Origin and formation of perithecia of *Claviceps paspali* Stev. et Hall

Nicolò Oddo (*) e Antonio TonoLO

Laboratori di Chimica Biologica, Centro Internazionale di Chimica Microbiologica

**Summary.** — This paper reports studies of the initial phases of perithecial development in *Claviceps paspali* (Stev. et Hall). Stroma of *Claviceps paspali* originates from the inner layer of sclerotium. The young binucleated sexual cells, the archicarps, arise from the inner layer of sphaeridium.

From archicarps unicellular, multinucleate ascogonia develop. Ascogonium produces multinucleate ascogenous hyphae which contain pairs of dicariotic nuclei; these hyphae form the centrum of the perithecium. No antheridia are visible. The following development phase is formation of asci from ascogenous hyphae through characteristic croziers, while perithecial wall develops.

A sudden increase in the number of nuclei in the ascogonium is not detectable, which suggests that plasmogamy does not take place. These observations point out that the occurring sexual event is autogamy, contrary to what occurs in *Claviceps purpurea* (Fr.) Tul. where plasmogamy between antheridium and ascogonium has been found and nuclear frequencies in copulating ascogonia sharply rise from 12 to 24 per cell.

On the basis of several features which differentiate *Claviceps purpurea* from *Claviceps paspali* (see «Conclusion and Discussion») it is suggested that *Claviceps paspali* might not be a member of the genus *Claviceps* but could be better classified as the type of a new genus: *Mothesia*.

**Riassunto.** (Origine e formazione dei periteci di *Claviceps paspali* Stev. et Hall). — In questo lavoro si descrivono le fasi iniziali dello sviluppo del peritecio in *Claviceps paspali* Stev. et Hall.

(*) Ospite dei Laboratori di Chimica Biologica.
Dallo strato più interno dello sclerizio si origina lo stroma (Tav. I, 2), al di sotto della superficie del quale si differenziano cellule binucleate intensamente colorate: gli archicarpi.

Dagli archicarpi si sviluppano gli ascogoni, unicellulari e multinucleati (Tav. II, 1, 2, 3). L'ascogonio produce ife ascogene multinucleate nelle quali i nuclei sono a dicarion; queste ife formano la regione basale del peritecio ancora immaturo. Il passo successivo dello sviluppo mostra la formazione degli aschi dalle ife ascogene per mezzo di caratteristi „uncini” (Tav. III, 1, 2), mentre si forma la parete del peritecio.

Non sono state osservate fusioni cellulari in nessuno stadio dello sviluppo, e ciò è in accordo con l'aumento dei nuclei per cellula sessuale, che si realizza senza discontinuità (Fig. 1, 2, 3). Queste osservazioni suggeriscono l'ipotesi di essere in presenza di un fenomeno autogamico, a differenza di ciò che avviene in Claviceps purpurea (Fr.) Tul. dove è stata osservata la plasmogamia tra l'ascogonio e l'antieridio (Tav. III, 4) e si trova una discontinuità nell'aumento dei nuclei per cellula sessuale, tra i valori 12 e 24 (Fig. 4).

In considerazione di queste differenze tra Claviceps paspali e Claviceps purpurea e di altre differenze relative alla struttura dello sclerizio, alle ascospore ed alla loro germinazione ed alla produzione di alcaloidi (vedi „Conclusion and Discussion”) si considera la possibile convenienza di creare un nuovo genere, Mothesia, con la specie Mothesia paspali (Stev. et Hall) comb. nov. (syn. Claviceps paspali Stev. et Hall) come tipo.

The modern classification of Pyrenomycetes (ALEXOPOULOS, 1962; GAÜMANN, 1964; LUTTRELL, 1951; MULLER & VON ARX, 1954; 1962; MUNK, 1957) is based, not only on the morphology of the perithecium, but also on the conformation and disposition of ascospores, on the structure of the ascus, and above all, on the initial phases of the development of the perithecium — the sexual phenomena that take place before the formation of the ascus.

In the order Clavicipetales fam. Clavicipetaceae (ALEXOPOULOS, 1962; GAÜMANN, 1964) the origin and structure of the ascogonium and antheridium have been studied in a few species only.

During the initial phases of perithecium development in Claviceps purpurea (Fr.) Tul. (KILLIAN, 1919) bulky and intensely stained cells (archicarps) may be seen in the sphaeridial plectenchyma. Subsequently these cells produce two branches of which one will be the ascogonium and the other the antheridium. After plasmogamy, ascogonia produce ascogenous hyphae. Nuclear fusion followed by meiosis then gives rise to the ascus and ascospores. In Claviceps microcephala (Wallr.) Tul. (VINCENT, 1917) only filamentous ascogonia between which plasmogamy occurs have been described. Antheridia are absent (parthenogamy; GAÜMANN, 1964).
A similar type of sexual development has been observed in *Cordyceps agariciformia* (Bolt.) Seaver (Jenkins, 1934), and in *Cordyceps militaris* (Linn.) Link. (Boodan Varitchak, 1927).

In *Epichloë typhina* (Pers.) Tul. (Docuet, 1960) no sexual differentiation can be observed. This suggests the possibility that autogamy may occur in this species.

Because of the importance of *Claviceps paspali* Stev. et Hall in the production of alkaloids in submerged culture (Tonoło, Scotti & Vero, 1961; Arcamone et al., 1961; Pacifici, Kelleher & Schwarting, 1962; Gröger & Tyler, 1963) study of the initial phases of perithecial development in this species was desirable. This paper reports such studies.

**MATERIALS AND METHODS**

*Source of sclerotia and their germination.*

Sclerotia of *Claviceps paspali* Stev. et Hall on infected plants of *Paspalum distichum* L. var. *paspalodes* Thell (Grasso, Lenzi & Tonoło, 1962) were collected in 1961 in Sabaudia (Latina; Italy). These sclerotia were stored for one year at room temperature. The following technique was employed for their germination: sclerotia were placed in Petri dishes on sterile sand moistened with tap water and stored at 5°C for one month. The Petri dishes were then placed at 24°C, the sand being moistened regularly. The emergence of the stroma (Bisby & Ainsworth, 1961) from the sclerotium began after 15 days.

The stromata were removed with a lancet and fixed. Various treatments involving different fixing fluids were used as indicated below.

**Fixation.**

The following fixing fluids were used:

(a) Fleming's strong (Sass, 1951): chromic acid 1 % 7.5 ml
    acetic acid 0.5 ml
    osmic acid 1 % 2.0 ml

(b) acetic acid-absol. ethyl alcohol 1:3

(c) osmic acid 1 % (vapour)

(d) absol. ethyl alcohol

**Embedding and sectioning.**

Stromata were embedded in paraffin (M.P. 55-58°C) with 0.5 % beeswax, and sections 4-6 µ thick obtained with a rotating microtome. Slides were also prepared by crushing ripe stromata.

The following staining methods were employed:

(a) Iron haematoxylin
(b) Meyer's haemalum
(c) HCl-Giemsa (ROBINOW, 1942)

Iron haematoxylin and haemalum did not give good results. The best results were obtained adapting to stromata slices the HCl-Giemsa stain that had previously given good results in staining nuclei of *Penicillium chrysogenum* Thom (CARILLI & TONOLO, 1959) and of *Penicillium purpurogenum* Stoll (TONOLO & CARILLI, 1959).

The technique used was as follows:

(a) Fix stroma in absol. ethyl alcohol for 24-48 h
(b) Embed in paraffin-wax
(c) Section, and unwrinkle slices by spreading on distilled water at 40°C
(d) Place paraffin sections on slides previously passed through a flame
(e) Dry sections in 40°C for 24-48 h
(f) Remove paraffin and hydrate slices in the descending series of alcohols (xylene I; xylene II; absol. ethyl alcohol-xylene 1:1; absol. ethyl alcohol; ethyl alcohol 80°; ethyl alcohol 60°; ethyl alcohol 30°; ethyl alcohol 15°; distilled water)
(g) Wash in distilled water for 1 h (changing water after 1/2 h)
(h) Fix sections with vapour from a solution of 1% osmic acid (the wet slide is placed, sections facing downwards, on the orifice of a large necked bottle, containing the osmic acid solution)
(i) Hydrolise for 12 min in 1 N HCl at 60°C
(j) Wash quickly in tap water
(k) Wash for 5 min in M/15 Sörensen buffer solution at pH 7.2
(l) Stain. Slides are placed for 1 h in the stain solution (2 ml of Merek's Giemsa in 80 ml of M/15 Sörensen buffer solution at pH 7.2)
(m) Wash quickly in distilled water
(n) Dry on the flame
(o) Mount in dammara (Merek)

All the observations were made with a Leitz Ortholux microscope with an immersion objective 100 X and a periplan ocular 10 X.

RESULTS

Cytology of the ripe sclerotium.

As has been described in early work (STEWART, 1957; GRASSO & TONOLO, 1964) the ripe sclerotium of *Claviceps paspali* Stevens et Hall consists of two clearly distinguishable layers (Plate I, I): the outer one (prosenchyma) with loose structure, the inner one (plectenchyma) with a more compact structure, formed of polygonal cells.

In the cells of the outer layer nuclei have most been seen. This observation suggests that it may consist of dead cells. The cells of the inner layer have only one nucleus, rarely two, placed in the centre of the cell.

*Origin and cytology of stroma.*

The stroma originates from the inner layer of the sclerotium after a phase of cell division. In the initial phase of development, the stroma differentiates into a sphaeridium and a stalk (Plate I, 2). The young cells of the stroma are oblong with 4-9 nuclei in each cell. After rapid cell division and growth the stroma emerges from the sclerotium. The elongation of the stroma results from cell division throughout the stalk, the basal region being particularly active. Archicarp formation takes place early in stroma elongation.

*Structure of stalk.*

During the initial phases of the development of the stroma there are no morphological differences between sclerotial and stalk cells. The distal end of the stalk, however, shows morphological differentiation. The early stages consist of cylindrical-prismatic cells 13 μ long, 3-4 μ wide and containing from 1-6 nuclei.

In the final stage the stalk consists of typical hyphal cells: their long axis is parallel to that of the stalk. The number of nuclei per cell is generally greater than in the younger stages and varies from 4 to 14; length is from 25 to 27 μ, while width is 4.5-5 μ. Important differences between inner and outer cells have not been observed.

Nuclear division and high nuclear frequencies per cell have been observed throughout the stalk. This suggests that growth is intercalary rather than apical. This contrasts with the growth of mycelial hyphae which is apical (TonoLO & URBANI, 1952).

*Structure of sphaeridium.*

Early in development it is possible to distinguish clearly sphaeridium cells from stalk cells (Plate I, 2). However, boundary cells between stalk and sphaeridium may exhibit intermediate features. Sphaeridial cells are more easily stained, are smaller (4-6 μ) and have from 2-6 nuclei.

Later in the development of the sphaeridium it is possible to distinguish clearly two layers: an outer layer, or ectostrona, 25 μ thick consisting of cylindrical-prismatic cells 13 μ × 5 μ with 1-4 nuclei, and an inner layer, or endostrona, consisting of polygonal isodiamic cells 6-7 μ in diameter with 2-3 nuclei. The differentiation into ectostrona and endostrona

is completed at approximately the same time as peritheciun development commences and the characteristic protuberances on the spheridial surface (which later bear the ostioles) appear.

*Origin and development of sexual cells.*

(a) *Archicarps.* — The archicarps (KILLIAN, 1919), from which sexual cells arise, have a rounded configuration, are readily stained and have large compact nuclei which are easily observed. They arise from undifferentiated cells of the endostroma, about 100 μ from the surface of the sphaeridium. They are first recognizable as binucleated cells 4-5 μ diam. Subsequently it enlarges to 7-8 μ diam. and contains up to 6 nuclei.

(b) *Ascogonia.* — In Figs. 1, 2, 3 the number of nuclei in archicarps and in sexual cells developed from archicarps are graphically recorded. As indicated in paragraph (a) the archicarps are the initial stage of sexual development and contain less than 6 nuclei. Cells with 6 or more nuclei are regarded as differentiated sexual cells, from which mature sexual cells will originate.

The sexual organs normal in Ascomycetes are the ascogonium with trichogyne and antheridium and various modifications of this basic pattern. In Claviceps paspali Stev. et Hall, however, male sexual organs (antheridia) have never been found nor has plasmogamy between sexual cells differentiated from archicarps been observed. Instead the archicarp develops into

![Figure 3](image)

Fig. 3. — The number of nuclei in sexual cells of Claviceps paspali in a mature sphaeridium. Note the absence of archicarps.

a large cell 5-8 μ long (Plate II, 1), hence the sexual event observed is an autogamic apandrogamy. This is established by the frequency of the nuclei in the cells concerned which are what would be expected to result from successive nuclear divisions: in Figs. 1, 2, 3 maxima are observed at 6, 12, 18 and 24 nuclei per cell, but along with virtually all intermediate values. If, however, sexual heterogamy or parthenogamy had occurred, intermediate frequencies would have been almost absent. This occurs in Claviceps purpurea (Fr.) Tul. (Fig. 4) where virtually no nuclear frequencies between 12 and 24

![Figure 4](image)

Fig. 4. — The number of nuclei in sexual cells of Claviceps purpurea. Note the absence of counts intermediate between 12 and 24 nuclei per cell.

per cell were observed, which suggests (Killian, 1919) that plasmogamy occurs between two cells with 12 nuclei each.

Hence, the round archicarp, with 5 or fewer nuclei develops into the first phase of the ascogonium which has 6 nuclei and is sometimes branched.
Branched (Plate II, 1) or unbranched ascogonia (Plate II, 2, 3) also occur later in development and have 12 nuclei. Mature ascogonia with 18 or 24 nuclei are all of the branched form. The final phase of ascogonium development is a cell with a maximum of 24 nuclei. At this stage the further differentiation of the ascogonium begins with the ascogenous hyphae and the perithecial wall.

**Origin and development of asci and ascospores.**

In slides prepared by crushing ripe stromata (Grasso & Tonolo, 1964) the development of ascogenous hyphae can be seen, and the formation of characteristic «croziers» with pairs of nuclei (dicaryotic stage) is easily distinguishable (Plate III, 1, 2). Subsequently in the young ascus diploidization occurs giving rise to a large diploid nucleus (Plate III, 3). Meiosis then occurs and ascospores are produced.

Ripe ascii are cylindrical, oblong, unitunicate with a typical apical swelling; they measure 100 - 132 μ × 5 - 7 μ. The ascus contain 8 ascospores; they are slender, wirelike, hyaline, pointed, with only one septum; they measure 60 - 90 μ × 1 - 2 μ. Each of the two cells of the ascospore has one nucleus.

**Germination of ascospores.**

Germination of ascospores occurs 36-48 h after their discharge from the ascus and begins with the swelling of one or both cells. In 70% of the ascospores only one cell swells and germinates; in the remainder both cells swell and germinate (Grasso & Tonolo, 1964).

**Origin and development of perithea.**

Endostromatic cells surrounding the ascogonium elongate and become oriented parallel to each other to form the perithecial wall, which is ca. 27 μ thick. Subsequently, cells within the perithecium lyse and the perithecial cavity forms.

**CONCLUSION AND DISCUSSION**

It has been suggested (Gaumann, 1964) that within the order Clavicipitales it is possible to trace three lines of evolution, as indicated below:

<table>
<thead>
<tr>
<th>Mycomalus Ascopogorus</th>
<th>Claviceps</th>
<th>Cordyceps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypocreella</td>
<td>Balansia</td>
<td></td>
</tr>
<tr>
<td>Oomyces</td>
<td>Ophiidotis</td>
<td></td>
</tr>
<tr>
<td>Hypomyces</td>
<td>Epichloë</td>
<td></td>
</tr>
<tr>
<td>Aspergillales</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It has not been possible to find in the literature a description of the sexual development of the genera *Oomyces, Hypocrella, Mycomalus, Ascopolyporus*. In the two species of *Cordyceps* studied (Boodan Varitchak, 1927; Jenkins, 1934) there is no antheridium and the ascogonium develops aphanously. The *Epichloë-Claviceps* line of evolution is one of increasingly advanced stromatic development, reaching a maximum in *Claviceps*. In *Balansia claviceps* Speg. and *Balansia cynodontis* Sid. the outer layer of the pseudostroma consists of separated hyphae, not typical plectenchyma. This pseudostroma is similar to the plectenchyma of the outer layer of the sclerotium in *Claviceps paspali*, and differs from the sclerotium of *Claviceps purpurea*.

The evolutionary sequence from *Epichloë* to *Claviceps* suggested by the character of the stromata is supported by the features of the sexual cells in these genera. In *Epichloë* the sexual cells have the primitive archicarpal configuration (Dogue, 1960).

The sexual characters of *Ophiodotis* and *Balansia* are unfortunately not known. In *Claviceps paspali* there is an advanced form of sexual reproduction; ascogonia are present. *Claviceps purpurea* is still more advanced in its sexual characters (ascogonia and antheridia are present) (Plate III, 4) and sclerotial development. Important features which differ in *Claviceps purpurea* and *C. paspali* are summarized in Table 1.

<table>
<thead>
<tr>
<th></th>
<th><em>Claviceps paspali</em></th>
<th><em>Claviceps purpurea</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sclerotium</strong></td>
<td>yellow-dark with an outer diffuse plectenchymatous layer</td>
<td>violaceous with an outer compact plectenchymatous layer</td>
</tr>
<tr>
<td><strong>sexual cells</strong></td>
<td>ascogonium but no antheridium and trichogyne (autogamy)</td>
<td>ascogonium and antheridium (eterogamy)</td>
</tr>
<tr>
<td><strong>ascospores</strong></td>
<td>single septum only</td>
<td>many septa (*)</td>
</tr>
<tr>
<td><strong>germination of</strong></td>
<td>germ tube emergence preceded by swelling of the whole cell</td>
<td>germ tube emergence preceded by a swelling of the cell but</td>
</tr>
<tr>
<td><strong>ascospores</strong></td>
<td></td>
<td>limited to the region near the nucleus</td>
</tr>
<tr>
<td><strong>alkaloid production</strong></td>
<td>only the α-ethoxyamide of lysergic acid</td>
<td>various derivatives of lysergic acid, mainly with polypeptide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>side chains</td>
</tr>
</tbody>
</table>

(*) Personal observations, unpublished.

A consideration of these characters and of the analogy between the morphology of the sclerotia of *Claviceps paspali* and pseudostroma of some species of *Balansia*, suggests that *Claviceps paspali* Stev. et Hall is best re-
garded not as a member of the genus *Claviceps*, but as belonging to a distinct new genus, *Mothesia* (*\(^*\)*), intermediate between *Balansia* and *Claviceps*, as indicated below:

<table>
<thead>
<tr>
<th>Mycomalus Ascopolyporus</th>
<th><em>Claviceps</em></th>
<th><em>Cordyceps</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypocrella</td>
<td><em>Mothesia</em></td>
<td></td>
</tr>
<tr>
<td>Oomyces</td>
<td><em>Balansia</em></td>
<td></td>
</tr>
<tr>
<td>Hypomyces</td>
<td><em>Ophiodotis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Epichloë</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspergillales</td>
</tr>
</tbody>
</table>

This new genus should include:

*Claviceps paspali* Stev. et Hall; type species;
*Claviceps gigantea* Fuentes, de la Isla, Ullstrup, Rodriguez;
*Claviceps grohii* Groves;
*Claviceps* sp. from *Pennisetum typhoideum* Rich;
*Claviceps* sp. from *Sorghum* sp.

because of homology of their cultural characters and related alkaloids' production in saprophytic culture.

Further studies about number of ascospores' septa and sexual characters in these species should give further support to the validity of this new genus.

We thank Prof. E. B. Chain, F. R. S., and Dr. L. Vero Barcellona for their interest, and Mr. M. Carlile Ph. d. for the interesting discussion and translation into English of the Italian manuscript.

5 ottobre 1966.

REFERENCES


(*\(^*\)* We dedicate this possible genus to Prof. Kurt Mothes, Director of the Institute of Plant Biochemistry, Halle, Saale, Germany.


Gnöger, D. & V. E. Tyler, Jr., 1963. Alkaloid production by Claviceps paspali in submerged culture. Lloydia, 26, a. 3.


Luttrell, E. S., 1951. Taxonomy of the Pyrenomycetes. The Curators of the University of Missouri, Columbia, Missouri.


1: Section of sclerotium of *Claviceps paspali* (200 ×)

a: loose textured outer layer; b: compact inner layer

2: Section of young stroma (80 ×)

a: region where ascoconia develop
PLATE II.

1: Young ascogonium with eight nuclei (1300 ×)
2: Ascogonia in various development phases (1300 ×)
3: Branched ascogonium with 18 nuclei (2200 ×)

PLATE III.

4: Antheridium and ascogonium of *Claviceps purpurea* Fr. (Tul.) (1500 ×)