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Histology of *Pinus maximinoi* cones infected by *Cronartium conigenum*¹

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Summary

Apparently healthy and *C. conigenum* infected female strobili of *Pinus maximinoi*, collected in Guatemala, were histopathologically studied by light microscopy. Scales from infected cones were fused and fertile scales had aborted seeds. Infected cone scales lacked fibre cells in the cortex, which are abundant in healthy cone scales. Intercellular spaces of cortex contained abundant hyphae compared to phloem and xylem.

The cell walls of infected tissues in the ovules were darkly stained, while those of comparable healthy tissue were colourless under Pianeze's IIIB stain. Infected ovules of different developmental stages with nucellus, or nucellus plus spongy tissue, were observed. The cells of each tissue type contained many haustoria and did not develop into female gametophyte and embryo. Ovule colonization by hyphae appeared after pollination and subsequently caused their early abortion.

1 Introduction

The cone-rust fungus, *Cronartium conigenum* (Pat.) Hedgc. & Hunt, causes an economically important disease which reduces cone and seed production in many native pine species extending from southwestern New Mexico to Belize and El Salvador (ANONYMOUS 1977; QUINARD and MARTINEZ 1987). After female strobili are infected, seed production is severely reduced or eliminated. This rust fungus has been reported to present problems in natural re-forestation of the affected pine species in the highlands of Guatemala (SCHIEBER 1967). The magnitude of cone infection and destruction by *C. conigenum* has been reported to range from 15 to 90% in affected pine forests (HEDGCOCK and HUNT 1922; JOHNSTON 1942; ETHERIDGE 1968). Heavy losses of cones and seeds have been reported for *Pinus chihuahuana* Engelm. in Arizona, and *P. montezumae* Lamb and *P. oocarpa* Schiede in Guatemala (QUINARD and MARTINEZ 1987). However, no specific information on the amount of pine-seed loss due to this disease has been recorded. FATZINGER and MILLER (1980) reported a seed loss of 25% in a seed orchard in Florida infected by a closely related cone-rust fungus, *C. strobilinum* (Arthur) Hedgc. & Hahn. A reduction in pine-seed crops by *C. strobilinum* by as much as 60% has been reported (FOSTER 1956). A similar or worse scenario for pine-seed losses due to cone infection by *C. conigenum* can be expected in affected forests.

Cronartium conigenum is heteroecious, requiring oaks as primary hosts and pines as secondary hosts for completion of its life cycle. At least 18 species of pines are secondary hosts (PETERSON and QUINARD 1967; ANONYMOUS 1977; QUINARD and MARTINEZ 1987), and several species of the genera *Quercus*, *Castanea*, *Castanopsis*, and *Lithocarpus*, of the family *Fagaceae*, have been reported as primary hosts (HEDGCOCK and HUNT 1922;

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HEDGCOCK 1939; HEDGCOCK and SIGGERS 1949). *Cronartium conigenum* is macrocyclic, producing uredinia, telia, and basidia on oaks, and pycnia and aecia on pines. The conelets are infected shortly after pollination (SCHIEBER 1967). In early spring, pycniospores are produced on infected cones followed by masses of orange-yellow aeciospores which form between March and early June in Guatemala (SCHIEBER 1967).

Female strobili infected by basidiospores of *C. conigenum* often develop into cones which die prematurely and remain attached to the branches for up to several years (GIBSON 1979). Cones infected by *C. conigenum* are softer and 3–4 times larger, with contorted lobes, in contrast to normal cones of the same age (SCHIEBER 1967; ETHERIDGE 1968; GIBSON 1979). Size variations among infected cones of up to 10 times that of healthy cones have been reported, and galled and mummified cones are frequently infested with insects (HEDGCOCK and HUNT 1922). In a survey conducted on slash-pine orchards in Florida, as much as 25% of the first-year strobili were lost due to rust, and many of them harboured coneworm larvae (FATZINGER and MILLER 1980). As much as 40% cone and seed loss due to insect infestation has been estimated for slash pine in Florida orchards (FATZINGER and MILLER 1980). These infected cones provide a brooding place for coneworms and result in additional cone and seed loss due to attack of healthy cones by new broods. Similar events of seed loss due to insect population build-up in *C. conigenum* infected cones may be prevalent in affected areas.

This disease has been recognized for many years (JOHNSTON 1942). The symptoms of the disease on affected cones are fusion of scales resulting from cellular hypertrophy and hyperplasia. These symptoms are adverse to seed development, however, the direct impact of the fungus on seed development has not been investigated. *Pinus maximinoi* H. E. Moore, formerly known as *P. tenuifolia* Benth., appears to have great promise for planting under tropical and semi-tropical conditions in a number of countries (DVORAK and DONAHUE 1988; PERRY 1991). The purpose, therefore, of this research was to perform a histological study of the cones of *P. maximinoi* infected by *C. conigenum*.

2 Materials and methods

Three (about 2-year-old) symptomatic cones of *P. maximinoi*, exhibiting brick-red colour, were collected from each of three trees from the highland region near Guatemala City along Highway CA-1. Three healthy cones of this species (of similar age) were also collected from the same locality in Guatemala. Cones were sliced in half longitudinally and fixed in a standard mixture of formalin:propionic acid:70% ethanol (1:1:18; JOHANSEN 1940). After 15 days, cone samples were washed and preserved in 70% ethanol. Scales were excised from the broadest part of the symptomatic cones, and 5 × 5-mm segments were taken from the umbo, scale base, and axis tissues of each of three scales from each infected and healthy cone. These segments, ovules from infected cones, and seeds from healthy cones were then processed for histological examination.

Cone materials to be processed for histological studies were dehydrated, infiltrated, and embedded in paraffin (Paraplast X-TRA, OXFORD, LABWARE, Division of Sherwood Medical, St Louis, MO, USA) according to methods described by JOHANSEN (1940) and JENSEN (1962), with slight modifications (RAYACHHETRY 1987). Sections 5–10 µm thick were obtained using a rotary microtome. Sections were stained using Heidenhain's hematoxylin (JOHANSEN 1940) or Pianeze's IIIB (VAUGHAN 1914). At least 10 randomly selected sections were studied for umbo, scale base, axes, infected ovules, and healthy seeds. Alteration of tissue characteristics and the presence of *C. conigenum* structures in cells of different tissues in the infected cones were noted, and representative photomicrographs were taken using a light microscope (Nikon UFX-II, Optiphot system, Nippon Kogaku, NY, USA).

3 Results

3.1 Morphology

The healthy cones were grayish-green and the cone scales were normal, i.e. not fused, and bore seeds at the scale bases (Fig. 1a,b). The infected cones were, however, brick-red, hypertrophied (2–3 times bigger than the normal cones) with ruptured surfaces and fused cone scales, and bore tiny ovules (Fig. 1c,d). The seeds from the healthy cones contained well-developed wings, seed coats, female gametophytes, and embryos (Fig. 1b,e). By



Fig. 1. a. A 2-year-old healthy *P. maximinoi* cone (8.5-cm long and 4-cm wide at the broadest part); b. A portion of a healthy cone split in half longitudinally showing normal cone scales and half of an apparently normal seed (arrow); c. A 2-year-old cone infected by *C. conigenum*, hypertrophied and still attached to a twig. Note rough cone surface with fused scales; d. A segment of the hypertrophied cone bearing aborted ovules (arrows) enclosed by completely fused cone scales; e. Cross section through the broadest part of a healthy seed illustrating well-developed seed coat (s), female gametophyte (fg), and embryo (e); f. Cross section through an aborted ovule still attached to the ovuliferous scale (os). Note poorly developed integument (i), nucellus (n), spongy tissue (t), and degenerating spongy cells (arrow) at the centre

contrast, the ovules in the infected cones of comparable age to healthy cones, did not develop into seeds (Fig. 1f).

3.2 Histology

3.2.1 Cone scales and axis

Epidermal tissue from the scales of healthy cones enclosed several layers of sclerenchymatous cells (Fig. 2a). The collateral vascular bundles in the centre of the scales were surrounded by a few layers of cortical cells. A large proportion of vascular bundles were composed of phloem (Fig. 2b). The scales of the infected cones were fleshy and the epidermis

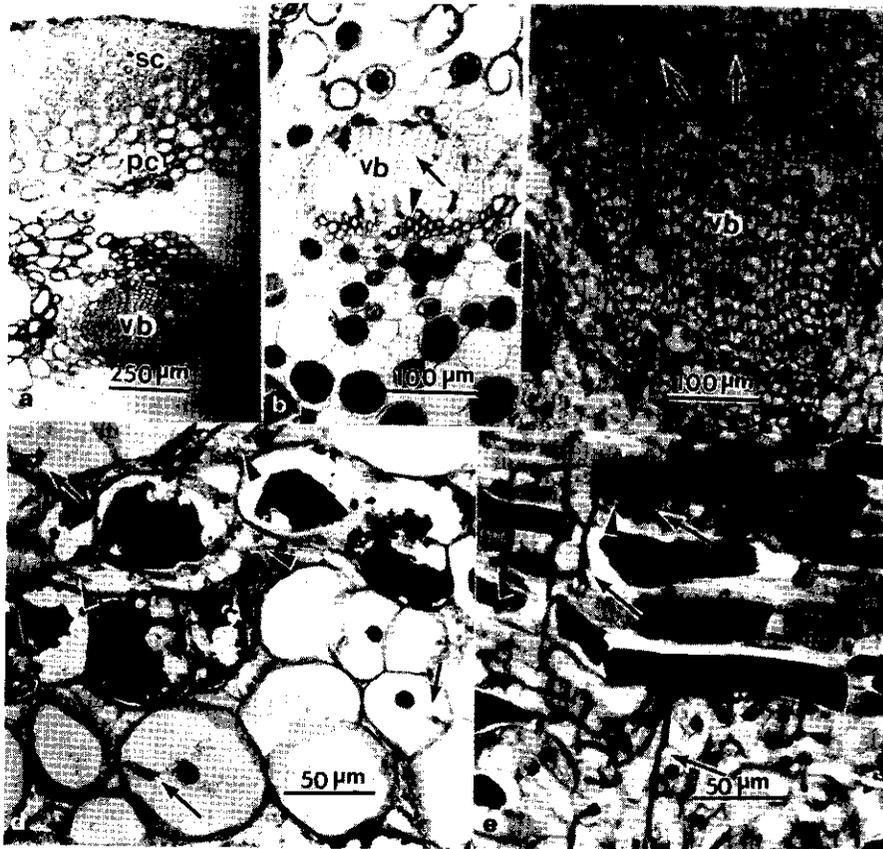


Fig. 2. a. Cross section through a healthy cone scale showing hypodermal sclerenchymatous cells (sc), cortical parenchyma cells (pc), and a vascular bundle (vb); b. A single vascular bundle (vb) of a healthy cone scale. Note normal phloem (arrow) and tracheids (arrow head); c. A vascular bundle (vb) of a diseased cone scale with collapsed cells (arrows) at the outer phloem; d. cross sectional view of a portion of cortex of a diseased cone scale. Note hyphal concentration (arrow heads) among darkly stained cell containing starch granules and haustoria (arrows) in the clear cells; e. Longitudinal view of a portion of phloem tissue from a diseased cone scale illustrating hyphae (arrows) and haustoria (arrow heads)

of the individual cone scales were indistinguishable from the remainder of the cortical parenchyma cells. No sclerenchymatous cells were observed in scale tissues of the diseased cones. Often, the distorted vascular bundles contained crushed phloem tissue (Fig. 2c). In the infected cone scales, intercellular spaces among cortical parenchyma, phloem parenchyma, sieve tubes, tracheids, and xylem parenchyma cells contained many hyphae. They were particularly abundant around the darkly stained (Heidenhen's hematoxylin) cells of cortical parenchyma containing starch granules (Fig. 2d). Hyphae were also concentrated around sieve areas of the sieve tubes (Fig. 2e) and often penetrated these tubes through sieve areas. Haustoria of different shapes and sizes were present in cells of these tissues (Fig. 2d).

The healthy cone axes were composed of cortex, phloem, xylem and pith tissues. Cellular characteristics, mycelial ramification, and haustorial behaviour among different tissue types of the axes of infected cones were similar to those described for cone scales.

3.2.2 Ovules

The seeds from healthy cones were apparently normal, with embryos surrounded by gametophyte tissue (Fig. 3a). The gametophyte tissue and embryos were separated by a corrosion layer as described by OWENS et al. (1982) in healthy cones of *P. contorta* Dougl. ex Loud. The gametophyte tissues were surrounded by a brown layer of papery tissue (remnant of nucellar tissue), which, in turn, were enclosed in well-developed seed coats (Fig. 3a). The embryos had seven cotyledons (Fig. 3a) at the chalazal end of the seed. The cells of the gametophyte and embryonic tissues contained darkly stained droplets (Fig. 3a,b) of lipoprotein, as described by OWENS et al. (1982). Cell walls in these tissue types were very thin (Fig. 3b).

Transverse sections (Figs 1f,3c) of ovules from infected cones revealed remnants of ovules at different developmental stages, as determined by comparison of the illustrations and descriptions of pine cones from other pine species (FERGUSON 1904; MATHEWS 1932; OWENS et al. 1982). Some ovules contained a well-developed nucellus surrounded by a poorly differentiated integument (Fig. 3c,d), while others contained a slightly advanced stage, i.e. spongy tissue, with degenerating cells in the centre (Fig. 1f). No detectable female gametophytes were observed in the ovules. Ovular integuments were still attached to the ovuliferous scale (Fig. 1f). Cells in the nucellus possessed thick walls and lacked darkly stained (Pianeze's IIIB) droplets, as in healthy tissues, but some cells contained other darkly-stained substances (Fig. 3c,d), observed in the cortical cells in scales of infected cones. The tissues of ovules were colonized by cone-rust hyphae, but not as abundantly as in the cortical parenchyma cells of the scales. Abundant simple haustoria were present in the cells of the integument and nucellus (Fig. 3d).

4 Discussion

Cronartium conigenum has been reported to negatively impact the natural regeneration of infected pine species (HEDGCOCK and HUNT 1922; JOHNSTON 1942; SCHIEBER 1967; ETHERIDGE 1968) in two ways: 1. Normal seed development; and 2. Seed release. Ovules from infected cones were only 10–20% of normal size, with soft integuments attached to the ovuliferous scales (Fig. 1f), compared to the seeds with well-developed seed coats from healthy cones at a comparable age (Figs 1e,3a). The fleshy texture of the fused scales of infected cones appeared to be mainly due to the total absence of sclerenchyma cells in the hypodermal region, which were present in the scales of the healthy cones of similar ages (Fig. 2a). Fleshy scales in the infected cones were also comparatively thicker (Fig. 1d), and mainly composed of parenchymatous cells (QUINARD and MARTINEZ 1987). Based on this study, seed release is not a factor because the ovules have already aborted following fungal

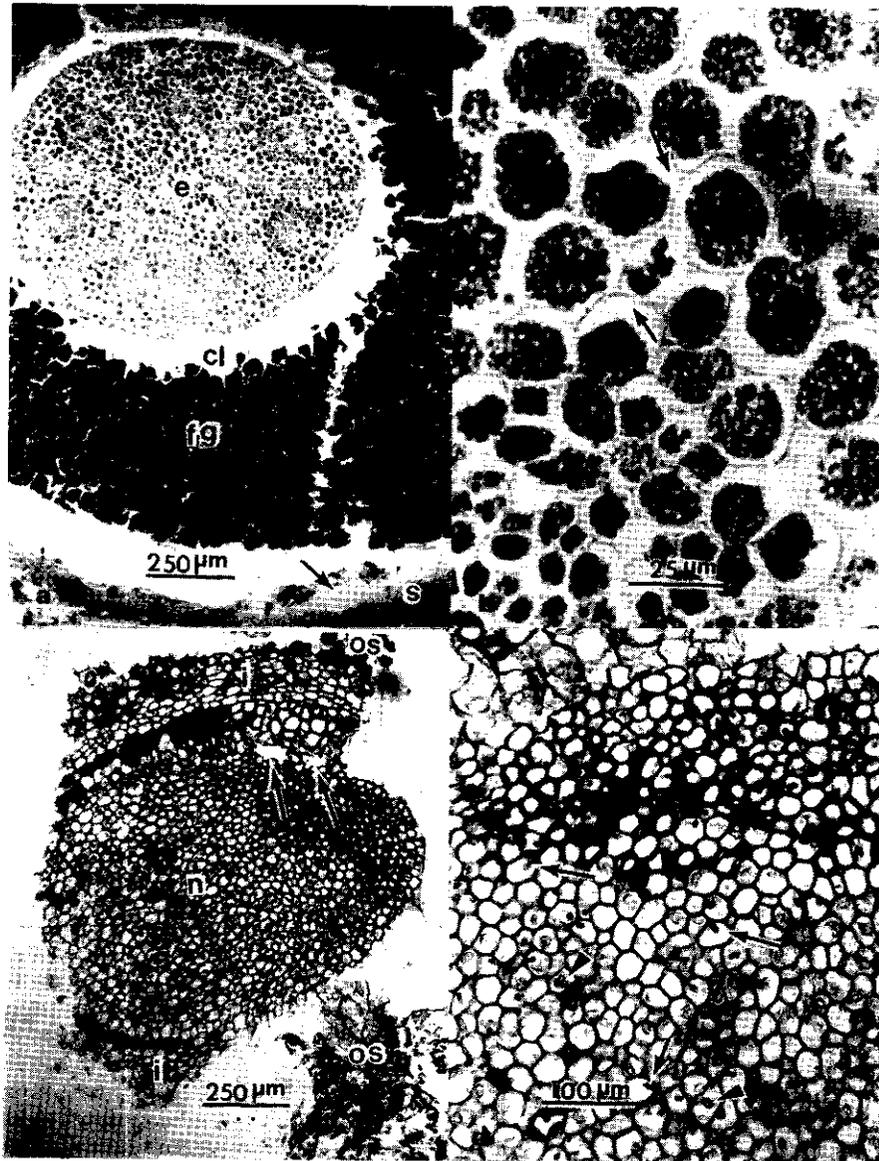


Fig. 3. a. A portion of the transverse section of a healthy seed through the epicotyl region of an embryo. Note seed coat (s), remnant of nucellus (arrow), female gametophyte (fg) with darkly stained cellular content, corrosion layer (cl), and embryo (e) with seven cotyledons; b. Cross sectional view of a portion of embryonic tissue of a healthy seed, note very lightly stained cell walls (arrows) enclosing darkly stained droplets and prominent nuclei; c. Cross sectional view of a portion of an aborted ovule with ovuliferous scale (os), poorly differentiated integument (i), nucellus (n), and remnants of pollen tubes (arrows); d. A portion of the cross section of an aborted ovule, note abundant haustoria (arrows) and host nuclei (arrow heads) in the nucellar cells

invasion. These observations indicate that the fungus essentially stops normal ovule and gametophyte development at a very immature stage. Infected ovules resemble first-year pine ovules prior to fertilization and embryo development. Thus, even if the scales were not fused, no healthy seeds would be present in the infected cones.

In infected cones, the fungus colonized cortex, phloem, xylem, and pith. In the intercellular spaces of parenchyma cells, the hyphae were abundant, where starch granules in the cells were prevalent (Fig. 2d). A similar mycelial concentration was reported in fusiform rust galls of *P. palustris* Mill., where starch granules occur abundantly in cortical parenchyma cells (WALKINSHAW and ROLAND 1990). In this study, the colonization of phloem tissue by hyphae was intense (Fig. 2e). A similar type of colonization of vascular tissue has been reported (BAKA and LOSEL 1992) for other rust fungi.

The most serious impact of *C. conigenum* is ovule abortion through colonization of developing tissues. In *C. strobilinum*, cone infection by basidiospores occurs during the period when the cone scales are fully opened for pollination (JEWELL 1957). A similar mode of cone infection by *C. conigenum* has been reported. In general, ovules of pines contain a well-differentiated integument, nucellus, and spongy tissue at the time of pollination (FERGUSON 1904). BRAMLETT et al. (1977) studied general seed development and viability of southern pines and reported that the seeds that aborted during the first year of cone development were characterized by a poorly developed seed coat compared to seeds aborted during the second year of cone development. The presence, therefore, of poorly differentiated integuments (Fig. 3c) on the infected ovules in this study was an indication of the cessation of further development of the infected ovules, occurring soon after the infection was initiated by basidiospores at the time of pollination. The infection of the ovules in the early stage of development also revealed that the basidiospores of the cone-rust fungus likely were directly deposited into the pollen chamber, germinated quickly, and colonized the nucellus. Since infected conelets showed an abnormal increase in size in March and April (SCHIEBER 1967), the fungus appeared to colonize scale and axis tissues quickly and to seal the ovules, as seen in Figure 1d. Ovule infection may precede the pollen germination in the pollen chamber at the micropylar end. Nevertheless, it would be interesting to know if the cells in the male prothallus are colonized by this pathogen.

In general, two cavities mark the active embryogenesis during the second year of cone development among healthy pine cones: 1. The outer cavity between the nucellus and the female gametophyte; and 2. The inner cavity between the female gametophyte and the central mass of embryonic tissue (FERGUSON 1904; OWENS et al. 1982). This study of seeds from healthy cones (Figs 1e,3a) supports these reports. In some infected seeds, the nucellus was well-developed inside a poorly developed integument (Fig. 3c,d), although the formation of the gametophyte seemed to cease. In other infected ovules, a spongy parenchyma was differentiated in the nucellus, but the macrospore mother cell seemed to halt further division to form the female gametophyte, and, hence, the spongy cells seemed to continue growth, eventually forming several layers of spongy tissue, which, after some time, started collapsing from the centre (Fig. 1f). The failure of nuclear division in macrospore mother cells, followed by subsequent division of spongy cells and eventual degeneration of spongy cells from the centre has also been reported in other pines (FERGUSON 1904).

In this study, the phenomenon of ovule abortion in the early stages of development may be attributed to the infection of the ovules by the fungus, as shown by the presence of haustoria (Fig. 3d). This reveals that ovule infection by this rust fungus started during the early stages of strobili development. No remnant of pollen was detected in the micropylar cavities of the developing infected ovules. However, the nucellus of some infected ovules contained a few abnormally larger spaces embedded in the nucellus (Fig. 3c). These larger spaces may be the remnants of pollen tubes.

During late embryo development, the outer and inner cells form starch and large droplets of lipoprotein throughout the female gametophyte and cells of the embryo (OWENS et al. 1982). In this study, similar darkly stained droplets were found uniformly distributed in

the cells of the female gametophyte and embryo of healthy seeds (Fig. 3a,b). However, cells in the nucellus from infected ovules lacked those darkly stained droplets (Figs 1f,3c,d). The cone-rust hyphae in these tissues may have utilized this material for respiration. Furthermore, the cell-wall thickness in the nucellus (Fig. 3c,d) and spongy tissues of infected ovules may be an effect of fungal colonization.

The results of this study show that cones infected by *C. conigenum* are useless for seed production.

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Résumé

Histologie des cônes de Pinus maximinoi infectés par Cronartium conigenum

Des cônes femelles de *P. maximinoi* apparemment sains ou infectés par *C. conigenum*, récoltés au Guatemala, ont été étudiés du point de vue histologique au microscope optique. Les écailles des cônes infectés étaient soudées et les écailles fertiles avaient des graines avortées. Les écailles des cônes infectés n'avaient pas de cellules fibreuses dans le cortex contrairement à celles des cônes sains. Les espaces intercellulaires du cortex contenaient des hyphes plus abondants que le phloème et le xylème. Les parois cellulaires des tissus infectés des ovules étaient sombres alors que celles des mêmes tissus sains étaient incolores (colorant Pianezze's III B). Il a été observé des ovules infectés à divers stades de développement, avec nucelle ou nucelle plus tissus spongieux. Les cellules de chaque type cellulaire contenaient beaucoup d'haustoria et ne se développaient pas en gamétophyte femelle ni embryon. La colonisation des ovules par les hyphes avait lieu après la pollinisation et en conséquence causait leur avortement précoce.

Zusammenfassung

Histologie von Pinus maximinoi-Zapfen mit Infektion durch Cronartium conigenum

Symptomfreie und durch *C. conigenum* infizierte weibliche Zapfen von *P. maximinoi* aus Guatemala wurden im Lichtmikroskop histopathologisch untersucht. Die Schuppen infizierter Zapfen waren miteinander verwachsen, und die Samen fertiler Schuppen waren abortiert. Infizierte Zapfenschuppen hatten im Gegensatz zu gesunden Schuppen keine Faserzellen im Cortex. In den Interzellularen des Cortex waren im Unterschied zu Phloem und Xylem zahlreiche Hyphen vorhanden. Die Zellwände der infizierten Gewebe in den Samenanlagen wurden durch den Farbstoff nach Pianese III B dunkel angefärbt, während die entsprechenden gesunden Gewebe farblos blieben. Infizierte Samenanlagen wurden in unterschiedlichen Entwicklungsstadien (mit Nucellus oder mit Nucellus und Schwammgewebe) beobachtet. Die Zellen dieser Gewebetypen enthielten zahlreiche Haustorien und entwickelten sich nicht zum weiblichen Gametophyten und Embryo. Die Pilzinfektion der Samenanlagen erfolgte nach der Befruchtung und führte zum Absterben in einem frühen Entwicklungsstadium.

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