Ultrastructure of *Hanseniaspora* ascospores

N. J. W. KREGER-VAN RIJ

Laboratory of Medical Microbiology and Laboratory of Electron Microscopy, Department of Biology, State University, Groningen, The Netherlands

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A comparative study of the ultrastructure in sections of the ascospores of six *Hanseniaspora* species showed three types of spores: (1) hat-shaped in *H. valbyensis* and *H. guilliermondii*, (2) spherical with an equatorial or subequatorial ledge, smooth or rough in *H. occidentalis* and *H. uvarum*, (3) spherical with warts in *H. osmophila* and *H. vineae*. Development and germination of the spores of *Hanseniaspora guilliermondii* is described in more detail.

INTRODUCTION

The genus *Hanseniaspora* includes species with three types of ascospores: *H. valbyensis* and *H. guilliermondii* have hat-shaped spores; *H. uvarum* and *H. occidentalis* have spherical or subglobose spores with an equatorial or subequatorial ledge, in *H. uvarum* the wall is slightly warty and in *H. occidentalis* it is smooth; *H. osmophila* and *H. vineae* have spherical spores with distinct warts. Kreger-van Rij and Ahearn (1968) have described the ultrastructure of the spores of *H. uvarum* and compared it with that of *H. valbyensis* and *H. guilliermondii*.

The present study primarily concerns development and germination of the ascospores of *H. guilliermondii*. In addition, development of the spores of *H. valbyensis*, *H. uvarum*, *H. occidentalis*, *H. osmophila* and *H. vineae* was examined.

MATERIALS AND METHODS

A strain of *Hanseniaspora guilliermondii* Pijper was isolated from a fresh fig bought in Jerusalem, by incubating a piece of it in malt extract for 2 days at

37 C, plating out on malt agar and incubating again at 37 C. A piece of the same fig incubated in malt extract at 25 C and subcultured on malt agar at 25 C produced a pure culture of *H. uvarum* (Niehaus) Shehata, Mrak et Phaff. All other strains were received from the Yeast Division of the Centraalbureau voor Schimmelcultures at Delft: *H. valbyensis* Klöcker, CBS 6681; *H. occidentalis* Smith, CBS 2592; *H. osmophila* (Niehaus) Phaff, Miller et Shifrine, CBS 313, the original strain described by Niehaus (1932); *H. vineae* van der Walt et Tscheuschner, CBS 2171, considered to be synonymous with *H. osmophila* by Miller and Phaff (1958).

Sporulating cultures were obtained as follows:
H. guilliermondii: on malt agar for 1–3 days at room temperature or at 25 C; *H. valbyensis*: on 5% Difco malt agar for 2 days at room temperature. When the culture was suspended in water, small clumps of cells could be isolated which contained many spores; *H. uvarum*: on 5% Difco malt agar for 2 days at 25 C; *H. occidentalis*: on 10% malt agar for 2 days at 25 C; *H. osmophila*: on 3% malt agar for 4 days at room temperature; *H. vineae*: on Gorodkowa agar for 2 days at room temperature.

Germinating spores of *H. guilliermondii* were obtained by inoculating a sporulating culture on malt agar and incubating for 3 h at 25 C, or by shaking the sporulating culture in malt extract for 2 h at 37 C.

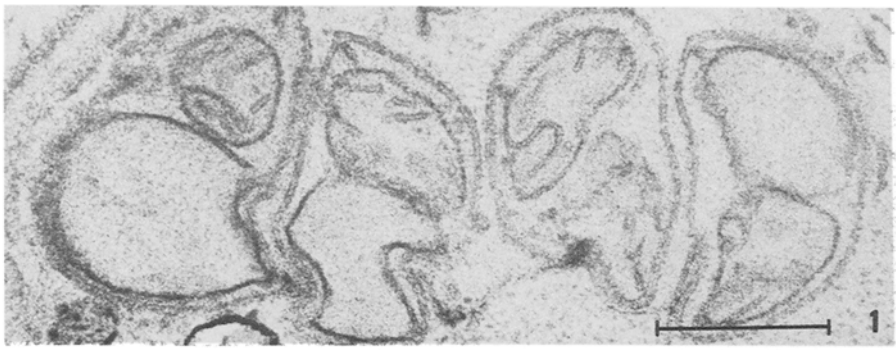
Malt extract 15° Balling was self-prepared from malt; malt agar was prepared with malt extract of 10° Balling, 3% and 10% malt agar with respectively 3% and 10% of this liquid.

For electron microscopy, the material was fixed with 1.5% $KMnO_4$ for 20 min at room temperature, dehydrated through an ethanol series, and embedded in Spurr's resin (1969). During dehydration, the fixed cells were stained with uranyl acetate.

RESULTS

The following stages of ascospore development of *Hanseniaspora guilliermondii* were observed in sections. A number of cells about to turn into asci had a lobed, probably meiotic nucleus, often with a membranous structure attached to it. A prospore wall consisting of two unit membranes enveloped each of the four nuclei formed after meiosis and other organelles (Fig. 1). The first devel-

The marker represents 0.5 μ.
Figs. 1–8. Sections through ascospores of *Hanseniaspora guilliermondii*.
Fig. 1. Four very young spores, each delimited by a prospore wall consisting of two unit membranes.
Fig. 2. Young ascospore with ledge (L).
Fig. 3. The wall of the young spore is thickened on the inside of the ledge.
Fig. 4. The rest of the spore wall develops.
Fig. 5. Ascus with bud (B). The wall of the ascospores has a light inner layer, a greyish part consisting of the thickening near the ledge and the ledge itself, and a thin dark outer layer.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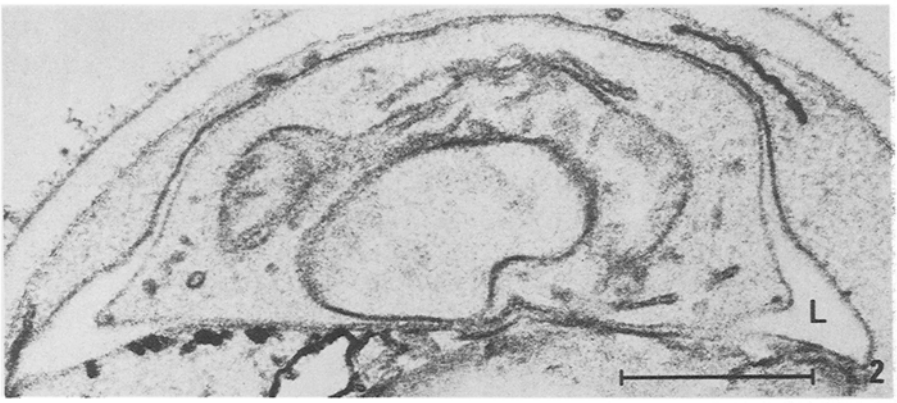
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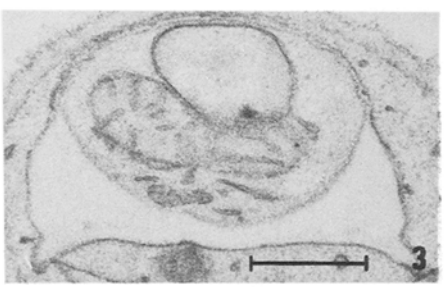
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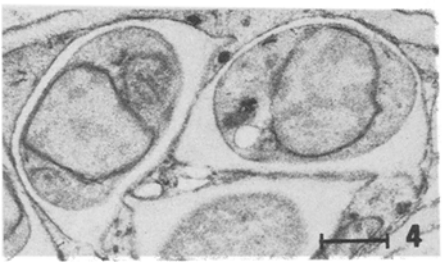
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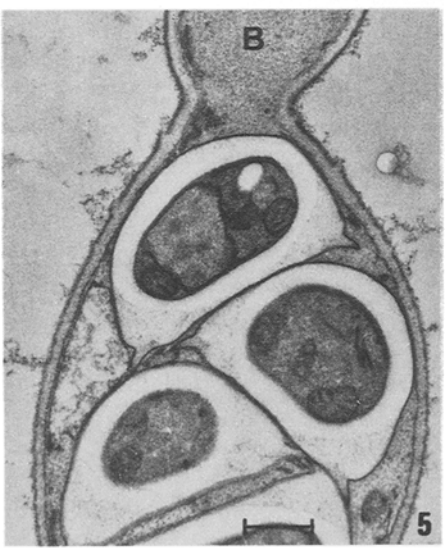
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opment of the prospore wall was a small ledge at the base of the hemispherical spore (Fig. 2). The next stage was a thickening of the wall at the site of the ledge (Fig. 3). The thickened part was electron-light and the small protruding ledge somewhat darker. After this, the rest of the prospore wall filled out with light material (Fig. 4). Compared with this, the first-formed thickening was greyish and clearly distinguishable from the light part (Fig. 5). In mature spores, still in the ascus or liberated from it, greyish material was visible around the protoplast within the light layer (Fig. 6). It formed a more or less distinct ring, lighter inside and darker at the edge. It is not known whether this layer is the partly changed light inner layer or a new layer.

During germination of the spores, the thin dark outer layer, which was the original outer membrane of the prospore wall, broke up and the greyish ring around the protoplast became the wall of the vegetative cell which emerged from the rest of the spore wall and formed buds (Figs. 7, 8). The rest of the spore wall consisted of the light part outside the grey layer, the thickened part near the ledge and the ledge itself.

Of the other *Hanseniaspora* species studied, *H. valbyensis* had ascospores of similar development and shape as *H. guilliermondii* (Fig. 9). The spores of *H. valbyensis* were also liberated from the ascus, but there were usually only two of them per ascus. *Hanseniaspora uvarum*, *H. occidentalis*, *H. osmophila* and *H. vineae* had one, seldom two spores in each ascus which did not come free. The asci were generally bigger than the vegetative cells and they had sometimes become spherical.

The wall of the ascospores of *H. uvarum* and *H. occidentalis* developed from a prospore wall on which a distinct ledge was formed early. The outer membrane of the prospore wall was darker than the plasmalemma at that time. After the formation of the ledge, the wall filled up with light material whilst the ledge was somewhat darker. At first, the wall was smooth and it remained so in *H. occidentalis* (Fig. 10). In *H. uvarum* small dark warts were formed (Fig. 11). In both species the ledge might be irregular; in sections of some spores it was observed on one side only and the width also varied.

In some, presumably mature, spores of *H. uvarum* and *H. occidentalis* a thin greyish layer was visible around the protoplast. However, it is not possible to say whether this constitutes the wall of the future vegetative cell without germination experiments.

The ascospores of *H. osmophila* and *H. vineae* were similar: without a ledge, but warty. The wall developed from a prospore wall; the outer membrane was darker than the inner one at an early stage. A light wall was formed between the membranes (Fig. 12). The outer part of the light layer became darker and warts arose, first as slight protrusions (Fig. 13), later more pronounced and irregular (Fig. 14). Some of them contained light spots. Mature spores had, apart from the warts, a broad dark outer layer and a light inner layer. Preservation of them was bad.

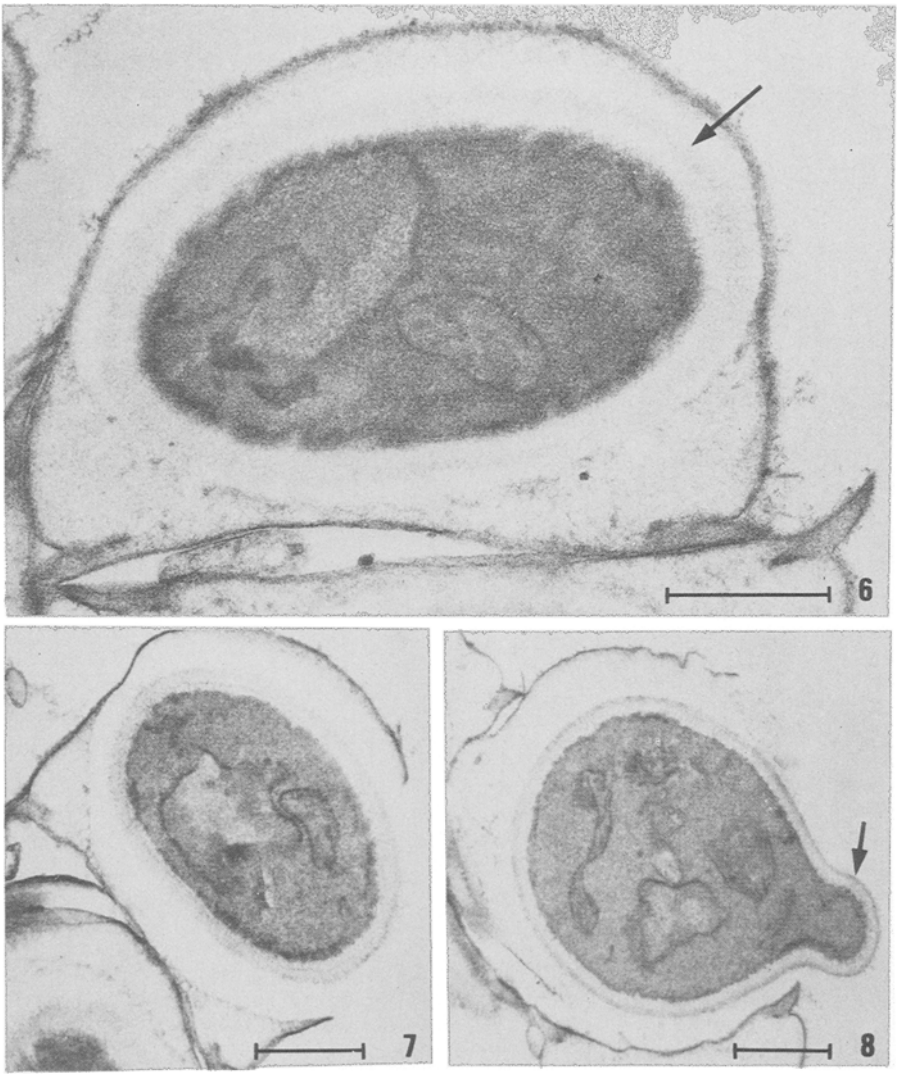

Fig. 6. Free, mature spore showing a thin ring of greyish material around the protoplast (arrow).
Fig. 7. Germinating ascospore. The dark outer layer has broken and the greyish inner layer has become the wall of the vegetative cell.
Fig. 8. Germinated spore with bud (arrow).

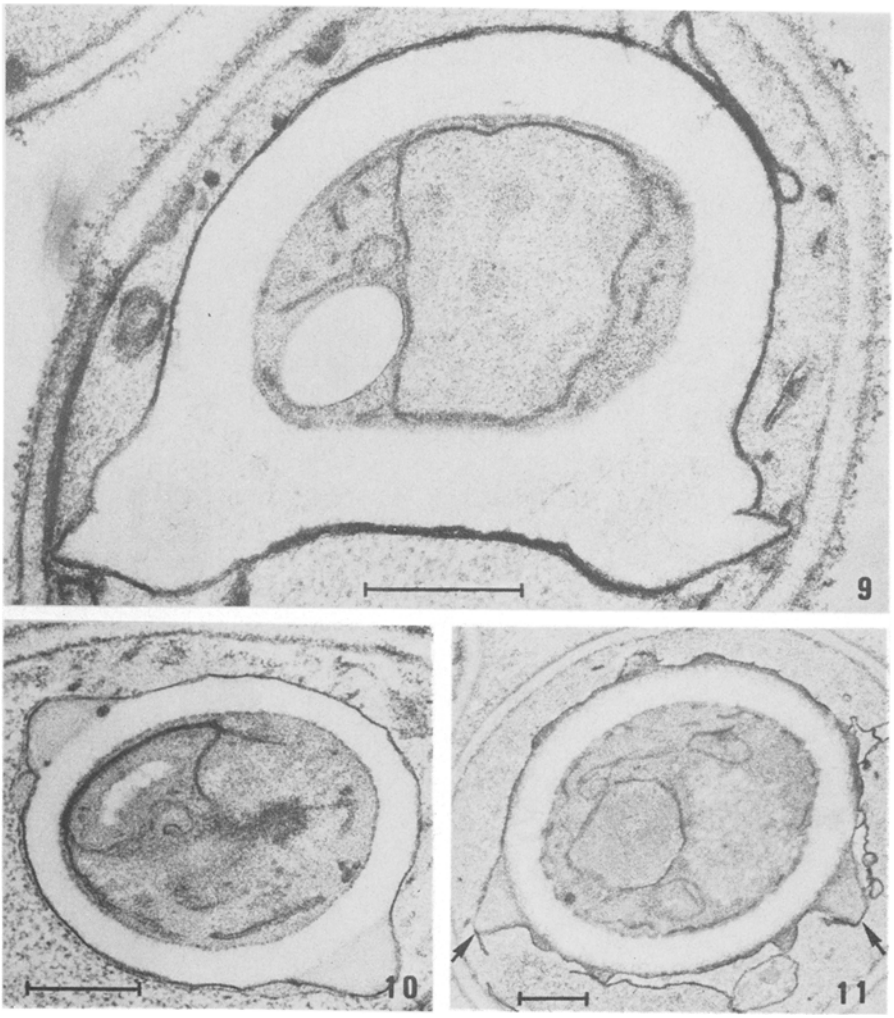

Fig. 9. Ascospore of *H. valbyensis*. The thickened part of the wall with the ledge is slightly darker than the rest of the wall. A greyish layer around the protoplast is not yet visible.
Fig. 10. Ascospore of *H. occidentalis*. The wall is light with a darker, narrow ledge and a thin, dark, smooth outer layer.
Fig. 11. Ascospore of *H. uvarum*. It has a ledge (arrows) and small, irregular dark warts.

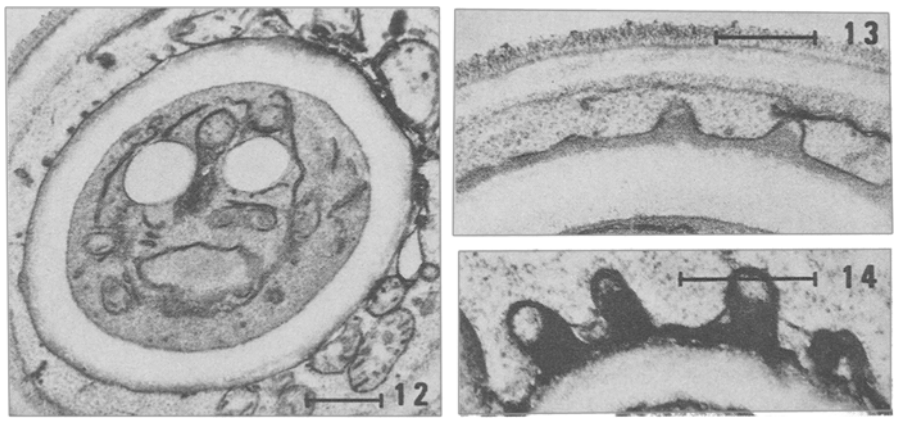

Fig. 12. Young spore of *H. vineae*. The wall is still smooth.
Fig. 13. Part of the wall of an immature spore of *H. vineae*. The wall has a broad light inner layer and a darker layer including the small warts around it.
Fig. 14. Wall of mature spore of *H. vineae* with large, irregular dark warts containing light spots.

DISCUSSION

Development (Fig. 15) and germination of the ascospores of *H. guilliermondii* follows a pattern which was also observed in a number of *Pichia* and *Hansenula* species with hat-shaped spores (unpublished), and may probably serve as a model for this type of spore.

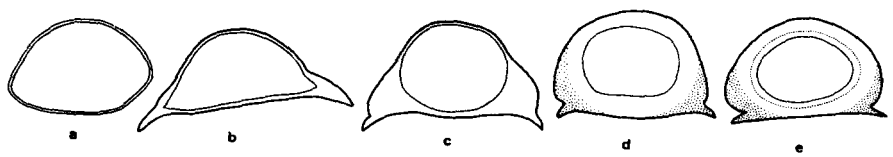

Fig. 15. Diagram of development of the wall of an ascospore of *Hanseniaspora guilliermondii*. a. prospore wall; b. a ledge is present; c. thickening of the wall on the inside of the ledge; d. a light inner layer has been formed, the ledge and the thickening near it are greyish; e. a layer of greyish material is present around the protoplast; it has developed at the inside of the light layer and it will become the wall of the vegetative cell after germination of the spore.

The ledge, if present on *Hanseniaspora* spores, was formed early, before extension of the prospore wall; this was also found in species of other genera such as *Saccharomycopsis capsularis* (Kreger-van Rij and Veenhuis, 1975) and *Schwanniomyces alluvius* (Kreger-van Rij, 1977). Warts were formed towards the end of the development of the spore wall. Turning of the inner layer of the spore wall into the wall of the vegetative cell during germination, which was shown for *Hanseniaspora guilliermondii*, has also been described for *Pichia pijperi* (Kreger-van Rij, 1969).

Although the shape and structure of the ascospores of the *Hanseniaspora* species, with the three different types described above, vary considerably, other taxonomic features show a close resemblance, such as vegetative reproduction by bipolar budding and the physiological properties. The latter are clearly different from species in other genera with bipolar budding. Another character similar for all *Hanseniaspora* species is the method of diploidization as described by Miller and Phaff (1958) and Phaff (1970). These authors found that the nucleus divided during germination of the spores and fused directly afterwards, so that the first bud on the germinating ascospore was diploid. In contrast with this proposed diploidization was the occurrence of buds on part of the asci now observed in all *Hanseniaspora* species (Fig. 5). The buds had an open connection with the mother cell and remnants of a separating wall were never found. Study of the nuclear behaviour may elucidate this phenomenon.

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