* 2013; Alwazani *et al.* 2016). Very little was known of its life cycle on this host.

Field and laboratory observations have shown that, following infection the disease follows a well-defined sequence of symptom expression (Fig. 1).

1. Minute chlorotic depressed spots often with a red fleck at the center.
2. Elevation of the spot center turning red or grey
3. Spot expands with the center forming a hard brown scab with pycnidia embedded in surface
4. Formation of a conspicuous yellow halo around or to one side of the scab and expansion of the lesion into the affected area.
5. Successive phases of expansion leading to large, zonate, grey, scab-like lesions destroying large areas of cladodes.
6. Decay of older lesions often dropping out leaving large shot-holes in the cladode.

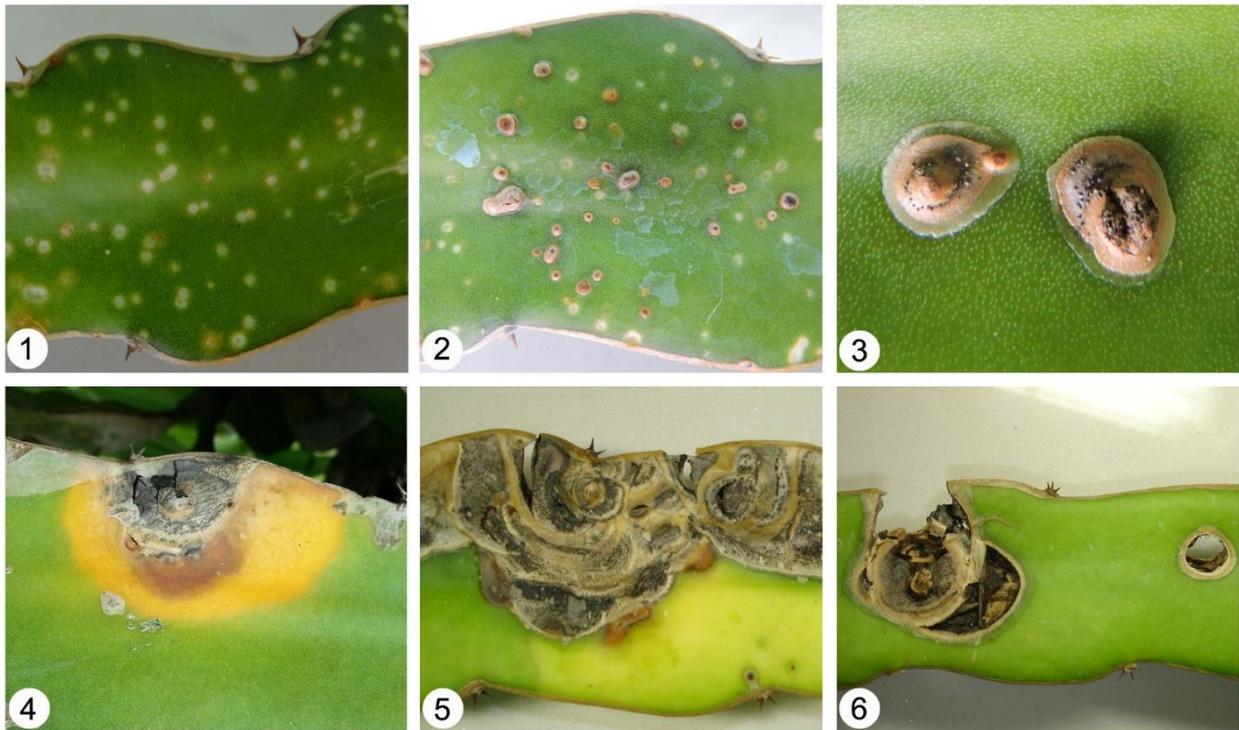


Fig. 1. Stages of symptom development of canker (*Neoscytalidium dimidiatum*) on dragon fruit.

The fungus produces two types of spores, pycniospores formed in ostiolate pycnidia embedded in the surface of mature lesions, and phragmospores formed by the breaking up of individual cells and clusters of cells of mature hyphae in the dead tissues of the lesion (Chuang *et al.* 2012; Lan *et al.* 2012; Mohd *et al.* 2013). In culture only phragmospores are formed and are produced in abundance on the surface of the culture medium.

Effective control of plant diseases typically relies on a detailed knowledge of the life cycle of the pathogen and the targeting of control measures to the most vulnerable stages of the life cycle. To fill the gaps in knowledge of the life cycle of this pathogen, biological and histological studies were made of naturally and artificially infected cladodes to elucidate the key features of infection and disease development. This knowledge was then applied to identify the potentially most effective control strategies.

The presence of large areas of chlorotic tissue surrounding developing lesions suggested the production of a toxin by the invading pathogen. A study was made to provide further evidence of toxin production by the pathogen.

Materials and Methods

Source of infected material. The study was made on naturally infected cladodes from the field and on artificially inoculated cladodes in the laboratory.

Artificial inoculations were made both on mature cladodes and on rapidly growing juvenile cladodes. Phragmospores were produced by inoculating Petri dishes of Potato Dextrose Agar (PDA) with blocks (c. 5x5 mm) of pure culture of *N. dimidiatum* and incubating at ambient temperature (c. 30°C) for approximately seven days. The spores were washed from the surface of the plates with sterile water and the suspension diluted to provide a concentration of approximately 1×10^6 colony forming units per ml.

Inoculations of mature cladodes were made on segments of cladode in plastic containers with wet tissue in the bottom. The spore suspension was misted onto the cladodes using a hand operated trigger sprayer to deliver an even coverage of small droplets. The cladode was allowed to air dry to allow the phragmospores to adhere to the cuticle then lightly misted again with sterile water. Each cladode was also misted daily to maintain a film of free water on the surface and the cladodes held in the laboratory until symptoms developed.

Inoculations were also made on rapidly growing juvenile cladodes (approximately 15 cm) growing on potted cuttings. After spray inoculation the plant was allowed to dry to adhere the spores to the epidermis then

lightly misted again with water. The inoculated plants were placed a plastic chamber humidified with a domestic room humidifier and held at ambient temperature (25 - 30°C) for 24 hours. They were then removed to a shade house and observed for symptom development. Being a modified stem, cladodes grow from a terminal growing point with tissue elongation and differentiation behind the growing point. In order to be able to differentiate between the area of the cladode that was inoculated and new cladode tissue formed after inoculation, a line was drawn with permanent marker pen approximately 10 mm below the tip of the cladode. After incubation the line provided a reference point for observing where in the new cladode the symptoms developed.

Microscopy methods. Samples were taken at different stages of symptom development. For observations of early-stage symptoms, the spot, together with a margin of healthy tissue of 4-5 mm², was excised with a scalpel. For more advanced lesions pieces of tissue spanning the necrotic lesion and adjacent healthy tissue were taken. Samples were either hand sectioned as fresh material and examined microscopically, or fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for processing for either light microscopy and scanning electron microscopy.

For light microscopy, small pieces of fixed tissue were transferred to fresh fixative and held under vacuum for 1 h. Tissue was then washed in buffer, dehydrated in an ethanol series and embedded in LR White Resin (London Resin Co, Reading UK). Structural observations were carried out on 1 µm thick section of resin-embedded material, stained in 0.5% toluidine blue in 0.1% aqueous sodium carbonate (pH 11.1), dried, and mounted in SHUR/Mount™ (Triangle Biomedical Sciences, Durham, NC). Sections were viewed using an Olympus Vanox AHB3 microscope (Olympus Optical Co Ltd., Tokyo, Japan).

For Scanning Electron Microscopy (SEM), tissue was placed in fresh fixative, held under vacuum for 1 h, washed in buffer and dehydrated in an ethanol series. It was then critical-point dried from CO₂ in a Baltec CPD030 critical-point drier (BalTec AG, Balzers, Lichtenstein). Samples were mounted on stubs, coated with gold and observed in a FEI Quanta 250 scanning electron microscope (FEI Company, Hillsboro, OR).

Isolation of the pathogen from diseased tissue. To check the location of the pathogen in the diseased tissue, isolations were made from lesions at different stages of development, and from the chlorotic zones surrounding the scabs. The surface of the affected tissue was swabbed with 95% ethanol to minimise surface contaminants. The epidermis of the lesion or halo was removed and small portions of tissue excised and transferred to Petri dishes of PDA containing 100 ppm each of penicillin and streptomycin to suppress bacterial contamination. The Petri dishes were incubated under ambient laboratory conditions of approximately 30°C.

Toxin production. Flasks (250 ml) of Potato Dextrose Broth were inoculated with blocks of pure culture and incubated on a shaker for approximately seven days at ambient temperature. The suspension was then strained through fine terylene cloth to remove most of hyphal material and the filtrate centrifuged to remove phragmospores and remnants of hyphae. The clear supernatant was decanted and passed through a sterile microfilter (pore size 0.2 µm) into a sterile container. Sterility of the filtrate was checked by spreading 20 µL onto the surface of Potato Dextrose Agar (PDA) and observing for microbial growth in succeeding days. The sterile filtrate was injected to a depth of approximately 2 mm below the epidermis of a mature cladode using a sterile 1ml insulin syringe. Negative controls using fresh sterile PDB and sterile water were included, as well as a positive control of unfiltered shake culture of the pathogen. The injection sites were marked on the cladodes and the plants observed over the following five days for symptom development.

OBSERVATIONS AND RESULTS

Symptom development

Mature cladodes. Symptoms were very slow to appear on inoculated mature cladodes. The first chlorotic spots appeared approximately 20 days after inoculation and slowly developed to brown lesions. Many of the inoculated cladodes rotted from other organisms over that time because of the high humidity conditions in the containers.

Juvenile cladodes. The first signs of infection on the inoculated plants appeared approximately five days after inoculation as minute depressions (dimples) on the surface of the cladode. Within 24 hours a minute red spot had formed in the centre of most dimples and affected areas became chlorotic to form clusters of small yellow spots (chlorotic flecks) over the surface, some with red spots in the centre (Stage 1 lesion). The majority of chlorotic flecks formed above the reference line drawn behind the growing point at the time of inoculation. This indicated that most of the infections had occurred on the very young tissue in the immediate vicinity of the growing point and the symptoms were expressing on cladode tissue that had elongated and matured after inoculation.

Individual chlorotic spots changed from yellow to red or brown and slightly raised (Stage 2 lesion) later becoming hard brown scabs, They eventually progressed to typical, light grey lesions with numerous dark spots,

the pycnidia visible on the surface (Stage 3 lesion) later producing chlorotic halos (Stage 4 lesion) and spreading cankers (Stage 5 lesion).

Microscopic observations of epidermal strips five days after inoculation showed that infection was initiated via dark appressoria formed at the tips of surface hyphae growing from germinating phragmospores (Fig. 2). The fungus penetrated directly through the cuticle and established within the epidermal cell below (Fig. 3). Infected cells responded by turning reddish-brown forming the tiny red spots visible to the eye in the centre of the chlorotic flecks. In some cases chlorotic flecks were observed beneath clusters of germinating spores without appressoria being formed.



Fig. 2. Appressoria of *Neoscytalidium dimidiatum* on the surface of mature cladode of dragon fruit.

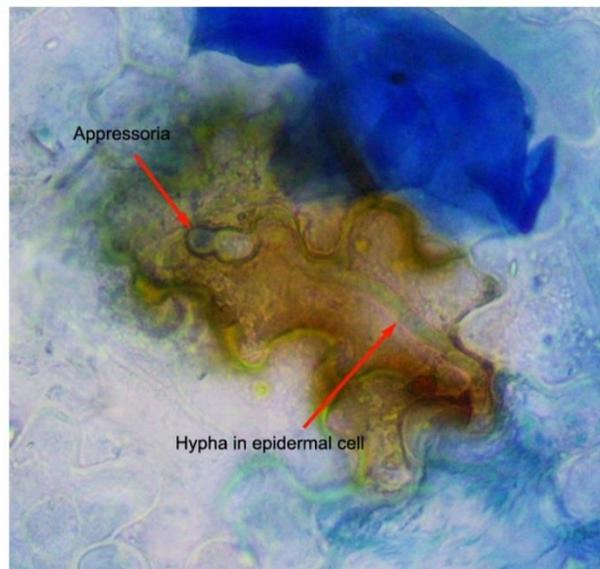


Fig. 3. Appressorium and infection hypha of *Neoscytalidium dimidiatum* on a juvenile dragon fruit cladode.

Internally, the first tissue change, corresponding with yellow depressed flecks on the surface, was the clearing of contents from the mesophyll cells below and around the point of infection (Fig. 4). This was followed by the softening and dissolution of the cell walls resulting in the formation of cavities and the collapse

of the tissue. During that phase, hyphae were either absent in the tissue or, when present, were sparse and confined to the epidermis and adjacent cells.

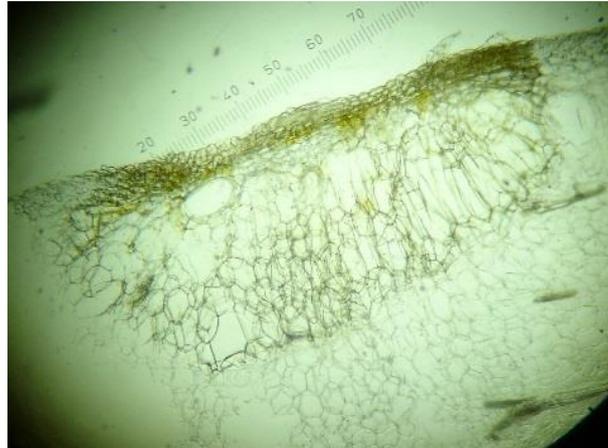


Fig. 4. Cross-section of the first stage of infection of a dragon fruit cladode by *Neoscytalidium dimidiatum* showing tissue disruption beneath and around the infection point.

Observations by SEM showed that as the lesions developed they also became elevated above the surface of the cladode (Fig. 5). Light microscopy showed that to be the result of the formation of a cambial layer and the generation of new tissue in the mesophyll below the damaged tissue. The walls of the layer of cells immediately adjacent to the diseased tissue became thickened and stained readily with Toluidine blue similar to normal epidermal cells (Fig. 6). That process resulted in the diseased tissue becoming physically walled off from the surrounding healthy tissue. At that stage the whole scab could be lifted out of the cladode leaving a clean crater lined with healthy cells.



Fig. 5. Scanning electron images of three stages of early symptom development of canker (*Neoscytalidium dimidiatum*) on dragon fruit. A. First stage depressed yellow fleck. B. Raising of infected site by tissue proliferation beneath infected area. C. Early scab partially lifted from cladode and pycnidia forming on surface.

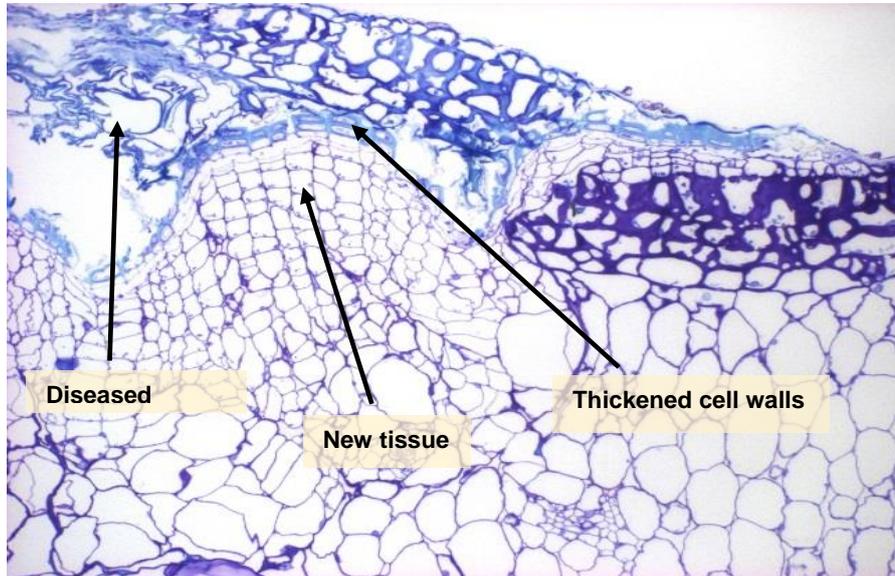


Fig. 6. Cross section of edge of lesion showing formation of new tissue beneath diseased tissue and thickening of cell walls at interface of healthy and diseased tissue.

Observations on artificially inoculated cladodes and on cladodes naturally infected in the field showed that not all lesions develop at the same rate. Some remain at the chlorotic fleck, red spot or early brown scab stages. Others progress directly to the grey scab stage with pycnidia at the surface. A number of lesions at the grey scab stage subsequently develop a narrow reddish or brown margin which expands outwards into a conspicuous yellow halo around the lesion (Stage 4). The halos later dry out to a firm grey scab with pycnidia on the surface. Successive phases of expansion result in the formation of large zonate cankers destroying significant areas of cladode (Stage 5). Pycniospores are extruded in masses from the pycnidia to the surface of the lesion and available for dispersal by rain splash (Fig. 7).

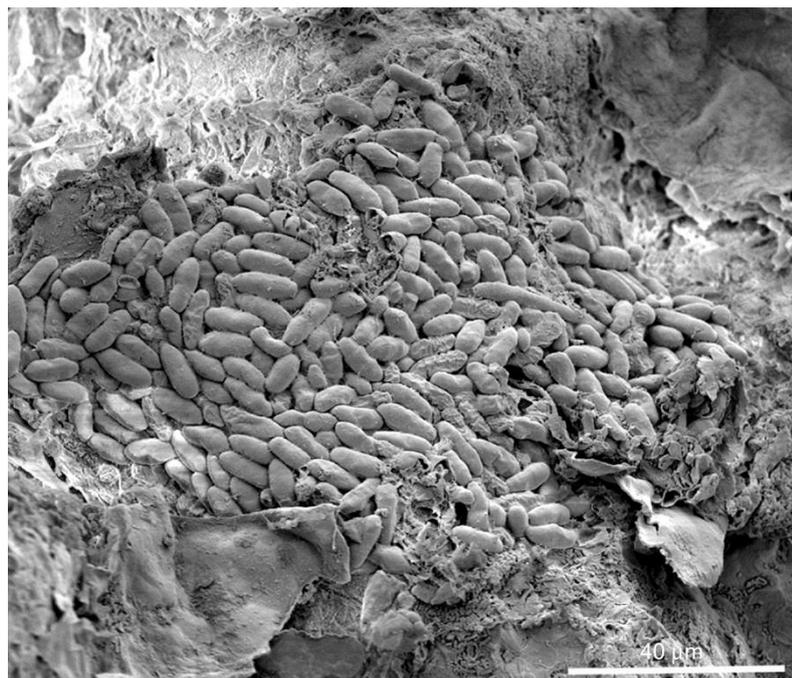


Fig. 7. Scanning electron microscope image of pycniospores of *Neoscytalidium dimidiatum* on the surface of a canker lesion.

The dead tissue of many older lesions drops out leaving large holes ('shot holes') in the cladode (Stage 6 lesion). The edges of the shot holes are lined with a mat of dark hyphae and phragmospores which remain viable for many months. In naturally infected cladodes in the field it is common to find symptoms confined to a well-defined band on the cladode with healthy cladode above and below. In those bands it is possible to find all stages of symptom development in close proximity.

The progression of the disease from the first chlorotic symptoms to the formation of pycnidia and the discharge of pycniospores is relatively slow. It can take over two weeks to progress from yellow fleck to the early scab stage and a further three weeks for pycnidia to be formed. It is only after that stage that some lesions advance rapidly from their margins by the formation of yellow halos

Location of the pathogen in lesions. Light microscopy at different stages of lesion development showed that initially mycelium is sparse and confined to the cells close to the epidermis. As the lesions progress to the brown scab stage hyphae proliferate in the dead tissue (which is often cracked), pycnidia develop and pycniospores are produced. The hyphae within the dead tissue become dark walled and individual cells disassociated as phragmospores (Fig. 8).

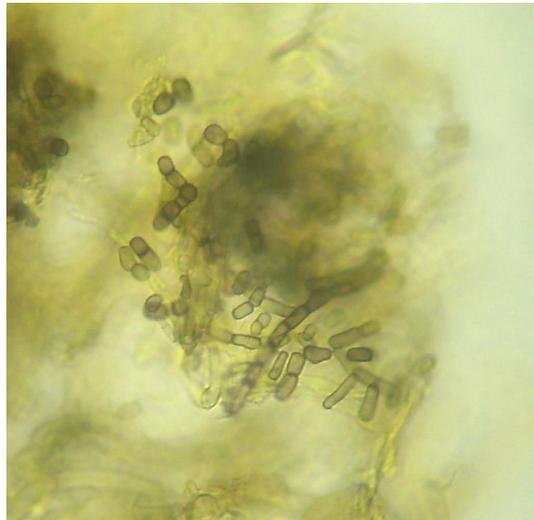


Fig. 8. Phragmospores of *Neoscytalidium dimidiatum* in tissue of mature lesion.

When isolations were made from the yellow halos at different distances from the margin of the lesion, the pathogen was consistently isolated close to the lesion margin, but not from the outer edge of the halo. Microscopic observation of the interface between halo and healthy tissue showed loss of cellular structure in the halo affected tissue but no hyphae. A cambial layer was formed at the margin of healthy tissue and the walls of cell adjoining the diseased halo were thickened in a similar way to that seen at the interface of healthy and diseased tissue in developing lesions (Fig. 9).

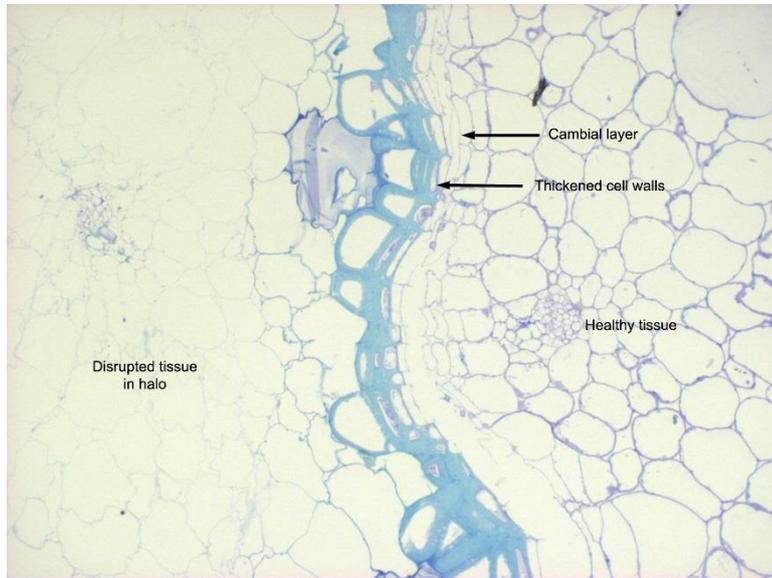


Fig. 9. Cross-section of interface of yellow halo (left) and healthy tissue (right) showing disrupted tissue in halo and cambium and thickened cell walls at interface.

Phytotoxin assay

When sterile crude culture filtrate was injected into healthy cladodes, the area surrounding the injection point turned chlorotic within 48 hours. No symptoms developed in cladodes injected with either sterile culture medium or sterile water. When the unfiltered fungal suspension was injected into the plant the site of injection rapidly became chlorotic followed by the death of the tissues and ultimately the production of a typical grey canker at the site of injection.

DISCUSSION

The infection process of *N. dimidiatum* involves the formation of an appressorium, direct penetration of the cuticle and colonisation of the underlying epidermal cell. The rapid clearing of cytoplasm, the softening of walls of cells, and collapse of tissue around and below the infection point in the absence of hyphae in the tissue, is consistent with a toxin having a role in pathogenesis. Similarly, the loss of cellular structure in the tissues of the yellow halos in the absence of hyphae suggests a diffusible toxin killing host tissue in advance of the pathogen. The ability of cell free culture filtrates to induce chlorosis in healthy cladodes provided further evidence of toxin production by the fungus. These observations suggest that toxin production is an important feature of the pathogenicity of *N. dimidiatum* on dragonfruit. The evidence to date suggests that once infection is established *N. dimidiatum* behaves primarily as necrotroph, producing a diffusible toxin which is able to overcome successive physical barriers produced as a resistance response, and killing tissues in advance of colonisation. This study of the life cycle has revealed several features of this disease which can be exploited for more efficient control.

N. dimidiatum commonly produces two types of spores in lesions, phragmospores formed by the disassociation of hyphal cells in the dead tissue, and pycniospores produced in shallow pycnidia just below the surface of younger cankers. Because the phragmospores are trapped within the hard necrotic tissues of the lesion and not readily available for dispersal, their role in the epidemiology of canker is probably limited.

Pycniospores are the most important means of dispersal and infection. They are released from pycnidia during wet weather and spread by rain splash. Thus canker is primarily a 'wet weather' disease. Dispersal and

infection can occur during any wet period of the year will be much more severe during the wet season when infection conditions occur very frequently

This study has shown that the tissue most susceptible to infection were the tips of rapidly growing cladodes. Few lesions were formed on tissue more than 3 cm below the growing tip at the time of inoculation. This site-specificity for infection can explain the discrete bands of symptoms often observed on mature cladodes in the field, with each band of symptoms the result of infection at the growing point from a specific infection period during cladode growth. Thus, the highest risk of infection in the orchard is during wet periods when plants are making maximum vegetative growth. There will be very few new infections, even during wet periods, if cladodes are not in a growth flush. Old mature cladodes are highly resistant to infection.

This new understanding of the life cycle of the canker pathogen allows us to identify the key steps to controlling the disease. They are:

Orchard hygiene. The elimination of sources of spores in the orchard is essential for successful control. The symptoms of canker are very obvious and infected cladodes can easily be located. Cankers can be cut out individually or, in the case of severe infection, the whole cladode can be removed. The extended period of up to five weeks between first symptoms and sporulation on new lesions provides an opportunity to prevent the pathogen from reproducing by physically removing young lesions from the cladodes. The cladodes of dragon fruit are highly resistant to dehydration. When diseased cladodes are left on the orchard floor or in drains, the lesions can continue to produce spores and infect the young growth. All infected material should be removed from the orchard. Infected cladodes should be shredded and composted for at least four months.

Fungicidal control. Research by staff of the Southern Horticultural Research Institute (SOFRI) on fungicidal control (unpublished data) has identified and field-verified a number of fungicides effective at controlling canker. These include the protectant fungicides mancozeb and copper, and the active ingredients difenoconazole and azoxystrobin which have penetrative (translaminar/curative) activity. Protectant fungicides can only kill the fungus on the surface of the cladode. Fungicides with ‘curative’ activity are able to penetrate into the cladode and kill the fungus after infection has occurred. These different modes of action allow fungicide control programmes to be designed according to weather conditions. During the wet season with numerous infection periods the fungicide programme should be dominated by fungicides with curative activity. During the dry season a programme of protectant fungicides and longer spray intervals could be adopted, potentially applying fungicides only in advance of forecast rain.

The main target for all fungicide applications should be the highly susceptible tips of actively growing cladodes. The application of fungicides to the mature cladodes in the canopy (‘full coverage’) will have no benefit as they are highly resistant to infection.

A combination of rigorous orchard hygiene to remove all sources of spores and a regular spray programme of fungicides with high efficacy against the disease will rapidly reduce the amount of disease in an orchard. At that stage it will be possible to reduce and modify the fungicide programme to reduce costs and the risk of exceeding maximum residue levels in the fruit. Experience at SOFRI has shown it is possible to restore a neglected, highly diseased, unproductive orchard to a healthy highly yielding orchard within 9 months.

CONCLUSION

Canker is a highly destructive disease which, if not controlled, has the potential to destroy orchards. By providing a greater understanding the biology and life cycle of the pathogen this study has delivered new knowledge able to be applied in an integrated control programme for canker based on rigorous hygiene and more efficient fungicide use to not only control the disease, but to potentially eliminate it from the orchard.

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