Disease cycle of *Stagonosporopsis tanaceti* in pyrethrum plants

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Abstract

Pyrethrum (*Tanacetum cinerariifolium*) is commercially cultivated in Australia for the production of pyrethrins. Ray blight caused by *Stagonosporopsis tanaceti* is considered a major limiting factor for pyrethrum production. Histopathology of infected pyrethrum plants identified the importance of crown infection where the infection hyphae were confined to the parenchyma cells of the epidermis, hypodermis and cortex without infecting the vascular tissues. Throughout the growth of pyrethrum plants, no vascular infection occurred in the petioles, flower stems and crowns. In infected leaf petioles and flower stems, pycnidia and extra-cellular anthocyanin material developed within 1-2 weeks of infection. The symptoms of infection included a dark brown discolouration or necrosis of infected cells caused by intra- or intercellular colonisation of hyphae. Secondary infection of leaves, flower stems and flower buds most likely occurred through wind, rain or water splash of pycnidiospores released from pycnidia in the leaves and petioles. Roots of the infected plants remained uninfected throughout the growth of the plants. Detailed understanding of the disease cycle of ray blight disease in pyrethrum plants will enable the development of more efficient, targeted control measures.

Key words: pyrethrum plant, petiole, crown, ray blight, pycnidia

Introduction

The perennial herbaceous pyrethrum plant (*Tanacetum cinerariifolium*) is cultivated in Australia with approximately 3,000 ha produced in Tasmania and Victoria (Bhuiyan et al. 2015). The major purpose of growing pyrethrum plants is to extract insecticidal esters known as pyrethrins. About 94% of pyrethrins are produced in the secretory ducts and oil glands of the seeds and a very minor quantity in leaves, stems and roots (Casida 1980; Zito 1994). Australia currently supplies approximately two thirds of natural pyrethrins worldwide. In Australia, pyrethrum plants are planted in early spring by using a direct seeding method and the first harvest occurs after approximately 15 months. After the harvest in spring/summer, the plants regrow to a rosette growth stage by autumn, where a dense canopy structure is formed and then in the following spring multiple stems are produced. Flowers are produced in the terminus of each lateral stem (Hay et al. 2015).

Pyrethrum production is affected by a complex of foliar diseases such as ray blight caused by *Stagonosporopsis tanaceti* (Vaghefi et al. 2012; Bhuiyan et al. 2015); tan spot caused by *Didymella tanaceti* and *D. rosea* (Pearce et al. 2016); anthracnose caused by *Colletotrichum tanaceti* (Barimani et al. 2013); pink spot caused by *Stemphylium botryosum* (Pethybridge et al. 2004) and winter blight caused by *Alternaria tenuissima* (Pethybridge et al. 2004). Among these diseases, ray blight is considered the most important foliar disease which causes defoliation, less branching and stunting of stems (Pethybridge et al. 2009). Ray blight is commonly found in almost all pyrethrum fields and may cause substantial loss of production. Although this pathogen is considered as a foliar pathogen, severe necrotic lesions are commonly seen in flower stalks with buds producing the typical ‘shepherd crook’ symptom. Rays of flowers also become infected, hence the name ray blight (Pethybridge et al. 2013).

Pethybridge et al. (2008) reported that extensive defoliation due to dying of leaves caused by *S. tanaceti* resulted in stunted plant growth. Bhuiyan et al. (2016) reported that *S. tanaceti* infected the outer layer of pyrethrum seed and was capable of causing pre- and post-emergence death of seedlings.

Stagonosporopsis tanaceti reproduces asexually by forming pycnidia and pseudosclerotia (Vaghefi et al. 2015b; Bhuiyan et al. 2016) and since the sexual stage has yet to be detected, pycniospores have been considered to be the main form of dispersal (Vaghefi et al. 2015a). Management of ray blight depends on minimum use of overhead irrigation to avoid spread of the pathogen, use of disease-free planting material, roguing of diseased plants followed by burning and use of fungicides (Pethybridge et al. 2005). Severely infected crops are usually terminated if no management practices are undertaken (Pethybridge et al. 2008).

Knowledge of the disease cycle of *S. tanaceti* is important for resistance breeding and implementing efficient control measures in pyrethrum plants. Until now, the infection process of *S. tanaceti* has only been studied in artificially inoculated leaf lamina of seedlings and seed (Bhuiyan et al. 2015 & 2016). The aims of this paper were to identify the infection process and colonisation of *S. tanaceti* in mature plant tissues during the growth cycle of pyrethrum plants and to describe the disease cycle of *S. tanaceti*.

Materials and methods

Plant material

Four-week-old pyrethrum seedlings (cultivar BR-1) raised from heat-treated, disease-free seeds were supplied by Botanical Resources Australia – Agricultural Services Pty Ltd. (BRA). Individual seedlings were transplanted to pots (10 cm diameter) with sterilized potting mix (Debco Seed Raising 25 L, Bunnings Warehouse, Australia). Seedlings were

grown at 25°C during day time and 20°C during night time in the glasshouse at the University of Melbourne with 12 h alternating dark and light using incandescent lights. The seedlings were watered twice/ day at 150 mL/ pot at each time. Slow release fertiliser (Osmocote®, Bunnings warehouse, Australia) was added to each pot at 10g/ pot.

Artificial inoculation of petioles and processing for histopathology

Agar blocks (1 cm^2) were cut from the leading hyphal edge of a 10-d-old culture of *S. tanaceti* (Tas-1) using a sterile scalpel. Each agar block was placed onto the middle of a petiole of three-month-old healthy plants then the petiole and inoculum were wrapped with parafilm (Parafilm M Laboratory film, Hach®, Australia). Two petioles from each of three plants were inoculated then each plant was covered with a transparent polybag for 24 h.

After removing the polybag, the plants were grown in the glasshouse at 25°C/ 20°C day/ night temperature with 12 h alternating dark and light using incandescent lights. Both the parafilm and the agar block were removed after 7 d. Then the inoculated petioles were cut from the plant 2 weeks after inoculation (wai) using a sterile scalpel. These petioles were surface sterilized with 1% (ai) sodium hypochlorite for 3 min, followed by rinsing twice with sterile distilled water (SDW) then air dried. Among the six infected petioles, three were sequentially cut into pieces (1 cm/ piece) then placed in serial order onto 1% water agar (WA) in 90 mm Petri dishes and incubated at 20°C for one week. Hyphae that emerged from the petioles were subcultured onto PDA for one week and then cultures were confirmed as *S. tanaceti* based on morphology and culture characteristics as described by Vaghefi et al. (2012). The extent of infection of each petiole was assessed based on the isolation of *S. tanaceti* from the tissue pieces plated onto WA.

The remaining three petioles were processed for histopathology taking 1 cm of tissue from the centre of the inoculation zone; and at 1 and 4 cm above and below the inoculation zone.

After surface sterilization, each tissue piece was cut into half with one half (0.5 cm) cultured as above to identify the presence of *S. tanaceti* and the other half (0.5 cm) processed for histopathology following the procedure described by Bhuiyan et al. (2015). Sectioned tissues were stained using Johansen's quadruple stain. The sectioned tissues were mounted on glass microscope slides then first immersed in safranin for 24 h, followed by rinsing with SDW. The sections were then stained with aqueous methyl violet (1%) for 10-15 min followed by rinsing with SDW and then by a mixture of equal parts of 95% ethanol: methyl cellosolve: tertiary butyl alcohol (TBA) for 15 sec. The sections were immersed in fast green FCF solution for 10 min after which they were briefly rinsed with equal parts of 95% ethanol: TBA: 0.5% glacial acetic acid. Finally, the sections were immersed in orange G for 3 min followed by two washes; firstly with equal parts of clove oil: methyl cellosolve: 95% ethanol and secondly with equal parts of clove oil: 100% ethanol: xylene. The sections were rinsed twice with xylene before mounting with Leica Surgipath Micromount followed by placing glass cover slips over the stained tissues. Stained tissue sections were observed with a light microscope (Leica DM 6000 B) and images captured using a Leica DFC 450 C camera.

Inoculation of three-month-old plants

Spore suspension of *S. tanaceti* (isolate TAS-1) was prepared from 10-d-old cultures, grown on V8 medium following the procedure described by Bhuiyan et al. (2015). The spores were adjusted to 10^6 spores/ mL using a haemocytometer.

Thirty three-month-old healthy plants were inoculated with the spore suspension until just before run-off followed by each plant being covered with a transparent polybag for 48 h. Controls were maintained by spraying 30 three-month-old plants with SDW. Both inoculated and control plants were grown in the glasshouse at 25°C/ 20°C day/ night temperature with 12 h photoperiod using incandescent lights, with a one-metre distance between treatments, for

six months. Then pre-flowering plants were transferred to benches outside the glasshouse during winter to enable vernalisation and exposure to natural environment conditions. Plants had supplementary water through drip irrigation.

Assessment of *S. tanaceti* infection process in inoculated plants

Petioles:

Five petioles from each plant showing dark brown necrosis, light brown necrosis and petioles with no symptoms were selected from plants 12 months after inoculation (mai). Five petioles were also collected from the uninoculated control plants. From each petiole, a 5 cm piece of tissue was cut using a sterile scalpel and immersed in formalin, 5 % acetic acid, 50 % ethanol and 35 % sterile water (FAA) for 12 h, then treated in an ethanol series of 20, 40, 60, 80 and 100% for 10 minutes in each ethanol concentration for tissue clearing (Bhuiyan et al. 2015). Then the cleared tissue pieces were mounted in glycerol and observed under a stereomicroscope (Leica Biosystems; model M 205 FA; Australia). The number of pycnidia per cm^2 of infected area was counted. For histopathology, three 1 cm tissue pieces were randomly cut from each of the dark brown and light brown necrotic regions of inoculated plants and green tissue of control plants followed by surface sterilization. Then half (0.5 cm) of each tissue piece was cultured to confirm the presence of *S. tanaceti* as described above and the remaining half (0.5 cm) was processed for histopathology.

Flower stems:

Fifteen flower stems showing typical ray blight necrosis at the distal end of the flower stems were collected from each of the glasshouse inoculated plants at 12 mai. Fifteen symptomless green flower stems from control plants were also selected. The length of the necrosis was measured and then 10 stems from each source were selected and separately cut into 1 cm pieces using a sterile sharp razor blade in a sequential order starting from the peduncle to 20 cm

downward followed by surface sterilization. After culturing these tissue pieces onto the WA medium then PDA, the extent of *S. tanaceti* infection (in cm) in each infected flower stem was recorded. The incidence of *S. tanaceti* infection in inoculated plants was calculated using the following formula and expressed as percentage.

$$\text{Incidence of infection (\%)} = \frac{\text{Number of } S.\ tanaceti \text{ infected flower stems}}{\text{Total number of flower stems observed}} \times 100$$

Another five stems of each source were processed for histopathology taking 1 cm tissue pieces from the region of necrosis at the distal end at 1 and 5 cm below the necrosis. Then half (0.5 cm) of each tissue piece was cultured to identify *S. tanaceti* infection and the other half (0.5 cm) was processed for histopathology.

Crown tissues:

Crown tissues (4-5 cm tissue between the root and shoot) were collected from each of three inoculated glasshouse plants (12 mai) and uninoculated control plants. Lesions, structural abnormalities and infection structures were recorded using a stereomicroscope. Three to 4 cm pieces of the crown tissues from each of three plants in each treatment were cultured and the remaining 1-2 cm used for histopathology.

Roots:

Five nodal and radicle roots were collected from inoculated glasshouse plants (12 mai) and uninoculated control plants. Tissue pieces (1 cm) from each source were divided equally into two pieces with one half cultured and the other half processed for histopathology.

Flower buds:

Fifteen buds each from healthy and brown discoloured flower stems at maturity stage (FMI 7; Suraweera et al. 2014) were collected from the inoculated glasshouse plants 12 mai. After

surface sterilization, 10 whole buds from each treatment were cultured to confirm the presence of *S. tanaceti*. The incidence of infection was determined as a percentage using the following formula:

$$\text{Incidence of infection (\%)} = \frac{\text{Number of } S.\ tanaceti \text{ infected flower buds}}{\text{Total number of flower buds observed}} \times 100$$

Another five flower buds were aseptically separated into bracts, petals and seeds which were also cultured onto media to determine the incidence of *S. tanaceti* in each flower tissue using the following formula and expressed as percentage:

$$\text{Incidence of infection (\%)} = \frac{\text{Number of } S.\ tanaceti \text{ infected bud tissues (bracts or petals or seeds)}}{\text{Total number of bud tissues (bracts or petals or seeds) observed}} \times 100$$

Infected field-grown plants

In order to study the infection process in plants grown under natural conditions, 15 infected plants were randomly selected from pyrethrum fields at Ballarat, Victoria in December, 2015. Infected plants were determined visually by the presence of the typical 'shepherd crook' necrotic symptom in the flower stems. Each plant was separated into flower stems, crown and roots using a sterile scalpel. Then 15 flower stems were each cut into 1 cm pieces using a sterile razor in a sequential order and each section cultured on 1% WA after surface sterilization. Following a similar process, 15 crown and root tissues were processed for culturing on 1% WA. Identification of *S. tanaceti* and the incidence of infection in these tissues were calculated following the identification technique and equation described above.

Results

Assessment of *S. tanaceti* infection process in inoculated petioles

Mycelial plug inoculation

Two weeks after inoculation, distinct brown lesions formed on all the inoculated petioles but the lesion size was restricted to only 1-2 cm around the inoculation site. Pycnidia were only seen in the upper surface of petioles at the inoculation site. The recovery of *S. tanaceti* was limited to 2-3 cm around the inoculation point with no infection hyphae recovered 4 cm away from the point of inoculation.

Histopathology at the inoculation site showed that infection hyphae penetrated directly into the epidermis then developed pointed to swollen hyphal tips before infecting the epidermal tissues (Fig. 1a). Colonisation of the epidermal, hypodermal and cortical tissues occurred intra- and inter-cellularly. In most cases, middle lamella tissue was colonised by the infection hyphae causing breakdown of the cell walls and the formation of digested cellular material in the infected tissue, resulting in brownish infected tissues. Infection hyphae were unable to penetrate the vascular tissues as the movement of hyphae were obstructed by the sclerenchyma celled bast fibre of the vascular bundles which eventually resulted in hyphal bending (Fig. 1b). Pycnidia developed mostly in the epidermis with the pycnidial wall sometimes extending towards the hypodermis (Fig. 1c).

Spore suspension inoculation

Stagonosporopsis tanaceti was only isolated from the dark and light brown petioles of the inoculated plants. In contrast, no hyphae were recovered from the green tissues of inoculated and control plants. Pycnidia occurred in the epidermal and hypodermal tissues (Fig. 2a) at an average density of 7 (±2) per cm^2. Masses of hyphae colonised the tissues of the epidermis, hypodermis and cortex which eventually degraded, with the vascular tissue completely free from infection hyphae (Fig. 2 b). Meanwhile, non-infected brown coloured tissue had no infection hyphae in the epidermis, hypodermis, cortex but the chloroplasts and cellular materials in these cells were degraded resulting in deformed cell structures. Also, vascular

bundles were intact and free from infection hyphae. Green petioles from both inoculated and control plants were free from infection hyphae.

Infection process by *S. tanaceti* in flower stems

The infected flower stems showed typical 'shepherd crook' symptoms with a constriction and delineation between the necrotic and the healthy tissues (Fig. 3a). The incidence of *S. tanaceti* in symptomatic flower stems was 75% in the samples collected from the field grown plants and 50% in the inoculated plants in the glasshouse at 12 mai. From both sources, *S. tanaceti* was recovered in segmental tissues cultured on PDA from the peduncle to 8-11 cm down the flower stem in which 6-8 cm of the stem showed brown necrotic lesions followed by 2-3 cm of symptomless (mostly green) tissue. Numerous pycnidia (5-10 pycnidia/cm^2) were seen in the necrotic region. However, pycnidia were rarely seen in the symptomless infected tissue. The epidermis and hypodermis, cortex, vascular tissues (except xylem vessels) in the necrotic regions were completely degraded and were replaced by hyphae and extra-cellular material. Meanwhile, only the xylem vessels were intact and free from infection hyphae (Fig. 3b). In contrast, tissues from the symptomless infected region (2-3 cm below the necrotic tissue) were colonised intra-cellularly by infection hyphae with minor degradation of epidermis, hypodermis and cortex. Vascular tissues were completely free from infection hyphae. Meanwhile, none of the tissues from 5-cm below the lesion were infected.

Infection process by *S. tanaceti* in crown tissue

The crown tissue of the inoculated and field-grown plants was surrounded by brown necrotic petiole bases (Fig. 4a) whereas green petiole bases surrounded the crowns of the uninoculated control plants at 12 mai (Fig. 4 b). The incidence of *S. tanaceti* from the crown tissues collected from field-grown and glasshouse inoculated plants was 80 and 70% respectively.

The uninoculated control crown tissues were composed of a single layer parenchyma celled epidermis and hypodermis, and 10 to 12 parenchyma celled cortical tissue followed by vascular elements, then pith tissue at the centre. The infection hyphae first colonised the epidermis (Fig. 4c), then progressed into the hypodermis resulting in degradation of both tissues (Fig. 4d). Then infection hyphae advanced into the cortical tissue (Fig. 4e) but not into the vascular elements and pith tissue (Fig. 4f). Pycnidia were present on the upper layer of almost all the infected crown tissue collected from the field-grown and inoculated pyrethrum plants.

Infection in roots by *S. tanaceti*

The nodal and radicle roots from field-grown, inoculated and control plants were not infected by *S. tanaceti*.

Infection in flower buds by *S. tanaceti*

The incidence of *S. tanaceti* in the brown discoloured flower buds collected from inoculated plants at 12 mai was 50%. The incidence of *S. tanaceti* infection in the bracts, petals and seeds was 50, 40 and 30% respectively. Pycnidia (an average of 3 (±1) pycnidia/cm^2) were also observed in the infected flower parts. However, green healthy flower buds and bud tissues were not infected.

Discussion

Stagonosporopsis tanaceti was found to infect petioles, upper flower stems, crown tissues and flower buds but not roots of pyrethrum plants in both mature field-grown plants and inoculated glasshouse-grown plants. Bhuiyan et al. (2016) also reported that infection of seed

was confined to the seed coat and not in the embryo and that pyrethrum seedlings became infected from infection hyphae in the seed coat infecting the developing hypocotyl of seedlings. Seedlings developed from heat-treated seeds were free from *S. tanaceti* infection.

Infection of *S. tanaceti* in the petioles of 3-month-old plants occurred by direct penetration of infection hyphae into the epidermis and subsequent cell infection was facilitated by hyphae with pointed or swollen hyphal tips. Similar kind of penetration by swollen hyphal tips was reported by Castell-Miller et al. (2007) for *Phoma medicaginis* in alfalfa (*Medicago sativa*). Direct penetration by *S. tanaceti* followed by intra- and intercellular colonisation was also observed by Bhuiyan et al. (2015) in artificially inoculated leaf lamina of pyrethrum seedlings. Colonisation by *S. tanaceti* was confined to the infection site of the petioles with development of localized lesions and pycnidia. The thick wall of bast fibre composed of sclerenchyma cells appeared to prevent infection of the vascular cells. According to Stone et al. (2008), hyphae of the ascomycete *Phaeocryptopus gaeumannii* were confined to the mesophyll tissues and never infected the vascular tissues of Douglas-fir (*Pseudotsuga menziesii* needles.

Numerous pycnidia developed in the infected petioles, leaves, crowns and flower stems of field grown pyrethrum plants. Therefore, it was very likely that the spores released from pycnidia in the lower leaves and petioles were dispersed through wind and water-splash, and then settled onto the flower buds before infection. Spread of spores using similar mode of dispersal is common in *Phoma* and *Leptosphaeria maculans* (Rai 1985; Howlett et al. 2001; Aveskamp et al. 2008). According to Pethybridge et al. (2003) in Australia in 2000 and 2001, there was a high correlation between infected flower stems and buds, and a high number of consecutive days with at least 0.1 mm of rain.

The pyrethrum plants were 3-months-old at inoculation and hence had not yet developed inflorescences however, at 12 mai about 50% of flower buds and the bud tissues were infected by *S. tanaceti*. These plants were exposed to natural rainfall events during vernalisation and subsequent flowering hence the flower buds were likely to have been infected from pycnidiospores splash dispersed from pycnidia at the base of the plants. Infection hyphae were only isolated from the terminus of flower stems. The typical "shepherd crook" symptom at the distal end of the inflorescence was due to necrosis of tissues, where most of the cells except the xylem vessels were completely degraded and replaced by mass of hyphae, pycnidia and extra-cellular anthocyanin like material.

Infected crown tissue plays an important role in the disease cycle of *S. tanaceti*. Spores released from the pycnidia that developed in leaves, petioles and flower stems were likely to have been splash dispersed to the leaves then transferred via water droplets to the crown region. The leaves and petals of pyrethrum plants are covered with hairs (hirsute) that cause water droplets to be repelled. The xylem vessels of the infected crown tissues were not infected and hence there would have been no movement of infection hyphae into the roots and the lower flower stems. The mode of *S. tanaceti* infection in the field-grown flower stems and crown tissues was similar to artificially inoculated plants.

Factors important to establishing a ray blight epidemic were described by Pethybridge et al. (2011) whereby seed transmission was identified as the key risk factor for initiating epidemics. They also described the monocyclic nature of ray blight disease when infection was confined to the seeds; otherwise the disease was polycyclic. In Australia, ray blight disease epidemics have occurred since 1999 where they were correlated with high rainfall during plant development and flowering stage (Pethybridge et al. 2011). Management of this disease in field-grown pyrethrum plants has relied on application of fungicides either as seed

treatment or as foliar sprays (Pethybridge et al. 2011). Nevertheless, ray blight infection still occurred in commercial farmer's fields (Hay et al. 2015). A careful study of the infection process of *S. tanaceti* in pyrethrum plants and secondary spread of inocula within the adjacent plants has improved the knowledge of the disease cycle which will enable the development of more effective control strategies for disease management. Seeds are the primary source of *S. tanaceti* inoculum, hence should be treated as the first step of disease management. Bhuiyan et al. (2016) showed that heat treatment was an efficient method of controlling *S. tanaceti* infection in pyrethrum seed. In field plants, pycnidia formed in leaves, petioles and crown tissues therefore, application of foliar fungicides such as difenoconazole at least twice (early growth stage and pre-flowering stage) in a year is recommended for controlling infection and build-up of inocula (pycnidiospores) before the plants reach the flowering stage. As the vascular tissues of infected pyrethrum plants were free from *S. tanaceti* infection, the application of systemic fungicides would not be efficient to control ray blight disease.

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Figure legends

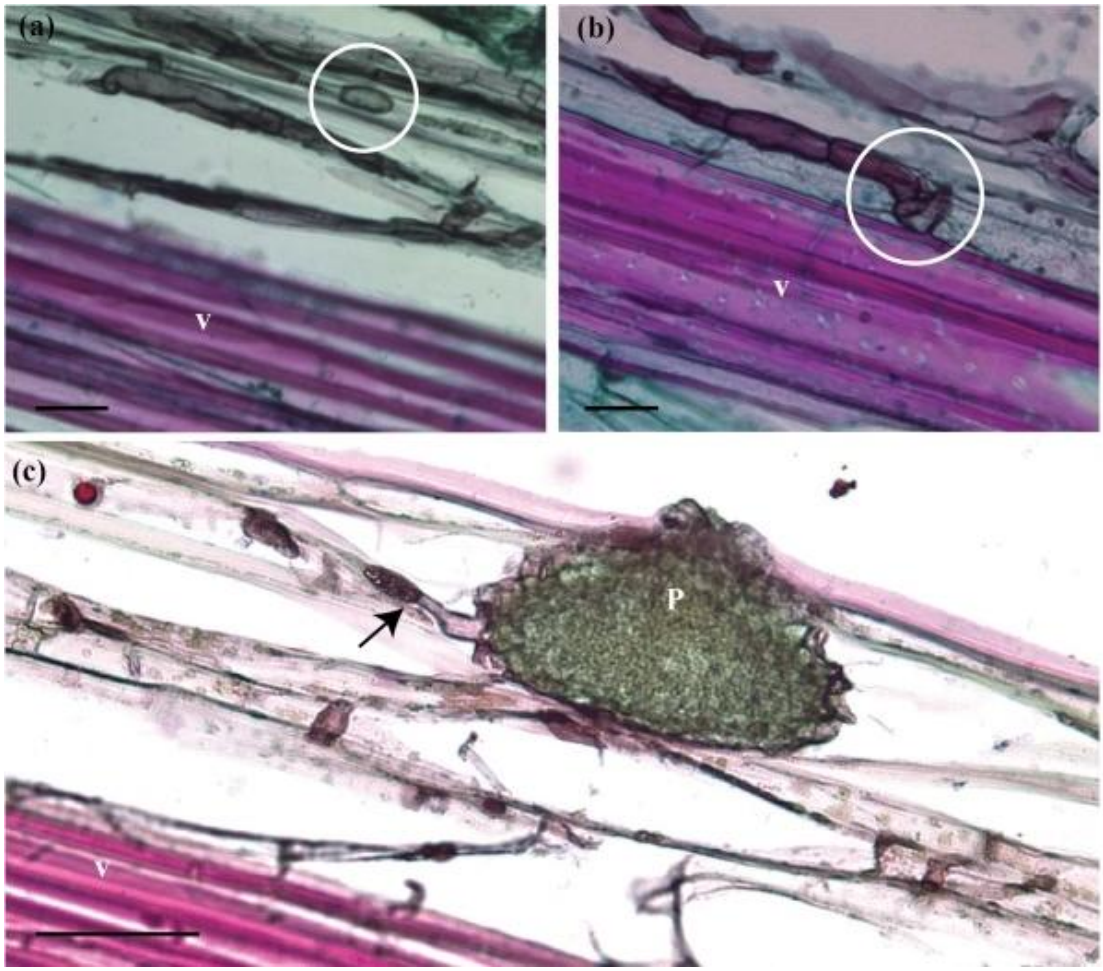

Fig. 1 (a) Infection hypha in the epidermal tissue with swollen hyphal tip (round circle); (b) Bending of hypha tip (round circle) at contact with the sclerenchyma celled bast fibre of the vascular bundle (v); (c) Pycnidium (p) located in the epidermis extended towards the hypodermis; hyphae (black arrow) from the pycnidia wall colonising the adjacent host cell. Scale bar 10 µm.

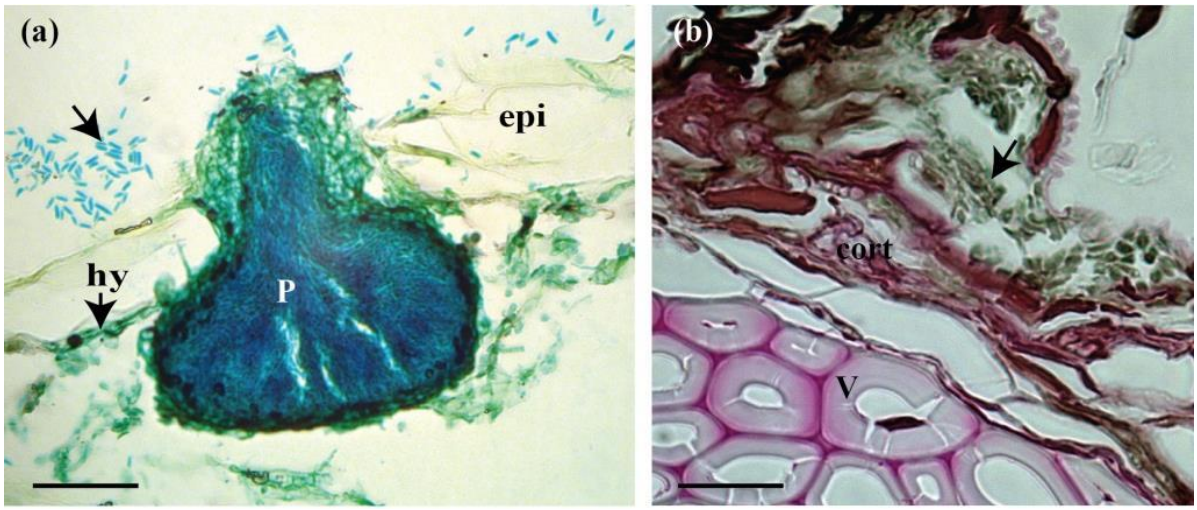

Fig. 2 (a) Pycnidium (P) burst through the epidermis (epi) and released spores (black arrow), hyphae (hy) from the pycnidium wall colonised the adjacent host cells; (b) Infection hyphae (black arrow) mostly colonised the epidermis and hypodermis and to a lesser extent the cortical tissues (cort) resulting in disintegrated epidermis, hypodermis and cortex; vascular tissues (V) were free from infectin hyphae and therefore remained intact. Scale bars in a & b= 10 µm.

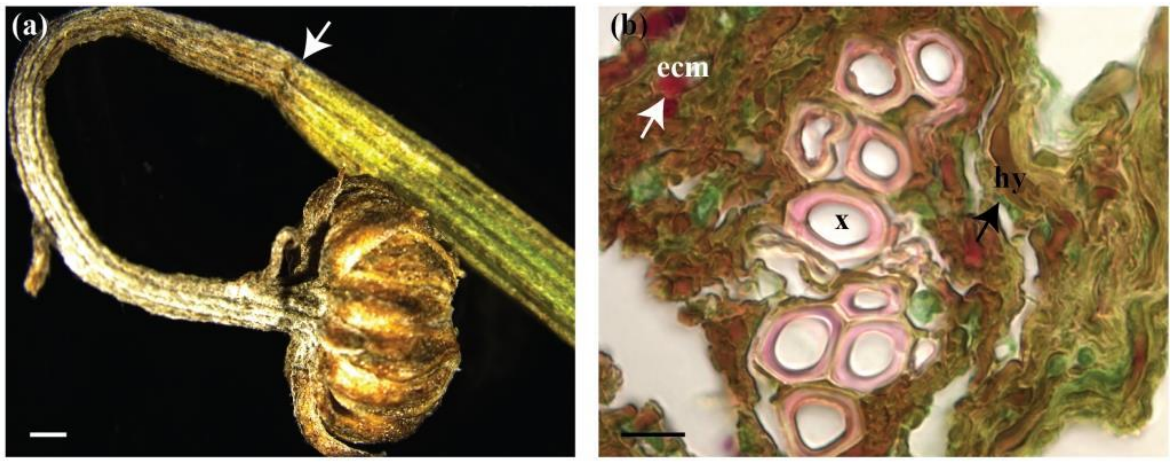

Fig. 3 (a) Infected flower stem showing typical 'shepherd crook' symptom and a constriction (white arrow) at the junction between the necrotic and symptomless infected tissue; (b) In cross-section of the necrotic region, all tissues other than the xylem vessels (x) were completely disintegrated and replaced by infection hyphae (hy; black arrow) and extra-cellular material (ecm; white arrow); Scale bars in a= 1 mm, b = 10 µm.

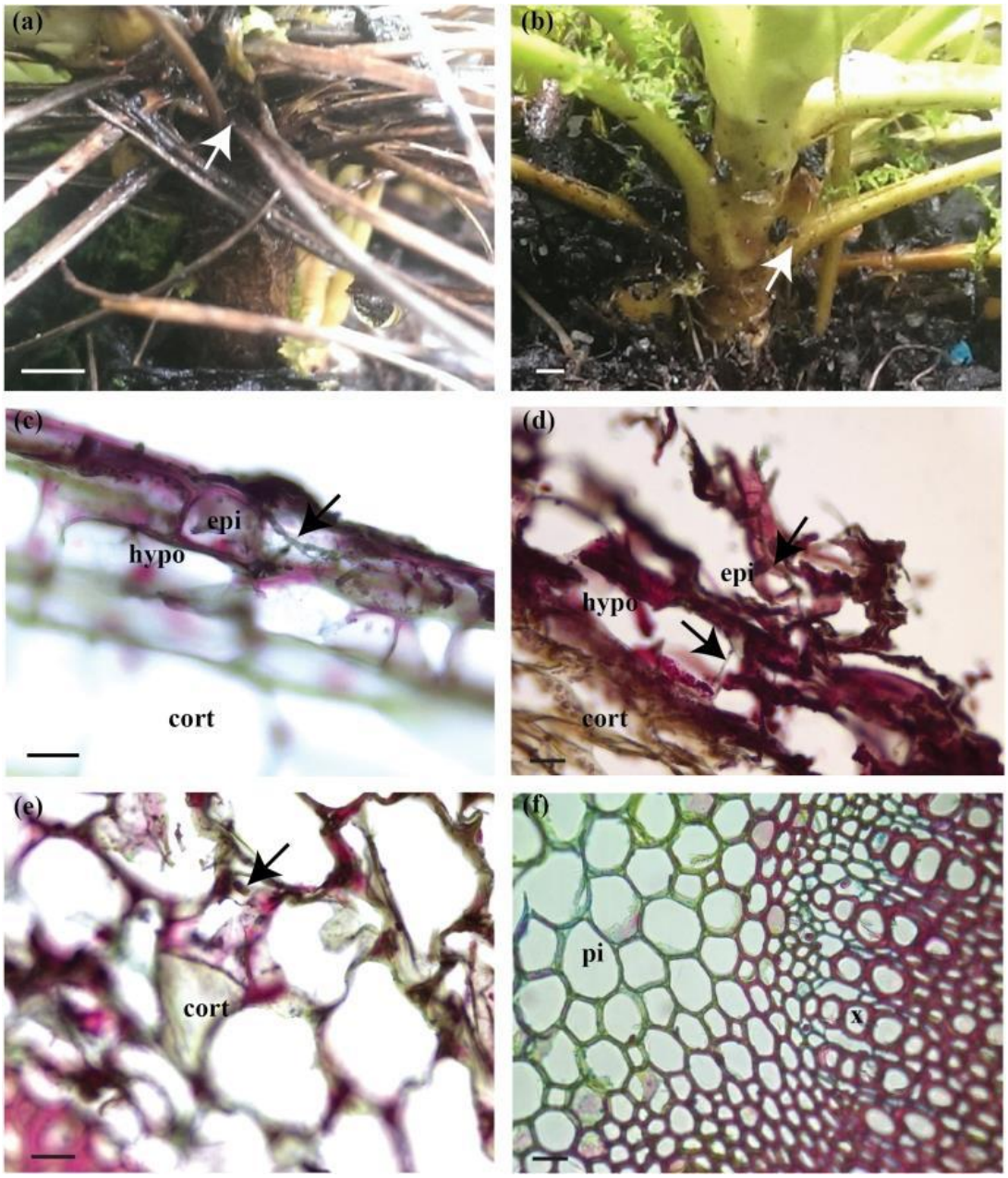

Fig. 4 Infection process of *S. tanaceti* in crown tissues; (a) Brown discoloured petioles (white arrow) surrounding the crown of the inoculated plants at 12 months after inoculation (mai); (b) Green petiole bases (white arrow) around the crown of the control plants; (c) Infection hyphae (black arrow) in the epidermis (epi), but not in the hypodermis (hypo) and cortical tissues (cort); (d) Degradation of infected epidermis (epi) and hypodermis by infection hyphae (hyp) (black arrow), cortical tissue (cort) were not infected; (e) Cortical tissues (cort) of inoculated plants were deformed due to the infection caused by infection hyphae (black arrow); (f) Both pith (pi) and xylem vessels (x) of inoculated plants were intact and free from infection hyphae.