Cytology & Development of *Preussia wilsonii*
THIS THESIS ABSTRACT


A new species of Preussia is described and its mode of ascocarp development - including the origins of the structure, its bitunicate asci, and paraphysoids - is elucidated. The cytology of the ascus and spore development is studied. The fungus was successfully cultured on numerous different media.

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The Cytology and Development of *Preussia wilsonii* Stotland Sp. Nov.

by

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The differences and the entire nature of plants are to be explained according to their parts, their reactions and their methods of propagation and life.

Theophrasti, Historia plantarum, L.A.
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1.

INTRODUCTION

A fungus isolated from soil collected in Mexico was determined as a new species of *Preussia* Fuckel. The life cycle, cytology, karyology, and centrum ontogeny of the fungus under surveillance were investigated in detail. The centrum structure was compared with the types described by Luttrell (1951). The fungus belongs to the family Sporomiacae of the Loculoascomycetes and is named *Preussia wilsonii* species nova. Its relation to other species of *Preussia* is discussed.
MATERIALS AND METHODS

The fungus was isolated from soil by Mr. Brendan V. Boylan in 1966, and maintained in the McGill Fungus Culture Collection (FP12-2). The soil sample had been collected in a dry irrigation ditch situated south west of Puebla on Mexican Highway 150.

Three techniques were used in this study:
1. squash mounts
2. growth on cellophane
3. paraffin embedding and sectioning.

1. **Ascocarp Squash**

   **Culturing methods**

   Squashes of ascocarp contents were used for all studies of nuclear behaviour. Except where indicated, the fungus was grown on clear "V-8" juice agar (Miller, 1955). The juice was centrifuged and filtered to remove the particulate matter, and then prepared in the standard manner.

   Cultures were grown in 100 x 22 mm plastic petri dishes at room temperature. The plates were placed under bell jars to reduce contamination and retard desiccation of the medium. Cultures containing mature ascocarps were placed in a refrigerator at 10°C for one or more days before squashing.

   **Fixation and staining**

   The cultures were removed from the refrigerator and fixed
after varying periods of time at room temperature. It was found that greater numbers of prophase, metaphase, and anaphase figures occurred after 15, 30 and 45 minutes at room temperature, respectively. A large block of agar containing numerous ascocarps was cut from the culture and fixed in Farmer's fixative (3:1 absolute ethanol/glacial acetic acid) for three hours. It was then transferred to 70% ethanol at room temperature for storage.

Dividing nuclei were studied with 1% aceto-orcein stain. This was prepared by diluting glacial acetic acid with tap water to make a 70% acid solution. The acid was boiled and 1% natural orcein (Edward Gurr Ltd., London, England) added to it. Subsequently the stain solution was boiled for 10 minutes, cooled, and then filtered.

Crozier nuclei were fixed in weak chrom-acetic fixative (Johansen, 1939) and stained with standard (refluxed) 45% aceto-carmine. One per cent lactophenol-cotton blue (Alexopoulos and Beneke, 1962) and HCl Giemsa stain (Kowalski, 1964) gave similar results.

Ascocarps were removed from stored agar blocks and cleared of attached hyphae by dissecting under a binocular dissecting microscope. Minuten needles (Wards, 1969) were used for all dissections. Five or six ascocarps were transferred to a slide and two or three drops of stain were added. The ascocarps were crushed with a flat needle, the contents squeezed out, and the walls removed with forceps. A number zero coverslip (Corning Glass Co.) was placed over the material on the slide, and the preparation was pressed between sheets of bibulous paper and briefly warmed over a spirit lamp flame. A half paraffin and half gum mastic preparation was used to seal the slides, and they were allowed to mature for 72 hours before use. For the study of ascus and paraphysoid
generation, the standard preparation method required some modification. The expressed ascocarp contents were teased apart with needles to separate the cells of the centrum. Acetocarmine or 1% lactophenol-cotton blue stain was added to the cell mass which was then squashed with extreme finger pressure. Subsequent slide making steps were the same as previously described. The contents of the centrum did not separate satisfactorily when aceto-orcein was used.

Zinc chlor-iodide proved to be the only stain capable of showing the bitunicate condition of the asci. Preparations using 10% KOH, lactophenol-cotton blue, 10% Iodine, or iodine-potassium iodide, failed to give positive results.

2. Growth on Cellophane

Ascocarp initials

Cellophane cultures were used for studying the initiation and early development of ascocarps. Wet, sterile 2 x 5 cm strips of wettable cellophane (no. 220 PT, courtesy of the Dupont Company) were placed on the surface of 2% water agar in petri dish cultures and allowed to dry. Water agar proved to be the only satisfactory medium for this technique, since all other media (e.g. \( \frac{1}{8} \) V-8 agar, \( \frac{1}{2} \) corn meal agar) produced the growth of aerial hyphae which made microscopic study impossible. A small piece of mycelium was placed on the cellophane and allowed to grow for approximately two weeks. The cellophane was then removed from the agar and placed in Farmer's fixative for 15 minutes, transferred to 70% ethanol, and finally placed on a slide and stained with lactophenol-cotton blue.

Riddell mount preparations (Riddell, 1950) did not produce
ascocarp initial cells, although the technique was tried on several occasions with various agar substrates.

**Spore germination**

Petri dishes with water agar and cellophane were prepared as for the study of ascocarp initials. An aqueous spore suspension was prepared from mature, crushed ascocarps. Drops of the suspension were placed on the cellophane together with three or four drops of sterile filtered 3% or 5% glucose solution (the 5% solution was more effective). The petri dish was held vertically to allow the fluid to flow down the cellophane, thus spreading the spores. Cellophane strips were fixed every half hour after inoculation for nine hours, then hourly for a further six hours, and subsequently at irregular intervals for a further 21 hours. The cultures were fixed in situ with Farmer's fixative and the solution was allowed to partially dry. The moist strips were then removed from the agar and placed on a glass slide, 1% aceto-orcein or lactophenol-cotton blue was immediately placed on the strip and a coverslip was added. The excess stain was removed with bibulous paper, taking care not to squash the preparation, and the slide was sealed.

3. **Paraffin Sections - Dehydration, Infiltration, Embedding**

Developmental studies of ascocarps were done with paraffin sections. Blocks of agar, 7 mm², on which abundant ascocarps were growing, were fixed for 20 hours in Randolph's modified Navashin's solution (CRAF) (Johansen, 1939). The tertiary butyl alcohol method was used for dehydration (Johansen, 1939). Paraffin infiltration of the material was done in the standard manner (Johansen, 1939), except
that 61°C "Tissuemat" (Fisher Scientific Company) was used instead of paraffin. This material was also used as the final embedding material. Tissuemat with a melting point of 54°C permitted the cutting of sections only 6 microns or thicker. The addition of piccolyte resin (Coil, 1968) did not result in sections thinner than 6 microns. Therefore 61°C Tissuemat was used. Cultures fixed were from 5 to 103 days old.

**Sectioning, staining, slide-making**

A Jung rotary microtome was used to cut 3, 4, 5, 7, and 12 micron sections. The sections were attached to the slides with Haupt's adhesive (Johansen, 1939). Before use, all slides and coverslips were cleaned in chromic acid, washed in ammoniated alcohol, rinsed in distilled water and dried.

Johansen's haematoxylin schedule was used for rehydration, staining, and permanent slide preparation (Johansen, 1939). Persistent paraffin deposits in sections necessitated three 15-minute xylene baths. The first bath contained warm xylene and the other baths were at room temperature. The iron alum haematoxylin (Heidenhein's preparation) was artificially ripened with 0.05 grams of sodium iodate (Gurr, 1960). The final mounting medium was Euparal. Lead weights were placed on the coverslips to express excess Euparal. The weights were removed after two days and the slides examined after five. Excess Euparal was scraped off with a sharp razor blade or removed with toluene.

**Photography**

All pictures were taken through a Carl Zeiss microscope with apochromatic objectives (unless otherwise stated). Chromosome pictures were made on Kodak High Contrast Copy film, and all other photographs were made on Kodak Panatomic-X film. The films were developed in
Kodak D-19 and Kodak Microdol-X developers respectively. Photographs were printed on Kodak Polycontrast paper F and developed in Kodak Dektol paper developer.
CULTURE CHARACTERISTICS

The fungus was cultured on various media to find a suitable substrate for subsequent studies. At least 10 replicates of each medium were used. All agar preparations were made with 2% Difco agar and the plates were grown in a dry, covered aquarium at room temperature. Colour terminology is taken from the Methuen Handbook of Colour (Kornerup and Wanscher, 1967).

**V-8 Juice agar**

Clear V-8 juice agar cultures produced the greatest number of ascocarps, found in the dense aerial mycelium, which was 0.5 to 1 mm high.

The colony margin of mature cultures was irregular. At first, mycelial growth was rapid but it slowed down with age and eventually stopped. The massed aerial mycelium was white and the overall colony colour was light mallow with a platinum center. Exuded pigment extended 0.5 mm into the agar. The colony's periphery was hyaline, and viewed from the obverse side of the plate, the colony was dark burgundy. (Fig. 38).

**YpSs agar** Emerson's yeast starch agar (Emerson, 1941)

Colony growth was moderate to heavy, though slower than V-8 cultures. The white aerial mycelium was 0.25 mm high. The colony was circular with an irregular margin and was greyish rose to platinum.
Numerous ascocarps were found buried in the mycelium.

**Czapek Dox agar (Difco)**

Ascocarps and aerial hyphae were not seen in cultures on this medium. Only surface and subsurface mycelia were produced. Surface mycelium (when seen under the dissecting scope) was hyaline to white. The colony colour varied from reddish-white in the center to hyaline at the margin. The obverse was light rose, and this pigment extended throughout the agar.

**Malt Salt agar (Christensen, 1965)**

This high osmotic pressure decoction is used for culturing fungus despoilers of stored grain. No growth was seen in all 12 plates, even though inoculation was carried out on two separate occasions.

**Water agar (Difco)**

Double distilled water was mixed with 2% Difco agar and plates were poured. Most of the numerous ascocarps produced were submerged in the agar, but some grew on the surface (Fig. 41). Aerial mycelium was never seen. Thin hyphae formed an irregularly dendritic colony margin. Pigment was lacking.

**C.M.M.Y. agar (Alexopoulos and Beneke, 1962)**

This medium supported rapid mycelial growth. Numerous ascocarps were found on the agar surface amid a moderate amount of aerial hyphae, which were up to 0.25 mm high. The colony was circular, with sparse aerial hyphae at the periphery. The colony was pale grey with a light geranium periphery (Fig. 39). The obverse was geranium with concentric circular bands of black bodies, which were ascocarps.

**P.D.A.**

A moderate number of ascocarps was found on the agar surface
of PDA plates. The slow-growing mycelium often grew into the agar, causing numerous deep furrows. Immediately under the agar surface, a 1.5 mm thick, crust-like layer was noted. The overlying mycelium was white, becoming mallow after eight weeks (Fig. 36). The obverse was claret to raspberry.

1/20 PDA

Standard PDA was diluted with distilled water and used as a substrate. A moderate number of ascocarps were formed on the agar surface, which had aerial hyphae only in the center (Fig. 37). The mycelium was 0.25 mm high after five weeks. Mycelial growth was as slow as on regular PDA, and produced an irregularly lobed colony margin. No furrowing was seen. The aerial mycelium was pale grey with a grey center and the underlying colony colour was cerise to ruby red.

Potato agar

This medium is prepared like PDA, but dextrose is not added. Abundant ascocarps were found on the agar surface in tufts of aerial mycelium. Peripheral ascocarps were all submerged 0.5 to 1 mm below the agar surface. An irregularly lobed colony margin was formed and the mycelium was mostly sessile with raised tufts of aerial hyphae 1 mm high which contained surface ascocarps. The colony had a light cerise background dappled with dark greyish-rose tufts. The obverse was light cerise.

Leonian's agar + Y (Leonian, 1924)

Leonian's agar was prepared with 4 g. per liter yeast extract. Few ascocarps were produced on this agar. Aerial mycelium, up to 0.5 mm high, was white and the colony was circular. Furrows were noted on most plates. A hard crust-like layer was found just below the surface as
in the PDA cultures. The agar surface eventually turned light madeira (Fig. 40).

**Horse Dung cultures**

Freshly collected horse manure was placed in large glass petri dishes and autoclaved for one hour. The dishes had previously been lined with four or five folds of wet paper towelling. Ten dung cultures were inoculated with a spore suspension and were periodically moistened with sterile distilled water to prevent desiccation. Profuse mycelial growth and ascocarp production was seen and some cultures showed the characteristic purplish exudate. No dehiscence of ascocarps was noted, though cultures were observed periodically for three months.

**V-8 broth, static cultures**

Clear V-8 broth was placed in 10 petri dishes and these were inoculated with mycelia and the plates left undisturbed. The fungus produced a floating mat of mycelium and a jelly-like exudate, one cm. thick at maturity. The grey aerial hyphae was one mm high, and the underlying mat was deep red. Numerous ascocarps were found immediately beneath the hyphae.

**V-8 broth shake cultures**

Ten 125 ml Erlenmeyer flasks were each filled with 50 ml V-8 broth and autoclaved. Each flask was inoculated and placed on a rotary shaker, set at low speed. The fungus formed numerous submerged hollow dark mycelial balls, some more than 2 cm in diameter. Ascocarps formed in the interior of these balls after 32 days, but eight weeks passed before ascocarps were seen on the outer surface of the mycelial spheres.
Summary

On the basis of these studies, malt salt agar, potato dextrose agar, and Czapek Dox agar were not used in subsequent experiments. V-8 juice agar was selected for the majority of studies, because of the profuse production of ascocarps. Water agar was selected for cellophane culture experiments, because no undesired aerial hyphae form on that medium. The colour of the exudate, of taxonomic importance in this genus, was fairly constant in most agar media, excepting PDA and Leonian's + Y agar.
ASCOCARP DEVELOPMENT

The fungus does not produce conidia in culture. Ascospores are the only reproductive cells produced on any substrate tested. The fungus is homothallic, since 10 single spore isolates produced asco-
carps.

Ascocarp initials develop in aerial, surface, and subsurface hyphae after two or three days of growth. The ascocarp initial cells are always intercalary, large, swollen and 4.0 to 10.0 μ wide and 4.0 to 6.5 μ long. In constrast, the somatic cells are 1.0 to 3.5 μ wide and 8.0 to 30.0 μ long.

The uninucleate ascocarp initial cells occur in chains of three to nine or sometimes more, formed by the laying down of cross walls in somatic hyphae and subsequently swelling. The first divisions are per-
pendicular to the long axis of the hypha (Fig. 1), and subsequent divi-
sions are internal and at right angles to the first division (Fig. 1). Divisions soon occur in all planes as the cells enlarge (Fig. 2). The ascocarp primordium becomes roughly spherical after about 10 to 12 divisions (Fig. 3). Only after this stage did hyphal fusions occur between elements of the ascocarp and the surrounding hyphae.

In five-day-old cultures, the primordial component cells are thin-walled, completely pseudoparenchymatous, and uninucleate (Fig. 4). The peripheral cells, destined to become the peridium, already have thickened walls and are beginning to darken (Fig. 4).

In eight-day-old cultures, the pseudoparenchyma cells become
less closely aggregated (intercellular spaces are seen), and cell differentiation begins.

**Crozier and Paraphysoid Formation**

The spherical pseudoparenchyma cells in the interior of the centrum, give rise to thin, emergent hyphae (Fig. 5, 15). Since numerous cells produced these hyphae, possibly all pseudoparenchyma cells have the potential to do so. These isodiametric cells may give rise to one, two, or sometimes three hyphal outgrowths (hereafter called thin hyphae) (Fig. 5). Many of the cells shrink as the thin hyphae elongate (Fig. 5). The hyphae, which are 1.0 to 2.5 μ wide and 7.0 to 25.0 μ long, soon elongate and branch (Fig. 5, 7, 13, 14) to form a network. These hyphae are the paraphysoids of the centrum. Soon after pseudoparenchyma differentiation, these same thin hyphae can form shorter and wider cells (hereafter called wide cells) (Fig. 6, 7, 14). These cigar-shaped cells, 1.0 to 4.0 μ wide and 4.0 to 14.5 μ long, are the crozier progenitors. Three or four contiguous wide cells bend over and become a crozier (Fig. 8, 9) or the wide cells may proliferate to form a great mass of wide cells (Fig. 7). These aggregations are ephemeral, probably because these cells also become croziers. Fifteen-day-old cultures have no wide cells, though the thin paraphysoids persist.

No ascogonia, antheridia, or other specialized cells were ever seen in this fungus.

Two difficulties made crozier progenitor studies almost impossible: 1) cells of the centrum proved difficult to separate for observation;
2) connections between thin hyphae, croziers, and asci often degenerate (Fig. 10).

Maturation of the Centrum

No asci or croziers can be found in ascocarps of cultures up to 10 days old. From the 11th day, cultures have recognizable asci and croziers (the latter appear as dark triangles) (Fig. 11, 12, 13). A definite hymenium of proliferating croziers is soon formed, half way up the centrum. The sterile hyphae at first lie in all directions in the locule (Fig. 11, 14, 15), but as the centrum matures the threads begin to grow vertically. These septate hyphae are always attached to both the top and the bottom of the expanding locule and elongate by intercalary growth. Asci grow up between the paraphysoids (Fig. 12) which soon begin to disintegrate.

Mature ascocarps (from the 25th day) are black, cardioid to subglobose, 185 - 800 μ non-ostiolate, and always unilocular. Mature ascocarps have asci in all stages of formation from croziers to mature asci (Fig. 16, 17). Asci containing mature spores are up to 168 μ long and 11.5 - 37.5 μ at the widest; the spore-containing portion is 46.0 - 75.0 μ long. The peridium is one or two cells wide with thick, heavily pigmented walls. These cells are 5.0 - 8.0 x 3.5 - 7.5 μ. A mass of undifferentiated pseudoparenchyma cells surrounds the locule. This layer is wider at the sides and base of the ascocarp than at the top, being 10 cells thick at its thickest and 5 cells at its narrowest (Fig. 11, 12). Older ascocarps have fewer pseudoparenchyma cells than younger ones. The cells are 5.5 - 16 x 4.0 - 9.0 μ. As ascocarps
approach maturity, all the paraphysoids and asci break down and only free ascospores remain.

Because the ascocarp has no ostiole, the ascospores are probably released by mechanical breakage or chemical disintegration of the ascocarp wall. Old ascocarps, in axenic agar cultures, have very soft walls which may facilitate ascocarp dehiscence.
ASCUS DEVELOPMENT AND CYTOLOGY

Asci are formed from numerous croziers in the typical manner (Alexopoulos, 1962). A binucleate cell of the ascogenous hypha elongates, bends over, and the two nuclei undergo conjugate mitotic division. The hooked cell lays down two septa delimiting three cells, a uninucleate ultimate, a binucleate penultimate, and a uninucleate antipenultimate cell (Fig. 19B). The penultimate cell may then grow and form an ascus. Croziers proliferate in a typical Ascomycete way (Alexopoulos, 1962). The ultimate cell grows downward and fuses with the antipenultimate cell (Fig. 19A), to form a binucleate cell. This cell grows out, bends over and repeats the crozier process (Fig. 19C). A large number of asci can originate from one proliferating crozier. The dikaryotic penultimate cell frequently repeated the crozier process instead of directly forming an ascus (Fig. 9, 10). This phenomenon has been reported in other fungi (Moreau and Moreau, 1928).

In ascus production, the nuclei of the penultimate cell eventually fuse, and the cell enlarges and elongates. The ascus eventually undergoes one meiotic division and then three mitotic divisions, in rapid succession.

The chromosomes of early prophase (of the first meiotic division) are extremely diffuse and cluster around the nucleolus (Fig. 21). By mid prophase, the nucleolus disappears and the chromosomes are considerably shortened (Fig. 22), and by late prophase, they resemble com-
pact little dots in both Meiosis I and II (Fig. 25). The chromosomes are most condensed at metaphase of Meiosis I (Fig. 23) and chromosome counts were usually done on material of this stage. The haploid chromosome number is 6 or 7. Nucleoli were never seen after prophase of the first meiotic division and centrioles were never seen.

Nuclei of the second meiotic division can lie in the long plane of the ascus (Fig. 25, 26), or at right angles to this plane (Fig. 24). Soon after meiosis, the first mitotic division occurs. At this stage, the nuclear orientation seems to be random (Fig. 27, 28, 29). The second mitotic division soon follows (Fig. 30). Asynchrony of nuclear division is first seen at the end of the second mitosis (Fig. 31).

Four longitudinal cleavage lines form at the eight-nucleate stage, starting free cell formation. Cross walls are laid down between nuclei immediately after the second and third mitotic divisions; mature ascospores are 4-celled and none ever had two nuclei in one cell. After the third mitotic division the chromosomes become diffuse and interphase nuclei are reconstituted (Fig. 32, 33).

The developing ascospores are hyaline at first with a crinkled wall (Fig. 34), but become dark with a smooth outline when mature (Fig. 35).

Up to the completion of the first mitotic division, many asci have one or two "clear" areas above and/or below the nuclei (Fig. 20, 22, 29). The nature and function of these areas is unknown and, to the author's knowledge, they have not been described in any other Ascomycete (Cain, 1971). The structure may have some role in spore wall formation.
The ascus is bitunicate and consists of two walls (Fig. 46), an outer ectoascus and an inner endoascus. It does not appear to be functional (in dehiscence) since there is no evidence of the capability for extension of the endoascus.
SPORE GERMINATION

Mature ascospores break up into their four component cells, which then function as separate spores. Ascospore cells that had been immersed for four hours in a 5% glucose solution were swollen, and earliest germination occurred at six and a half hours.

The percentage germination, after 24 hours, was 96%. Germination was seen on the germinal slit in 50% of observed ascospore cells. One or two hyphae emerged from each and septa were laid down within half an hour of germination. The germinal hyphae were vacuolate (Fig. 44, 45) and many formed branches soon after germination.

More mature hyphae sometimes formed extensive coils, whose function (if any) was not determined. Ascocarp initial cells were seen in hyphae 72 hours and older.
DISCUSSION

The object of this study was to:

find the new isolate's place in Luttrell's scheme of classification (Luttrell, 1951, 1955),
to elucidate salient points in the life cycle of this new species of Preussia,
and to compare this fungus with other species of its genus and related genera.

Preussia wilsonii is clearly a member of the Loculoascomycetes (sensu Luttrell) since its sexual structures are not ascohymenial and the asci are definitely bitunicate.

Excepting Preussia cylindrica (Malloch and Cain, 1972), all species of Preussia are homothallic. In contrast, the mode of ascocarp development differs widely within this genus and in closely related genera. Meristogenous development is the most common method encountered and has been reported in this study, and in Preussia typharum, Preussia funiculata, Preussia isomera (Kowalski, 1965, 1966, 1968), and Sporormia leporina (Arnold, 1928). Kowalski (1964, 1965) found that Preussia dispersa (Clum) Cain [as Pycnidiohophora dispersa Clum] and Preussia typharum form ascocarps from an intercalary group of three contiguous, uninucleate, reproductive cells, whereas Arnold (1928) saw ascocarps of Sporormia leporina arise from single, swollen, uninucleate cells scattered throughout the vegetative hyphae.
The ascocarps of *Preussia dispersa*, *Preussia funiculata*, and *Preussia isomera* (Kowalski, 1964, 1966, 1968) are formed from single, large swollen cells which are multinucleate. Routien (1956) saw ascocarps arise from single swollen cells located near the tips of hyphae in *Muellerella nigra* Routien [*Preussia nigra* (Routien) Cain].

The number of nuclei in these cells was not described. *Preussia wilsonii* ascocarps are meristogenous, developing from a contiguous group of modified somatic cells. The number of nuclei of these cells was not seen.

Some *Preussia* species are not meristogenous. Fusion of somatic hyphae forms ascocarps in *Preussia flanaganii* (Boylan, 1970) and *Preussia multilocularis* (Maciejowska and Williams, 1963).

The Beatus (1938) and Kowalski (1966) studies of *Preussia funiculata* conflict in many important points of the organism's development. For example, Beatus claimed that the ascocarps of *Preussia funiculata* (Preuss) Fuckel [*as Perisporium funiculatum* Preuss] arose from the fusion of an antheridium and an ascogonium, which were located on hyphal apices. Kowalski did not see ascogonia or antheridia in this fungus and showed that ascocarps develop meristogenously from swollen hyphal cells. It is conceivable that these workers in fact studied different *Preussia* species. Croziers have been described in all *Preussia* species whose ontogeny has been studied. Not all members of the Pleosporales produce croziers. Wehmeyer (1954, 1955) failed to find crozier development in both *Pleospora trichostoma* and *Pleospora armeriae*.

In only a few species of *Preussia* has initial crozier formation
been unequivocally discerned. It was not seen in the *Preussia nigra* (Routien, 1956) and *Preussia multilocularis* studies and was conjectured in *Preussia flanaganii* (Boylan, 1970), where deeply staining cells in the centrum's center were seen. Kowalski (1965) found that croziers initially arose from a few enlarged, multinucleate, ascogonial cells in the *Preussia typharum* ascocarp. In the cases of *Preussia dispersa* and *Preussia funiculata* (Kowalski, 1964, 1966), numerous, enlarged, multinucleate cells (located in the center of the centrum) gave rise to the first croziers. Preliminary crozier development in *Preussia isomera* (Kowalski, 1968) is similar, but the enlarged cells are uninucleate.

Numerous pseudoparenchyma cells, physically indistinguishable from their neighbouring cells, produced crozier initials in *Preussia wilsonii*. Most of the pseudoparenchyma cells of the centrum have the potential to form croziers or paraphysoids or both.

Centrum organization in *Preussia* species ranges from ascocarps composed of an irregular mass of cells with scattered asci and paraphyses, to sexual structures with organized cell types and a definite hymenium. The asci of *Preussia cylindrica* (Malloch and Cain, 1972) and *Preussia flanaganii* (Boylan, 1970) are scattered throughout the centrum, whereas those of *Preussia isomera* (Kowalski, 1968) are more organized, radiating out in all directions from the center to the peridium. Asci in *Preussia dispersa* (Kowalski, 1964) are found scattered in fascicles in the centrum, as are the asci of *Preussia multispora* (Saito and Minoura) Cain, (Stolk, 1955, Thompson and Williams, 1966 [as *Pseudoportunium multisporum* (Saito and Minoura) Stolk]). All the other *Preussia* species that have been subjected to developmental studies have asci in a hymenial layer. These species are *Preussia nigra* (Routien, 1956), *Preussia mul-
tilocularis (Maciejowska and Williams, 1963), Preussia typharum and
Preussia funiculata (Kowalski, 1965 and 1966), and Preussia wilsonii.

Bitunicate asci have been demonstrated unequivocally in only
three species of the genus Preussia: Preussia wilsonii, Preussia nigra
(Routien, 1956) and Preussia multilocularis (Maciejowska and Williams,
1963).

In Luttrell's classification bitunicate asci are essential for
membership in the Loculoascomycetes, of which Preussia is a member.

It is the author's opinion that the ascus wall number should
not be so important a consideration in the taxonomy of the cleisto-
thecial ascomycetes. The frequent difficulty in ascertaining the num-
ber of ascus walls and the non-functioning of this spore release mechanism
(the bitunicate ascus degenerates, in most cleistothecial ascomycetes,
prior to spore dispersal) makes this taxonomic character of little prac-
tical value.

Preussia wilsonii has all the characteristics of the genus
Preussia and differs significantly from all described species of the
genus. It most closely resembles Preussia typharum but differs from
it in chromosome number, mode of ascus initiation, uniloculate centrum,
and non-production of sterile ascocarps. Preussia wilsonii and Preussia
typharum differ significantly in the measurements of ascospore cells,
somatic and fertile hypha size, and ascocarp diameter.

The author hereby proposes to add this new isolate to the genus
Preussia as a new species.

Ascocarpis subglobosis 185 - 800 μ diam., in agaro superficialibus, inclusis vel inter hyphas aerias. Ascocarpis nigris, non ostiolatis, congregatis vel separatis, uniloculatis. Peridio uno vel duo strato cellulis composito. Ascis bitunicatis, clavatis, usque ad 168 μ long., 46.5 - 75.0 μ diam., stipitibus longis., octosporis. Ascosporis triseptis, septis transversis. Ascosporis cellulis subjunctis maturis, cellulis terminalibus ovalibus 5 - 7 x 6 - 10 μ, cellulis medianis 5 - 7 x 5 - 8 μ. Hilo germinali obliquo.
Preussia wilsonii, Sp. nov.

Ascocarps subglobose, 185 - 800 μ in diameter, superficial or immersed in agar, sometimes growing in aerial hyphae above the agar, black, shining, glabrous, leathery, non-ostiolate, densely aggregated or separate, strictly uniloculate. Peridium thick-walled, one or two cells thick. Asci bitunicate, up to 168.0 μ long, 46.5 - 75.0 μ wide at the widest, clavate with a long narrow stipe. Ascospores eight per ascus, always trisepate, with transverse septa. Ascospore cells separate at maturity, end cells longer, more oval 6.0 - 10.0 μ long, 5.0 - 7.0 μ wide, median cells shorter, 5.0 - 8.0 μ long, 5.0 - 7.5 μ wide. Germininal slit longitudinal, extending obliquely entire length of each segment. No conidia, spermogonia, ascogonia or trichogynes. Preussia wilsonii grows on most common agar media, horse dung, and hemp seeds immersed in water.
SUMMARY

1) The new isolate first described here has characters which clearly place it in the genus *Preussia* as a new species.
2) Three to nine or more contiguous swollen, almost spherical cells give rise to the ascocarp.
3) The fungus is uniloculate.
4) Pseudoparenchyma cells give rise to thin hyphae which become paraphysoids. Thin hyphae also develop into wide cells and croziers.
5) Croziers are formed in the typical manner. A definite hymenium is formed.
6) Asci, paraphysoids, and croziers disappear when the ascocarp is mature, only spores remain.
7) No pycnidia or conidia were ever seen in culture.
8) The ascus' nuclear cycle follows a pattern similar to that found in other Euascomycetes.
9) Fusion of two haploid nuclei occurs in the ascus. A meiotic division is soon followed, in rapid succession, by three mitotic divisions.
10) Free cell formation takes place after the first mitotic division.
11) Mature ascospores are four-celled with one nucleus per cell. The ascus contains 32 cells, in all. At maturity the cells of the ascospores separate and become individual spores.
12) The haploid chromosome number is six or seven.
Fig. 1  Ascocarp progenitor cells. The upper hypha is divided in one plane, the lower (more advanced) in two planes. Note the normal vegetative hypha above the fertile cells. Cellophane culture (x 1,080).

Fig. 2  Ascocarp initial divisions have occurred in three planes. Cellophane culture (x 1,080).

Fig. 3  An older ascocarp initial, which has now become spherical. Cellophane culture (x 1,080).

Fig. 4  Very young ascocarp with uninucleate pseudoparenchyma cells, and peridial cells just becoming thick-walled and darker. Paraffin section (x 216).

Fig. 5  Ascocarp pseudoparenchyma cells differentiating into thin hyphae (paraphysoids). a) cell with one outgrowth  b) cell with two outgrowths  c) progenitor cell is degenerating  d) hyphal outgrowth is branched. Dissected squash (x 1,000 phase contrast).

Fig. 6  A progenitor cell (P) is attached to a thin hypha and two wide cells (W). Dissected squash (x 1,040).

Fig. 7  Wide cells (W) and paraphysoids (PS). Dissected squash (x 490).

Fig. 8  Pseudoparenchyma cell (P), with a thin hyphal outgrowth, producing a proliferating crozier (c). An extraneous cell (EXT) lies above the thin hypha. Dissected squash (x 1,480).
Fig. 9  A thin hypha (th) is connected to an ascus (a) and a proliferating crozier (c). The arrow denotes a connective hypha. Dissected squash (x 1,120 phase contrast).

Fig. 10  Degenerating connecting cells (bars) join an ascus and several proliferating croziers together. Connecting cells could barely be seen with bright field optics. Dissected squash (x 1,120 phase contrast).
PLATE II
SECTIONED MATERIAL

Fig. 11 Vertical section of a young ascocarp, with croziers (the triangles at the base of the centrum). Note the clear area beneath the fertile cells. (x 430).

Fig. 12 Vertical section of a young ascocarp. Croziers and a few immature asci can be seen at the lower left of the centrum. Note the difference in pseudoparenchyma thickness above and to either side of the centrum (x 430).

Fig. 13 Vertical section of a young ascocarp. Note proliferating croziers (lower left), and the paraphysoid which can be traced from top of the centrum to the base (line). Some branched paraphysoids can be seen. (x 430).

Fig. 14 Vertical section of a part of the centrum and ascocarp wall, showing a branched thin hypha (th) which is attached to a wide cell (W) and a darkly staining cell (an ascus?). (x 1,350).

Fig. 15 Vertical section of a part of the centrum. Thin pseudoparenchyma cells (bars) giving rise to thin hyphae. (x 1,080).

Fig. 16 A mature ascocarp, with asci in all stages of development. (x 325).

Fig. 17 A typical ascocarp. Note the ascogenous hyphae. (x 210).

Fig. 18 An 85 day old ascocarp with only ascospores in the centrum. (x 165).
PLATE III
CYTOLOGY

Fig. 19 Three dikaryotic croziers; in a, the penultimate cell is elongating and the ultimate and antipenultimate have fused (bar). In c, the crozier is proliferating. (x 2,370).

Fig. 20 Two developing asci, note the single diploid fusion nucleus in each. (x 1,320).

Fig. 21 A nucleus in early prophase of Meiosis I. Note the degenerating nucleolus and the diffuse chromosomes. (x 1,320).

Fig. 22 Mid prophase of Meiosis I, the chromosomes are more compact and the nucleolus has disappeared. (x 1,320).

Fig. 23 Metaphase of Meiosis I. (x 1,560).

Fig. 24 Metaphase of Meiosis II, the nuclei are at right angles to the long axis of the ascus. (x 1,320).

Fig. 25 Late prophase of Meiosis II; the nuclei are on the long axis of the ascus. There appear to be six or seven chromosomes. (x 1,320).

Fig. 26 Metaphase of Meiosis II. (x 1,320).

Fig. 27 Mid prophase of first division of mitosis. (x 1,320).

Fig. 28 Metaphase of mitosis I, the bar indicates the position of the fourth nucleus. (x 1,320).

Fig. 29 Late metaphase of mitosis I; note the lighter area (bar) above the nuclei. (x 1,320).
Fig. 30  Telophase of the first mitosis. The nuclei are scattered. (x, 1320).

Fig. 31  Anaphase and telophase of the second mitosis. Synchrony of nuclear division is lost at this stage. Note the cleavage lines. (x 1,320).

Fig. 32  The cells of the future ascospores are now delimited by cross-walls. (x 1,080).

Fig. 33  An ascus at the end of the third and last mitotic division. All but one of the ascospores (bar) have interphase nuclei. Spore walls are present but unclear in this preparation. (x 1,760).

Fig. 34  An ascus with mostly hyaline immature ascospores. Note the wrinkled walls. (x 1,000).

Fig. 35  Mature dark-coloured spores with germinal slits (bars) are visible in this ascus. (x 900).
Fig. 36  PDA.  Note the deep furrowing in the center and the irregular colony margin.

Fig. 37  1/20 PDA.  There are abundant aerial hyphae in the center.

Fig. 38  V-8 juice agar.  The numerous ascocarps are covered by abundant mycelium except at the periphery.

Fig. 39  C.M.M.Y. agar.  Note the ascocarps at the periphery.

Fig. 40  Leonians agar with yeast extract.  The furrows in the colony center are evident.

Fig. 41  Water agar.  There is no aerial mycelium though ascocarps are formed and are hyaline (and invisible in this figure).  The bar indicates an artifact.
Fig. 42  Hemp seen with abundant ascocarps and mycelium. The culture is 63 days old. (x 10).

Fig. 43  Germinating ascospore. (x 1,240).

Fig. 44  A germ tube with abundant vacuoles. Note germinal slits. (x 1,320).

Fig. 45  Twenty hour old germinated spore. Note the cross-walls and vacuoles. (x 528).

Fig. 46  A bitunicate ascus; the long bar denotes the ectoascus, the small bar may be the ectoascus of another ascus. a) is an artifact. Phase contrast (x 820).
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