# Scanning Electron Microscope Examination of Sugarbeet Flowers and Fruits Infected with Phoma Betae *

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## INTRODUCTION

Phoma betae (Oud.) Frank (= Pleospora bjoerlingii Byford) is the only important seedborne fungal pathogen of sugarbeet (Beta vulgaris L.) (10, 11). The fungus can survive in the soil for at least 26 months after the sugarbeet crop is planted (4). A high percentage of beet seed may become infected with P. betae when produced in regions with adequate rainfall (3). Infected seeds, germinating in cool damp soil, produce seedlings that fail to emerge, or die following emergence. Infected seedlings that survive are stunted and retarded in growth until warm weather permits recovery (3).

P. betae attacks almost every part of the sugarbeet and causes seedling black leg symptoms (7, 10, 11), leaf sport (19), root rot and crown rot in storage, and sometimes in the field (6, 8, 10, 20).

This fungus is found whereever sugarbeet is planted commercially. Seed dissemination is the method by which the fungus is spread over long distances from regions of seed production to areas of sugar production. This research was undertaken to gain more information on how infection of the flower and fruit occurs.

The structure usually called a sugarbeet seed technically is a fruit composed of a single true lentil-like seed, lying within dead corky tissue (pericarp or the fruit wall). Throughout this

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study, the seed (dark brown, shiny, lentil-like structure) surrounded by dried suberized corky tissue will be termed the fruit. The seed occupies a horizontal position and is curved in such a manner that its lower part is covered with the receptacle. The latter will be called the fruit cavity wall. The structure covering the upper part of the seed will be called the operculum. The pericarp, with the dried floral parts still attached, will be termed the florocarp.

## MATERIALS AND METHODS

One hundred naturally infested fruits were surface sterilized using 90% ethanal for 1-2 min., then washed with sterile distilled water and dryed on filter paper. These fruits and an additional 100 untreated fruits were then separated into their fruit walls, seed coats and embryos, which were plated onto selective media (5) and water agar (15).

Sugarbeet roots that had been stored for at least 80 days at 5 C were planted in autoclaved soil and placed in a growth chamber, with 8 hr. darkness at 16 C $\pm$ 4 C and 16 hr. of incidence and fluorescent light at 21 C $\pm$ 4 C. The inflorescences were cut 10 to 15 days before maturation of the fruits. The cut ends were placed in flasks filled with sterile distilled water, and the flasks were placed in a plastic-box moist chamber at room temperature with 8 hr. darkness and 16 hr. of incidence light under aseptic conditions. The flowers were inoculated in the moist chamber by spraying them with a conidial suspension of P. betae. Flowers were harvested after 24 or 48 hr., and 5 days after inoculation. Samples then were prepared for scanning electron microscope (SEM) examination as follows: naturally .infected fruits were harvested from a seed production field near Salem, Oregon in 1977. Over 95% of the seed were infested with P. betae as determined by us. The seed were superficially treated with 90% ethanol for 1.5 - 2 min., soaked in running distilled water for 1/2 to 1 hr., dried on sterilized filter paper, then immersed in a fixative solution of 5% glutaraldehyde in Millonig's phosphate buffer at pH 7.4 for 1 hr., and post-fixed overnight in 2% osmium tetroxide. Subsequently, the specimens were washed in a phosphate buffer at pH 7.4. The fruit wall was separated from

the seed while in the buffer. Pericarps (opercula and the fruit cavity walls) were dehydrated in a graded ethanol series and cryofractured in liquid nitrogen with a razor blade precooled in liquid nitrogen. Samples were critically point dried, mounted on metal stubs by using silver adhesive paint, and sputter coated with gold. Specimens were examined with a SEM operating at 20-25 KV. Images of secondary electrons were recorded on Polariod type 55 positive-negative film.

Artificially infected flowers were prepared by the same procedures. Inflorescences with black lesions caused by P. betae also were prepared similarly for SEM examination.

## RESULTS

Direct and microscopic examination of plates showed that P. betae was highly associated with the fruit wall up to > 95%, seed coat < 7% and embryo < 2%.

Light microscopic and SEM examination showed that the sugarbeet seed is covered with layers of sclerenchyma cells. There are three openings to the seed: 1) an apical pore in the upper part of the pericarp (operculum); 2) an eccentrically-oriented pore at the base of the lower part of the pericarp (the fruit cavity wall), previously described as the basal pore (9, 12, 17); and 3) a peripheral zone (9) of dehiscence between the upper and lower part of the pericarp.

The pericarp is composed of roughly isodiametric closely-packed sclereids with markedly stratified thickenings, frequent converging pits, and small lumens with occasional crystalline inclusions except at the basal pore (9, 17) and the apical pore. Sclereid cells were thicker in the lower than in the upper part of the pericarp. The exocarp is an unicellular layer of parenchyma cells which dries during the maturation of the fruit. The mesocarp consists of several layers of parenchyma cells which are usually dried by the time the fruits are mature. The endocarp darkens and becomes hard and dries before complete maturation of the fruit. Both the exocarp and the mesocarp are rubbed off along with the other exterior floral parts during seed processing, leaving the seed enclosed in the endocarp, which is commonly known as the pericarp.

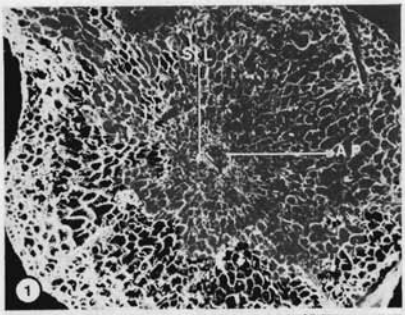


Figure 1. Operculum (outer view) of the pericarp with a central apical pore (AP). Note that the stigmal lobes (StL) persist at the apical pore. (X 20).

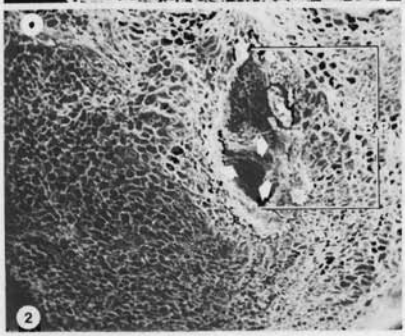


Figure 2. Basal part (outer view) of the pericarp (fruit cavity wall), with eccentric basal pore (BP). Note hyphae (arrows). (X 20).

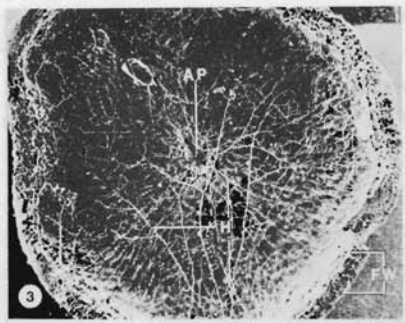


Figure 3. Operculum (inner view) of the pericarp with a central apical pore (AP). Hyphae (H) cover the entire inner surface of the operculum. (FW) fruit wall. (X 20).

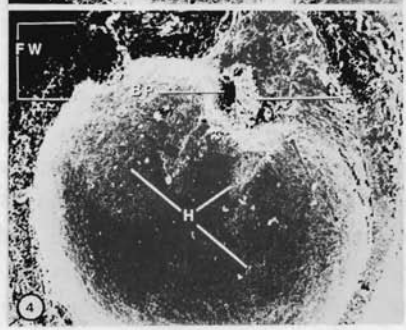


Figure 4. Basal part (inner view) of the fruit wall with the remnant funiculus (F), and the associated basal pore (BP) underneath. Note the fruit cavity is covered entirely with hyphae (H). (FW) fruit wall. (X 25).

The basal pore is located close to the radicle of the embryo. The basal pore is filled with loose cells characteristic of dead, dried parenchyma and conducting tissue. The apical pore is densely clothed with papillate dried cells. The basal pore is almost 10 times larger than the apical pore, but, nevertheless, some apical pores were seen with the naked eye.

Naturally infected fruits were so heavily infected that the hyphae of Phoma betae covered all the inner surface of the pericarp (Figures 3 and 4). Hyphae on the outer surface of the pericarp were observed mostly at the basal pore (Figure 2). Phoma betae and other saprophytes colonized the fruit cavity wall (Figure 4) and the inner surface of the operculum (Figure 3). Some of the stigmatic lobes persisted on the operculum even after the fruit had been processed (Figure 1). Hyphae grew over the funiculus and passed through the dried parenchyma cells near the vascular tissue. Hypha were seen penetrating the fruit cavity through the peripheral zone. Septate, flattened hyphae characterized the resting hyphae located between the seed coat and the pericarp (Figure 5). Figures 1-5 show the exterior surface and the interior of the pericarp of a sugarbeet fruit naturally infected with Phoma betae.

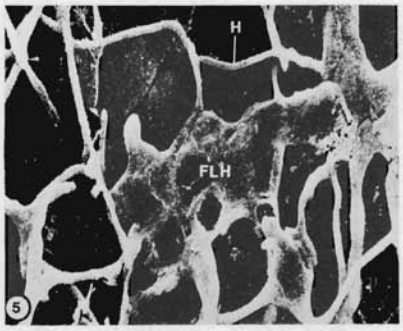


Figure 5. Close view of the hyphae (H) that covered the interior of the pericarp. Note flattened hyphae (FLH) characteristic of the mycelium growing between the seed coat and the pericarp. (X 240).

Anthers and stigmal lobes of artificially inoculated flowers were infected (Figure 6 and 7). Stigmal papillae were completely invaded and surrounded with hyphae. In cross section of the flower (Figure 8), hyphae were seen covering the outer surface, penetrating the exocarp; hyphae became intercellular as well as

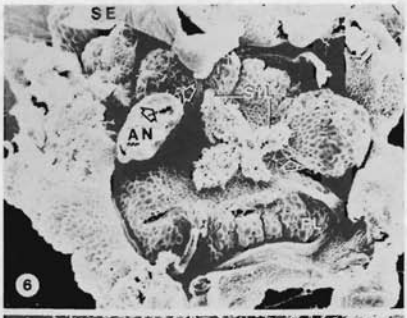


Figure 6. Flower parts include sepals (SE), filment (FL), anther (AN) and stigmatic lobes (StL). Note hyphae (arrows). (X 15).

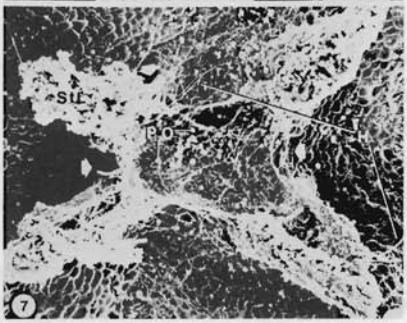


Figure 7. Ungerminated pollen (PO) and hyphae (H) on the surface of a sugarbeet flower near the stigmatic lobes (StL) and the apical pore (arrows) partially covered with the bases of the stigmatic lobes (StL). (X 35).

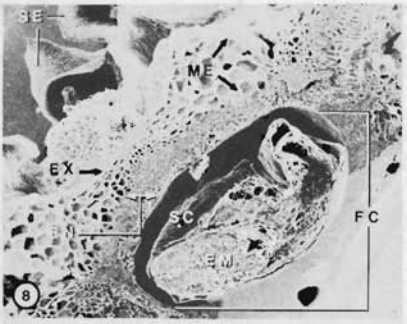


Figure 8. Cross section of immature flower showing embryo (EM), seed coat (SC), fruit cavity (FC), endocarp (EN), mesocarp (ME), exocarp (EX), and sepals (SE). (X 15).

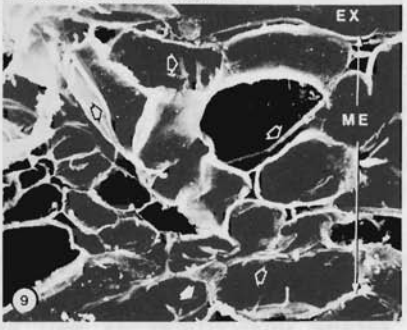


Figure 9. Intra- and inter-cellular hyphae (arrows) have penetrated the exocarp (EX) and the mesocarp (ME) five days after inoculation. Note hyphae passing from cell to cell (solid arrow). (X 240).

intracellular in the mesocarp cell layers (Figure 9). Figure 10 shows a branched hypha with internal cytoplasm that has emerged from the inner side of the apical pore. Figures 6-10 show scanning views of the outer and inner side of sugarbeet flower artificially infected with Phoma betae.

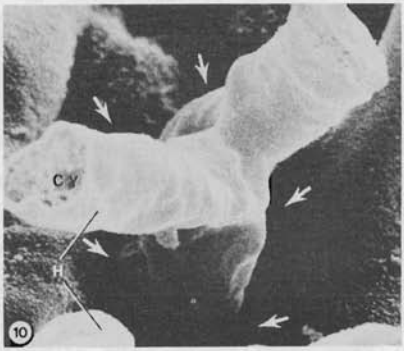


Figure 10. View of infected flower 5 days after inoculation, showing the apical pore (arrows). Forked hyphae (H) penetrated the apical pore into the fruit cavity. Fractured hyphae show the cytoplasm (CY). (X 5200).

Examination of the black lesions that formed on the seed stalks after inoculation showed hyphae extending over the outer surface of the stalk. Nongerminated spores and hyphae mixed with pollen grains were associated with the black lesions. In a cross section through the stalk, an intercellular hypha appeared to penetrate the epidermal layers directly, with continued colonization inside the cortical parenchyma cells. No appressoria or haustoria were seen.

## DISCUSSION

Leach and MacDonald (14) demonstrated that the removal of cortical tissue covering the pericarp during seed processing reduced the percentage of infected fruits; however, infection was still high even after seed processing in some seed lots (14). They categorized the seed lots infected with P. betae as follows: 1) Type A, little or no P. betae (< 5%); 2) Type B, P. betae mostly superficial (5 - 20%); 3) Type C, moderate to severe infection (30 - 60%); and 4) Type D, seeds heavily infected (> 60%). The naturally infected fruits used in our study fall into Type D.

P. betae was associated with the inner fruit wall much more frequently than were other fungi. Isolation from the seed coat

gave a very low frequency of recovery of P. betae, and other fungi were not present. This indicated that P. betae invaded first or has a competitive advantage over other saprophytes. Attempts to isolate P. betae from the cotyledons also gave a very low frequency of recovery. Isolations from the cotyledons that were successful could have been due to contamination of the cotyledons by P. betae in the seed coat during the delicate procedure of separating these two tissues.

When Warren (21) mixed rye pollen with the conidia of P. betae there was an expansion of sugarbeet leaf spots. Anthers and stigma lobes were heavily infected with P. betae (Figures 6 and 7). Hyphae grew over the stigma papillae, and pollen grains were present with the hyphae on the outer surface of the flower (Figures 6 and 7) and stalks. Nectary excretions and pollen grains probably played an important role in the heavy amount of hyphae on the stigma lobes. They also could have stimulated spore germination.

Physiological studies on the effects of water, and chemical agents showed that loosening the operculum or removing it improved seed germination (12, 18). Perry and Harrison (17) claimed that the only passageway for water through the pericarp was the basal pore. Coumans (9) also added that water reached the seed through the basal pore as well as the peripheral zone, and affected seed germination. No one has previously shown the important role of the apical pore as an avenue of entry for air or fungi. The apical pore must be considered as one of the possible ways of entry for fungi, air, and also water.

There are three sites at which a fungus can penetrate the fruit without encountering a mechanical barrier: 1) the apical pore; 2) the basal pore; and 3) the peripheral zone. The microscopic examination described here shows that this does occur. Direct penetration also occurs. Infection hyphae were seen penetrating the exocarpic unicellular layer, eventually becoming inter- and intracellular within the mesocarpic parenchyma cell layers (Figure 9). Hyphae were not seen to penetrate the highly suberized sclerenchyma cell layers of the endocarp.

Hyphae were seen in the dried parenchyma of the funiculus and at the basal pore of naturally infected fruits (Figure 2). Stem lesions of P. betae developed near flowers after inoculation These observations suggest that the fruit could become infected through the basal pore by hyphae progressing from the stem lesions. Also this possibility of infection through the basal pore could increase when the seed stalks are cut and wind-rowed to dry in the field. This late infection could be serious if the fungus is present in the soil and moisture is adequate.

Hyphal penetration through the peripheral zone of the sugar-beet fruit was not seen after artifical inoculation, whereas such penetration was observed in the naturally infected fruits. Flowers used in artificial inoculations were not mature enough for the peripheral zone to have developed.

The stigma unfolds about 7 hr. after the flowers open (1, 2). Infection might occur any time after the flowers are opened, or after the flowers are mature. Infection through the apical pore may occur as early as pollination. Hyphae may penetrate and colonize the fruit cavity before the empryo has matured and the ovule has reached full size. When the embryo continues to enlarge, it would then press the hyphae against the pericarp. This could account for the unique and peculiar flattened hyphae found between the seed coat and the fruit wall in the naturally infected fruits (Figure 5).

If infection happened during embryo maturation, hyphae could reach the fruit cavity either by direct penetration through the exocarp or mesocarp or through the apical pore. Penetration through the apical pore may develop from lateral growth of hyphae that have penetrated the exocarp, the mesocarp, or both (Figure 11).

Ingress through natural openings (basal pore and peripheral zone), obviously, cannot occur until some late stage in fruit maturation, when the openings develop during dehydration of the fruit. Infection of the florocarp could occur early, however. This infection could be reduced when the florocarp parts are removed in seed processing (14). If the environmental conditions remain adequate, particularly moisture, then the fungus could

continue to penetrate the florocarp and progress to the fruit (Figure 11). Once the pathogen is inside the fruit cavity, the fruit would be classified as deeply infected, or Type D.

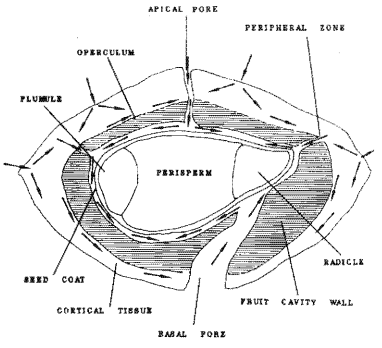


Figure 11. Diagram of a mature sugarbeet seed enclosed in the florocarp. Arrows indicate the possible routes of ingress of P. betae through the different layers and openings of the pericarp.

Leach and MacDonald (14) found that the most effective fungicides for seed treatment gave satisfactory control with processed seed that carried the b or C type infection (14), but were only partially effective with fruits that carried the D type of P. betae infection or with unprocessed seed that carried C type of infection (14). Once the fungus has penetrated deeply inside, there is no way to reduce the amount of infected fruits to a satisfactory level by either seed processing alone, or by seed treatment with insoluble fungicide. The location of P. betae in the fruit cavity of type D-infected seed explains why volatile mercury, or thiram [bis(dimethylthio-carbamoyl)disulfide] seed soaks (16) are the most effective sugarbeet treatments. Direct contact of the fungus with a nonsystemic fungicide can only be accomplished in this way. Without the natural openings previously mentioned, such methods of seed treatment also would be ineffective.

Invasion through the basal pore suggests that seed infection might occur by systemic activity of the fungus. Koch (13) recently claimed systemic infection of seed stalks and seed by P. betae. This must be confirmed, and if true, an ultrastructural examination of this infection should be pursued. Also, further ultrastructural studies are needed in order to obtain information about how primary infection occurs in different stages of flower ontogeny.

## LITERATURE CITED

(1) Artschwager, E. 1927. Development of flowers and seed in the sugarbeet. J. Agric. Res. 34:1-25.

(2) Artschwager, E. and R. C. Starrett. 1933. The time factor in fertilization and embryo development in the sugar beet. J. Agric. Res. 47:823-843.

(3) Bennett, C. W. and L. D. Leach. 1971. Sugarbeet diseases and their control, PP. 223-285. In Advances in sugarbeet production: principles and practices. The Iowa State University Press, Ames, Iowa. 420 p.

(4) Bugbee, W. M. and O. C. Soine. 1974. Survival or Phoma betae in soil. Phytopathology 64:1258-1260.

(5) Bugbee, W. M. 1974. A selective medium for the enumeration and isolation of Phoma betae from soil and seed. Phytopathology 64:706-708.

(6) Bugbee, W. M. 1975. Dispersal of Phoma betae in sugarbeet storage yards. Plant Dis. Rep. 59:396-397.

(7) Byford, W. J. 1972. The incident of sugarbeet seedling disease and effects of seed treatment in England. Plant Pathol. 21:16-19.

(8) Cormack, M. W. and J. E. Mcffatt. 1961. Factors influencing storage decay of sugarbeets Phoma betae and other fungi. Phytopathology 51:3-5.

(9) Coumans, M. 1978. Role du pore basal dans la germination du glomerule de Betterave sucriere. Biologia Plantarum 20:114-118.

(10) Edson, H. A. 1915. Seedling disease of sugar beets and their relation to root-rot and crown-rot. J. Agric. Research. 4:135-168.

(11) Edson, H. A. 1915. Histological relation of sugarbeet seedling and Phoma betae. J. Agric. Research. 4:55-58.

(12) Heydecker, W., R. S. Chetram, and J. C. Heydecker. 1971. Water relations of beet root seed germination, effects of the ovary cap and of the endogenous inhibitors. Ann. Bot. 35:31-42.

(13) Koch, F. and M. Paragio Laku. 1979. Investigation regarding infection and pathogenesis of the sugar beet infected with Phoma betae (Pleospora betae Bjoerl.) Zuckerindustrie 107:611-617. (Translated from German.)

(14) Leach, L. D. and J. D. MacDonald. 1976. Seedborne Phoma betae as influenced by area of sugarbeet production, seed processing and fungicidal seed treatment. J. Am. Soc. Sugar Beet Technol. 19:4-15.

(15) Mangan, A. 1971. A new method for the detection of Pleospora bjoerlingii infection of sugar beet seed. Trans. Brit. Mycol. Soc. 57:169-172.

(16) Maude, R. B., A. S. Vizor, and C. G. Shuring. 1969. The control of fungal seed-borne diseases by means of a thiram seed soak. Ann. App. Biol. 64:245-257.

(17) Perry, D. A. and J. G. Harrison. 1974. Studies on the sensitivity of the monogerm sugarbeet germination to water. Ann. App. Biol. 77:51-60.

(18) Peto, F. H. 1964. Methods of loosening tight seed caps in monogerm seed to improve germination. J. Am. Soc. Sugar Beet Technol. 13:281-286.

(19) Pool, V. W. and M. B. McKay. 1915. Phoma betae on the leaves of the sugar beet. J. Agric. Research 4:169-171.

(20) Tompkins, C. M. and S. B. Nuckolo. 1928. Development of storage disease in sugarbeets. Phytopathology 18:939-941.

(21) Warren, R. C. 1972. Attempts to define and mimic the effects of pollen on the development of lesions caused by Phoma betae inoculated onto sugarbeet leaves. Ann. Appl. Biol. 71:193-200.