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HISTOPATHOLOGICAL STUDIES OF SUNFLOWER SEEDLINGS INFECTED WITH *MACROPHOMINA PHASEOLINA*

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ABSTRACT

The mode of infection of charcoal rot *Macrophomina phaseolina* was studied employing light and transmission electron microscopy. When this pathogen was inoculated in sunflower seedlings, it changed the color of root tips. Staining of semi thin section of roots with methylene blue separated fungal mycelium from plant tissue. Initially, infection was restricted to root epidermal cells but in later stages the infection was also observed in cortical cells. At this stage cell configuration including organelles became distorted. Micrographs also revealed sclerotia and development of appressoria. Intercellular spaces were occupied by the fungal hyphae, which appeared amorphous, with intracellular invasion occurring later. It is envisaged that the development of fungus within the host plant is accompanied by the production of certain exudes, which helps fungus in penetration and destruction of the host.

Key words: Histopathology, sunflower, *Macrophomina phaseolina,* ultrastructure, sclerotia

INTRODUCTION

Sunflower *Helianthus annuuu* L. is an important crop on account of its high quality oil and fairly modest production requirements. Our present edible oil requirements are around 1.7 million tones per annum, 0.54 million tones of the total requirements (29%) are produced locally and remaining 71% (1.22 million tones) has to be imported. Sunflower is being cultivated in Pakistan as a potential crop to decrease the deficit between local requirements of edible oil (Ahmad, 1998).

Macrophomina phaseolina Tassi (Goid) the cause of charcoal rot is cosmopolitan in distribution and is potential threat to crop production in arid regions (Hoes, 1985). Brown to dark brown spots appears on stem. The plants become weak, dry and show light gray or ashy black discoloration of stem. Poorly filled heads can be seen and pre-mature ripening and drying of stalks occur, which also become normally discolored. Pith disintegrates and vascular fibers appear redden, covered with sclerotia of disease causing fungi (Chan and Sackston, 1973; Kunwar *et al*., 1986). The fungi perpetuate on the infected host plant debris in dry soils for a long period, but in moist (wet) soil, it can not survive for longer. Pathological action of the parasite causes the rotting of the roots and the destruction of vascular tissues leading to a premature ending of the vegetations, poor seed filling and low yield (Acimovic, 1978). In Pakistan charcoal rot is reported to be the major limiting factor for sunflower production (Mirza and Beig, 1983; Bhutta *et al.,* 1983; Bhutta *et al.,* 1995). In the present investigations histopathology of sunflower cells/ mechanism of fungal infection up to ultra structural level were studied.

MATERIALS AND METHODS

Seeds and virulent isolates of fungus *M. phaseolina* were placed simultaneously on sterilized potato dextrose agar (PDA) plates in laminar flow cabinet. Fungus was placed in the middle of the plate and seeds at the periphery. Plates were incubated at 28.5^0C. Infected roots were selected after 5 days of incubation.

For ultra structural studies root tips of healthy and infected seeds were placed in phosphate buffer with 7.2 pH and cut into pieces of about 4mm under dissecting microscope. Samples were placed in fixative i.e. 2% Gluteraldehyde (GAD). After overnight fixation the root tips were washed three times with phosphate buffer and then fixed in 1% Osmium tetraoxide (OsO_4) for 1 h Again three washes were given with buffer (10-15 min. each). Dehydration was carried out in different concentrations of acetone with increasing order. The infiltration was achieved by gradually increasing the concentrations of resin or embedding media as follows:

1 ml resin EPON 812+3 ml Acetone for 1 h
2 ml resin EPON 812+2 ml Acetone for 1 h
3 ml resin EPON 812+0 ml Acetone for overnight.

After overnight treatment in pure resin, tissues were embedded in resin in flat rubber molds and then placed in vacuum drying oven at 40 lbs. pressure for 30 min. at 37^oC to remove the air bubbles from the blocks. Samples were allowed to polymerize by subjecting them to a gradual increase of temperature at 37^oC, 47^oC and 67oC. Blocks were

then trimmed with common blades under (Bausch and Lomb) dissecting microscope. The semi thin and ultra thin sections were cut with ultra tome LKB Bromma 2088. Semi thin sections were stained with 1-% aqueous toluidin blue and studied under NIKON optiphot research microscope. Ultra thin sections were contrasted with Reynolds lead citrate and Uranyl acetate (Reynolds, 1963). The observations were made under Jeol transmission electron microscope (JEOL100sK) and the photographs were taken on Kodak 4489 EM film.

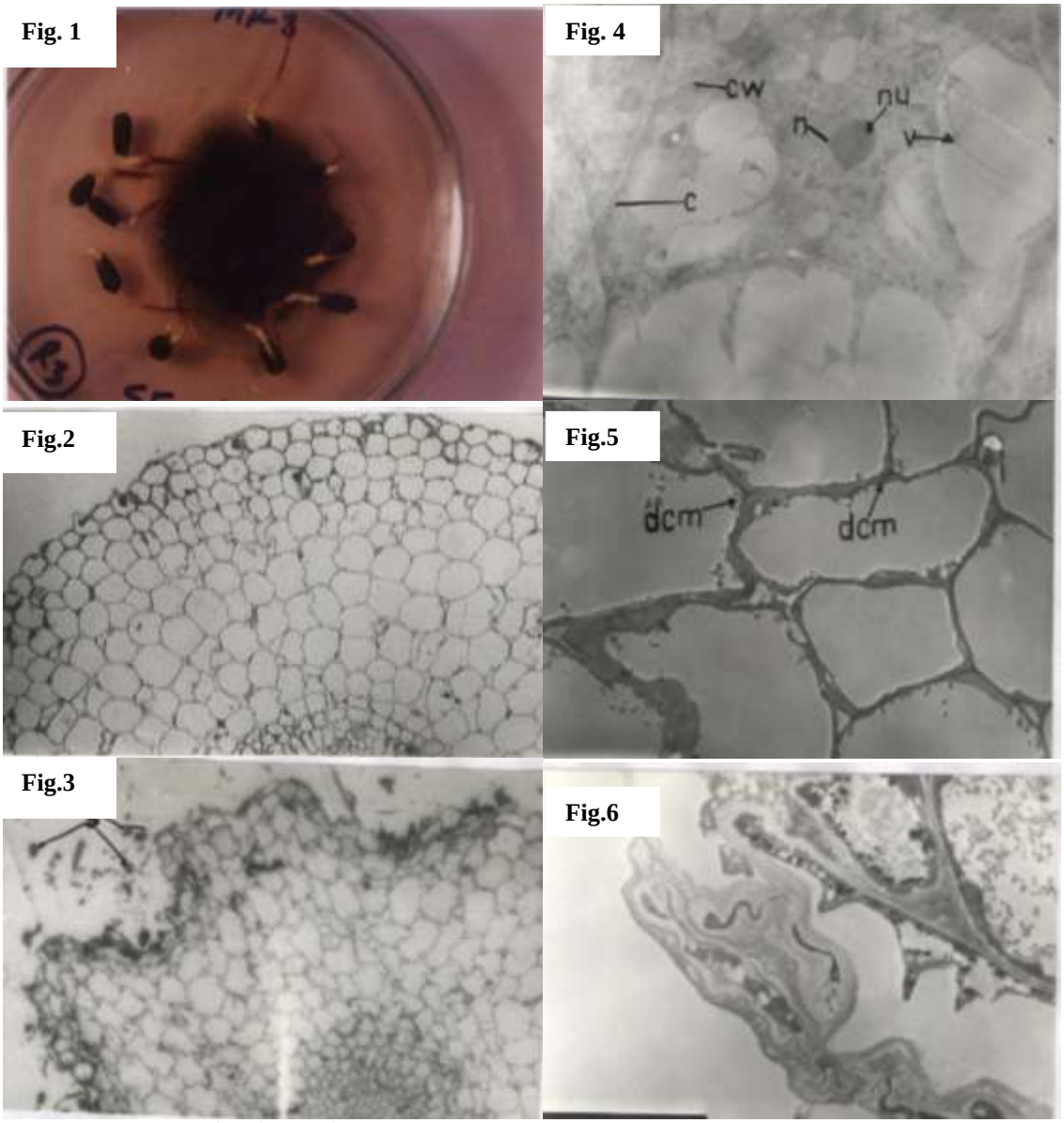


Fig 1. Germinated seeds of Sunflower infected by *Macrophomina phaseolina.*

Fig 2. Transverse section (T.S) of a healthy root tips as seen under light microscope,,showing healthy epidermal cells which are well shaped and symmetrical (410X).

Fig 3. Transverse section (T.S)of infected root tips showing heavily damaged cells on the periphery, individual cells can't be distinguished and appear to be depleted and digested by the fungus. The resulting space filled with fungal hyphae and spores. Numerous sclerotia (s) can be observe along with dead epidermal and cortical cells, less infected cells were seen shrunken and deformed(410X).

Fig 4. An electron micrograph of a healthy cell showing cell organelles, including nucleus (n), vacuole (v), nucleolus (nu), cytoplasmic membrane (c) and cell wall (cw) (8000X).

Fig 5. Electron micrograph of infected cells showing intercellular invasion of fungus *Macrophomina phaseolina*, where fungus is primarily confined to intercellular spaces and middle lamella. The individual cells are vacuolated and devoid of cell contents. Cell membrane is also disrupted (dcm) (5600X).

Fig 6. A portion of figure 5 further magnified showing the disintegrated middle lamella (ml), disrupted cytoplasmic membrane (dcm), which is being separated from the cell wall (8000X).

RESULTS

Roots of the germinated seeds were invaded by the fungus within 24-48 hours of inoculation. Fungal hyphae were observed firstly on the surface of the roots. The tips of the invaded roots turned dark brown in color, after about 72 hours of inoculation. Beyond this dark brown portion of the roots, the discoloration was also observed, extended up to several millimeters (**Fig. 1**). After 90 hours, the infected roots did not show further growth. At this stage ad axial epidermal layer under microscope was found completely distorted. It was not possible to distinguish individual cell (**Fig. 3**) when compared with transverse section of healthy root (**Fig.2**). The resulting space seems to be filled with the fungal bodies and dead cells. Numerous sclerotia can be observed along with the dead cells (**Fig.3**). At some places the fungus has penetrated deeper and destroyed eight to nine layers of cortical cells leaving behind a sort of depression (**Fig. 3**) filled with sclerotia. The adjacent cells were found either shrunken or deformed (**Fig. 3 & 5**).

At ultra structural level, the cellular contents of infected cells were found completely destroyed (**Fig. 6**). The middle lamella was disintegrated and cell walls were partially broken. The cell membranes of these cells were also found disrupted and has separated from the cell walls as compared to the healthy cells (**Fig.4**) with well defined cell organelles nucleus (n), vacuoles (v), mitochondria (m), ribosome (r) etc. The micrograph of a healthy cell (**Fig. 7**) shows smooth middle lamella and intact cell membrane. When the fungal hyphae come in contact with the epidermal cells they form a pear shaped aspersoria (**Fig. 10**). This development in hyphae helps the fungus to penetrate directly into the epidermal cells. Initially the fungus penetrates and establishes itself into the intercellular spaces of the epidermal and cortical tissues (**Figs. 5 & 9**). In these intercellular spaces, several hyphal branches can be observed, which were protruding and proliferating, forming a series of short bud like structures (**Fig. 9**). From these intercellular hyphal strands the intracellular invasion occurs. This can be observed in figure 8 in which the fungus is probing into the healthy cells by forming a bud like structure. The nucleus of this healthy cell can also be seen. The fungus after penetration into the cell brings about the disintegration of the cellular contents. In Fig. 10 a virtually disintegrated nucleus can easily be identified. The contents of the heavily infected cells were found disintegrated and cannot be distinguished as they have turned into granular form (**Fig.6, 10, 11 & 13**) whereas heavily infected cells were completely destroyed and can not be distinguished (**Fig. 12**) as they were digested by the fungus. The resulting space is filled with fungal hyphae and spores (**Fig. 2, 3 & 12**). As a result of this cellular destruction, root tissues turned dark brown in color and its growth stopped.

DISCUSSION

Normally the fungus penetrates through the root hairs and epidermal tissues of the roots at early stages of plant growth and development from cotyledon to 5-7 pairs of leaves. The mycelium of the fungus spreads in the root tissues of the infected plants. Later on from budding to maturity the mycelium spread through the vascular system of the infected plants, when the mycelium reaches the basal part of the stem, they induce the occurrence of light brown spots on that plant. The spots gradually turn dark brown and then black. They form a ring around the stem leading to a rapid wilting specially after flowering stage which results into premature ending of the vegetation, poor seed filling and low yield (Tihonov and Nedeljkov, 1978; Acimovic, 1978).

It is an established fact now that the fungus survives in the soil by means of sclerotia, which are usually the interwoven mass of mycelium. Single hyphal strand on the host epidermis swells and become thick walled, rather like chlamydospore (Wyllie and Brown, 1970; Ammon *et al.*, 1975). They also found that sclerotia are formed only on the surface of infected roots as soon as the fungus penetrates deep, the sclerotia could also be observed in the internal tissue. Similar results are obtained in the present study as far as the presence of sclerotia is concerned (**Fig. 2 & 3**).

The fungal hyphae radiate and spread over the surface of host roots without any recognizable pattern. The tips of the hyphal strands produce flask shaped appressoria on coming in contact with the epidermal cells (Ammon *et al.*, 1972; Ammon, *et al.*, 1975; Kunwar *et al.*, 1986; Rao and Mukerji, 1972). When the tip of the hyphae is pressed against the host surface the appressoria formation takes place. It is initiated by either a binding or cessation of further hyphal elongation. The factors responsible for the cessation and stimulation of appressorial formation remains to be unanswered even in the present investigations. Similar doubts are expressed by earlier investigator (Ammon *et al.*, 1975). The factors such as virulent pathogen, chemical substances in the susceptible host tissue and the mechanical pressure may be involved, which have been demonstrated to be necessary for appressorial development of *R. solani* on radish (Flentje, 1959). In other crops such as soybeans, Ammon *et al.* (1971,1972 and 1974), found that the initial growth of the fungus is primarily confined to intercellular spaces and the middle lamella, whereas intra-cellular invasion takes place later.

Fig. 7.

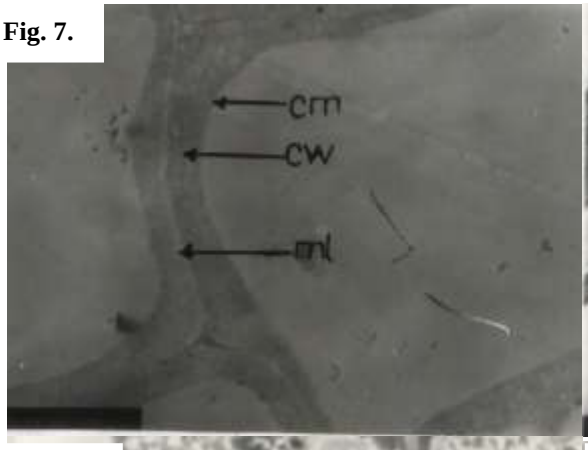


Fig. 10.

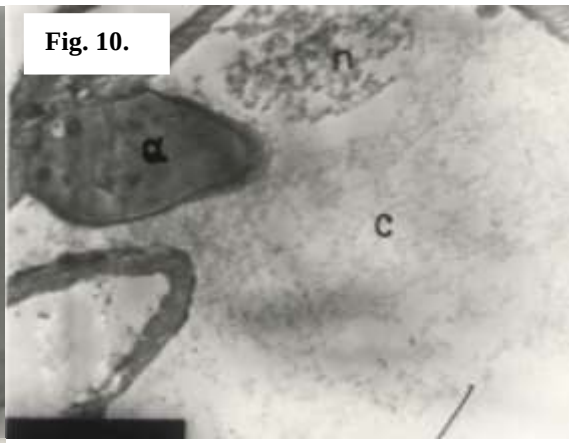
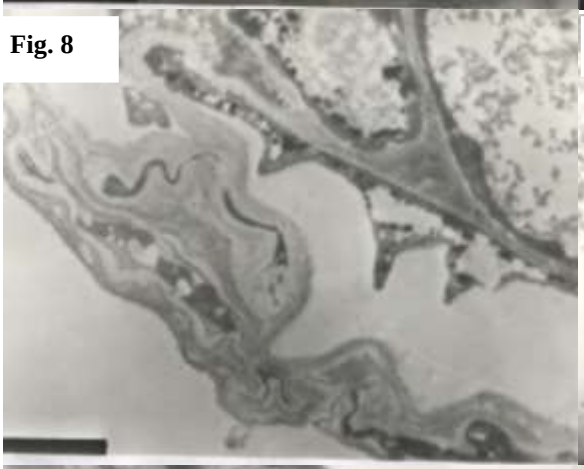


Fig. 8



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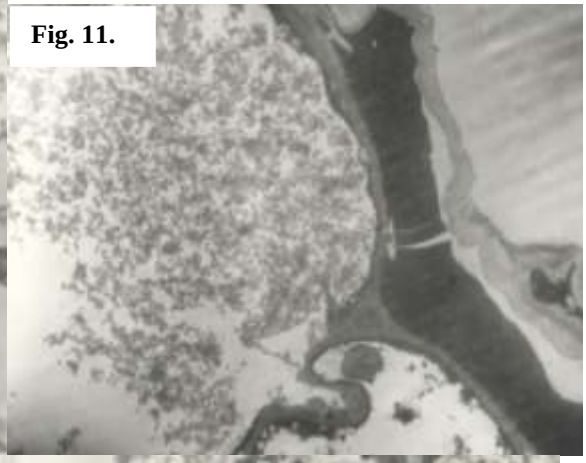

Fig. 9.

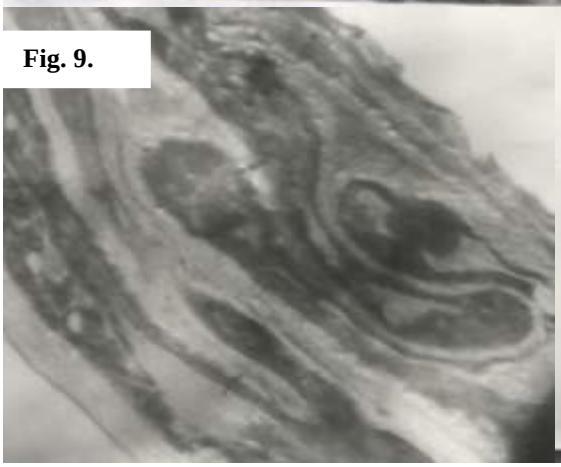

Fig. 12.

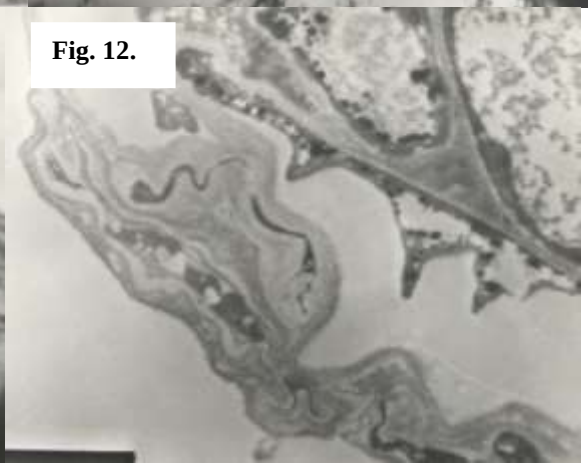

Fig. 7. Micrograph of non-infected cell showing cell wall (cw) and middle lamella (ml), cell membrane (cm) (8000X).
Fig. 8. Micrograph showing intercellular fungal hyphae (if), probing into healthy cell whose nucleus (n) can be observed (8000X).
Fig. 9. A portion of Fig. 8 further magnified to show the intercellular growth of *Macrophomina phaseolina.* Several hyphal branches protruding and proliferating forming a series of short bud like structures (20000X).
Fig. 10. A portion of the cell invaded by the the fungus (*M. phaseolina*) by forming appear shaped appressoria (a) and avirtually disintegrated nucleus (n). Cytoplasm (c) of this cell appears completely disintegrated (14000X)
Fig. 11. Electron micrograph of of an infected cell whose contents have been digested and depleted. They can not be distinguished and turned into granular form as compared to healthy cell Fig. 4 (10000X).
Fig. 12. Micrograph of acell which appears to be completely destroyed by the fungus and the resulting space, seems to be filled with the fungal hyphaeand spores (1000X).

Fig. 13.

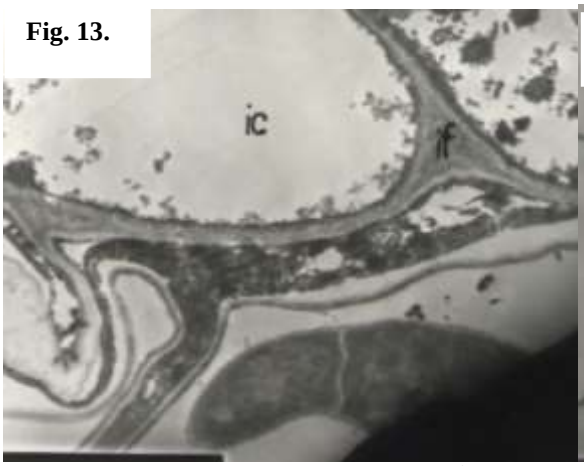

Fig. 14.

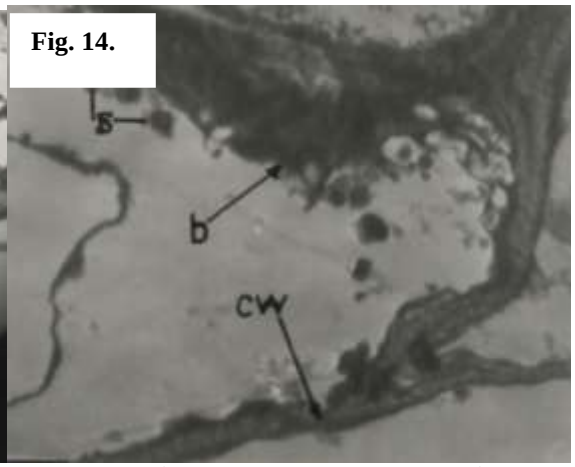

Fig. 13. Electron micrograph showing intercellular fungus (IF) and two heavily infected cells (Ic) which are devoid of cellular contents and disrupted cell membrane (dcm) (10000X).

Fig. 14. Electron micrograph of another highly infected cell showing cell wall (cw) broken at several places. Cell is highly vacuolated and resulting space filled with the sclerotia (s) and broken fungal bodies (b) (10000X).

In an ultrastructural study of this fungus on soybeans by Wyllie and Brown (1970) it was observed that several hyphal branches in the intercellular spaces do not have any specific structural features. This penetration of the fungal hyphae in the host cells is aided by the chemical softening and mechanical pressure (Ammon, 1972; Chan and Sackston, 1973).

Thorn berry (1938) found in his study that the fungus *M. phaseolina,* produce some translocatable toxins. These toxins were found to be non host specific and they might be enzymes. However, Chan and Sackston (1972) and Dhingra *et al*. (1974) investigated the mechanism of pathogenesis and found that these toxins are certains cellulytic and pectolytic enzymes, which bring about the chemical changes in the organization of cellular contents before the physical penetration of fungus into cell. Chan and Sackston (1970) found the discoloration of root tissue adjacent to the dark brown infected tissues of the root, might be a possible chemical effect of fungus before its penetration.

Present research supports the earlier findings i.e. the discoloration of the root tissues adjacent to the dark brown portion of the root. Furthermore the cells adjacent to the infected one were found shrunk. This situation might be due to the chemical effect of the pathogen before actual penetration (Ammon *et al.,* 1971). Subsequently, the middle lamella disintegrates, plasmalemma separates from the cell wall, the cytoplasm became highly vacuolated and mitochondria were disorganized (Ammon *et al*., 1974). Present ultrastructural study indicated that the infected cells become de-shaped, disintergrated and resulting space is filled with sclerotia. The disruption of the cellular contents and tissue disintergration is believed to occur by the enzymes produced by the fungus in association with the mechanical pressure (Ammon *et al.,* 1971 and 1972), which leads to the death of the host plant.

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