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Hormonema schizolunatum, a new species of dothideaceous black yeasts from phyllosphere

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Abstract

Two black yeast isolates from plants from the Canary Islands (Spain) are described and illustrated. Absence of Woronin bodies at simple septal pores, local coralloid terminal hyphal cells, indeterminate thallus maturation, the presence of budding cells and local conversion to meristematic growth all indicate a relationship to the Dothideaceae (Dothideales, Ascomycota). Morphological properties were consistent with the genus *Hormonema* Lagerberg & Melin, as defined by presence of percurrent conidiogenous loci alongside undifferentiated hyphae, and results of PCR-ribotyping supported this classification. The isolates were judged to belong to a hitherto undescribed species, characterized in particular by curved conidia soon developing transverse septa. The physiological profile of this species is also described.

Introduction

In the course of a study on yeasts inhabiting plants on the Canary Islands (Spain), several isolates with a peculiar macroscopic appearance were found. Colonies initially were whitish or pink, gradually turned purple and eventually became black. Yeast cells predominated in initial cultures, while old cultures converted to meristematic growth or to mycelium. The hyphae produced conidia by percurrent conidiogenesis from flat scars. This morphology is consistent with the black yeast genus *Hormonema* Lagerberg & Melin. Several isolates were subsequently identified as *H. dematioides* Lagerberg & Melin, but two isolates with lunate conidia were judged to represent a hitherto undescribed species. This taxon is presented below.

Materials and methods

Strains and culture conditions

Host plants were identified according to the monographs of Bramwell and Bramwell (1974) and Hohenester and Wells (1993). Strains CBS 706.95 and 707.95 were isolated from giant reed, *Arundo donax* L. (fam. Poaceae) harvested in Fuerteventura and from Canarian sage, *Salvia canariensis* L. var. *candidissima* Bolle (fam. Lamiaceae) taken from the Barranco de Arguineguín, Gran Canaria, respectively. Amounts of 5–10 g of plant material were cut to pieces of about 1 cm^2 and suspended in 50 ml sterile basal growth medium (Middelhoven et al. 1991), diluted 1/10 in sterile water with 1 $g.l^{-1}$ Tween 80. After shaking at 25 °C for 60 min, the suspension was poured into sterile culture tubes which were left overnight at 4 °C. Most of the supernatant was discarded. The lower 3–5 ml was shaken in order to resuspend the settled yeast cells. This suspension was streaked onto YMA plates (pH 4.5). After 3, 5 and 12 days different colonies were transferred to YMA plates. The cultures were maintained on YMA slants.

Table 1. Standard physiological pattern of *Hormonema schizolunatum*

	706.95	707.95		706.95	707.95
D-Glucose	+	+	D-Galacturonate	w	w
D-Galactose	w	w	DL-Lactate	-	-
L-Sorbose	+	+	Succinate	w	w
D-Glucosamine	-	-	Citrate	-	-
D-Ribose	w	w	Methanol	-	-
D-Xylose	+	+	Ethanol	+	w
L-Arabinose	+	+	n-Hexadecane	-	-
D-Arabinose	+	+	Propane-2,3-diol	-	-
L-Rhamnose	+	w	Butane-2,3-diol	-	-
Sucrose	+	+	Nitrate	+	+
Maltose	+	+	Nitrite	+	+
α,α-Trehalose	+	+	Ethylamine	+	+
methyl-α-D-Glucoside	-	-	L-Lysine	+	+
Cellobiose	w	+	Cadaverine	+	+
Salicin	-	-	Creatine	w	-
Arbutin	-	-	Creatinine	-	-
Melibiose	+	w	Glucosamine	-	-
Lactose	-	-	Imidazole	-	-
Raffinose	+	+	n-Hexadecane	-	-
Melezitose	+	+	5% $MgCl_2$	+	+
Inulin	-	-	10% $MgCl_2$	+	+
Sol. starch	+	+	5% NaCl	+	+
Glycerol	+	+	10% NaCl	+	+
meso-Erythritol	+	+	9% NaCl/0.5% glucose	+	+
Ribitol	+	w	50% Glucose	w	w
Xylitol	+	+	0.01% Cycloheximide	-	-
L-Arabinitol	+	w	Mycosel	-	-
D-Glucitol	+	+	Urease	+	+
D-Mannitol	+	+	DBB	-	-
Galactitol	-	-	30C	w	w
myo-Inositol	-	-	37C	-	-
keto-2-D-Gluconate	-	-	Fermentation	-	-
keto-5-D-Gluconate	-	-	Gelatin	-	-
Glucono-δ-lactone	-	-	DNAse	+	+
D-Gluconate	-	w	w/o Vitamins	-	-
D-Glucoronate	w	w			

Morphology and physiology

Light microscopy was based on cultures grown on 4% malt extract (MEA4), oatmeal (OA) and potato-carrot (PCA) agars. Slides were made in water or in Melzer's reagent. Isolates were examined twice for their cultural, morphological and physiological properties according to the standard methods adopted for yeast identification (Van der Walt & Yarrow 1984) and repeated once by methods adapted for black yeasts (De Hoog et al. 1995), respectively. Comparison with described taxa was performed using a physiological database on dothideaceous and herpotrichiellaceous black yeasts available at the CBS. For assessment of growth on potentially toxic compounds the slant culture method (Middelhoven et al. 1991; Middelhoven 1993) was used. Non-toxic compounds were tested in liquid mineral growth medium (Middelhoven et al. 1991) which has the composition of Difco Yeast Nitrogen Base but with a tenfold higher phosphate concentration. For electron microscopy, cells grown in standing liquid 1% glucose/peptone/yeast extract (YPG) were

Table 2. Physiology of *Hormonema schizolunatum*: unusual compounds

	706.95	707.95		706.95	707.95
Butylamine	-	-	Phloroglucinol	-	-
Putrescine	-	-	Pyrogallol	-	-
Xylan	+	+	Orcinol	-	-
Pectin	-	-	Saccharate	-	w
Polygalacturonic acid	-	-	Gallate	-	-
Carboxymethyl cellulose	-	-	Vanillate	-	-
3-Hydroxycinnamate	+	-	Ferulate	-	-
Phenol	-	-	4-Hydroxyphenylacetate	-	-
Hydroquinone	-	-	3-Hydroxybenzoic acid	+	-
Quinate	-	w			

Figure 1. *Hormonema schizolunatum*, CBS 707.95, 3-day-old culture on PCA. Hyphae producing conidia repetitively from short butts.

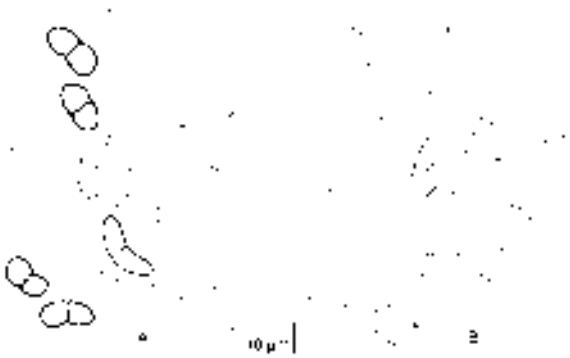

Figure 2. *Hormonema schizolunatum*, conidia. A. CBS 707.95, 3-day-old culture on PCA, conidia gradually converting to meristematic cells. B. CBS 706.95, 4-day-old culture on PCA.

washed with water, fixed in potassium permanganate (1.5% w/v) for 20 min, suspended in agar, dehydrated, post-stained with saturated uranyl acetate in absolute ethanol, washed with absolute ethanol, embedded in Epon, cut, poststained with lead citrate and observed in a Jeol transmission electron microscope model JEM-1200 EX II.

Cell wall composition

Cells were grown for 5 days in liquid 4% malt extract at 24 °C under continuous shaking at 150 rpm, harvested by centrifugation and washed twice with physiological saline. Hydrolysis was carried out in 1 N HCl under N_2 for 12 h at 100 °C. The hydrolysate was trimethylsilylated with Tri.Sil (Pierce Chem. Co., Rockville, U.S.A.) and analyzed with a gas-liquid chromatograph with fused silica capillary columns using helium as carrier gas. The oven temperature was 100 °C for 3 min, followed by heating to 250 °C at a rate of 7.5 °C.min^{-1}; total analysis time 15 min.

DNA isolation

Two ml of cell suspension was centrifuged at 14000 rpm and cells were washed twice with sterile water. The resulting pellets (approximately 100 μl) were stored at - 70 °C. For DNA isolation, pellets were dissolved in 0.5 ml TES buffer (0.1 mM Tris-HCl, pH 8.0; 10 mM EDTA, 2% SDS); approximately 400 μl of glass beads (diam 0.45–0.50 mm) were added. After homogenization, the mixture was vortexed for at least 10 min. Proteinase K was added and

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Figures 4–7.
Figure 4. Hormonema schizolunatum, CBS 707.95, 1-day-old culture on 0.2% YPGA. Young, unswollen conidia.
Figure 5. Hormonema schizolunatum, CBS 707.95, 3-day-old culture on 0.2% YPGA. Conidia converting to chlamydospore-like cells.
Figure 6. Hormonema schizolunatum, CBS 706.95, 1-day-old culture on 0.2% YPGA. Hyphae converting to meristematic cells.
Figure 7. Hormonema schizolunatum, CBS 707.95, 3-day-old culture in 1% YPG. Transmission electron microscopy.

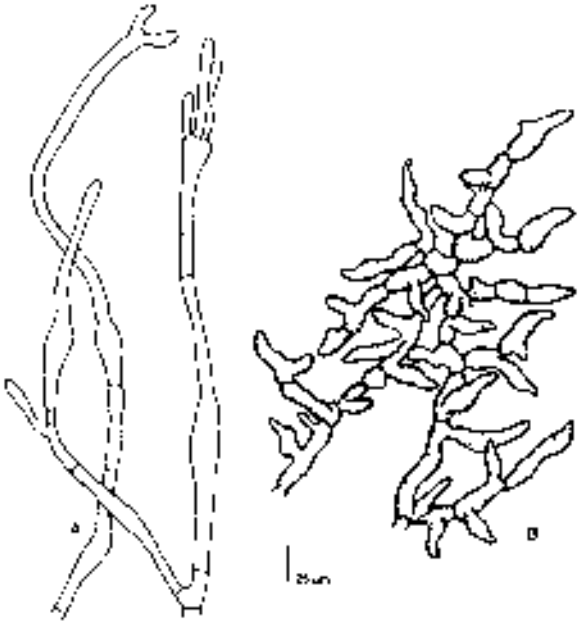


Figure 3. Hormonema schizolunatum, branching systems. A. CBS 706.95, 3-day-old culture on MEA4, expanding hyphae with di- and trichotomous apices. B. CBS 707.95, 10-day-old culture on OA. Coralloid hyphae.

the mixture was incubated for 30 min at 55–60 °C. After centrifugation, the supernatant was transferred to a new tube. Subsequently the procedure of Möller et al. (1992) was followed, starting with the addition of CTAB (cetyltrimethylammonium bromide) in high salt concentration. The resulting DNA pellet was dissolved in water and the DNA concentration was determined spectrophotometrically.

DNA amplification

Polymerase chain reaction (PCR) was performed in 50 μl volumes of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate, 50 pmol of each primer, 10–100 ng nuclear DNA and 0.25 U of Taq DNA polymerase (Super Taq; Sphaero Q, Leiden, The Netherlands). Primers NS1, NS24, ITS1 and ITS4 (White et al. 1990) and 5.8S-LR7 (Yurlova et al. 1996) were employed. Forty amplification cycles were performed: 94 °C, 1 min; 48 °C, 2 min; 74 °C, 3 min (delay was 1 min) in a Biomed thermocycler (type 60).

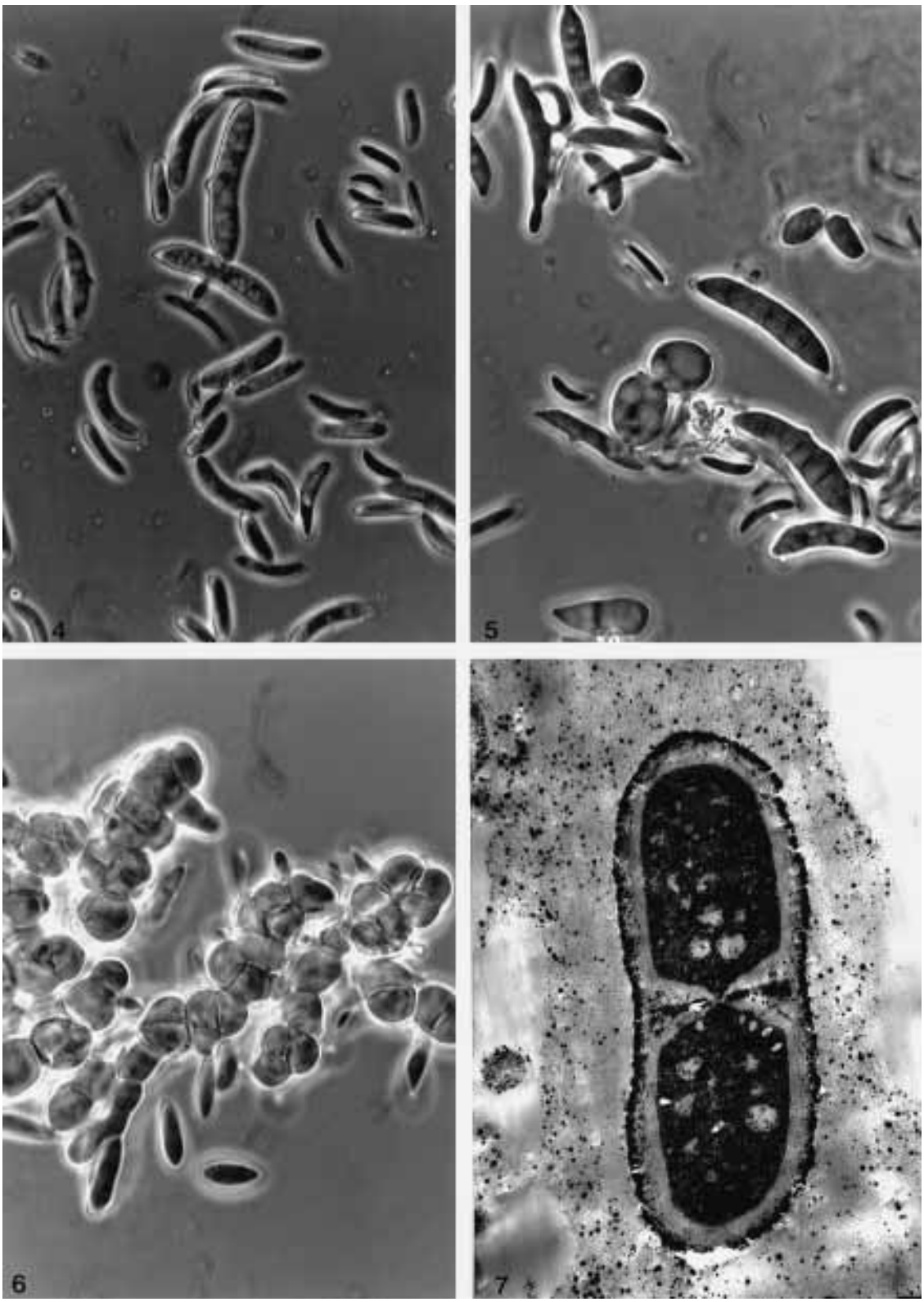
Restriction analysis

Amplicons were digested with restriction enzymes (1 U) for at least 2 h. The following enzymes were used: HinfI, HaeIII, RsaI, NdeII, HhaI, DdeI, TaqI and HpaII. Digests were subjected to electrophoresis on 1.2% agarose gels (TAE buffer) containing ethidium bromide.

Results

Young colonies of isolates CBS 706.95 and 707.95 were characterized by an abundance of banana-shaped yeast cells showing bipolar budding. In a later stage, yeast cells inflated, developed one to four septa and frequently became melanized and thick-walled. Colonies of CBS 707.95 stained black within a few days, but those of CBS 706.95 remained pale or pink for a week or more, gradually developing a purple margin and eventually staining black. However, remarkable similarity was noted in physiological properties (Tables 1 and 2). In addition, PCR-ribotyping of SSU and partial LSU ribosomal genes as well as intrageneric spacers revealed identical profiles (Figure 8). This led us to neglect the cultural differences and to assign the two strains to the same species, which is described below.

Coloniae (CBS 707.95) in agaro maltoso primum madidae, albidae vel pallide olivaceo-griseae, deinde siccantes, cerebriformes, in medio atrae; ad radium 10 mm post 10 dies, subinde ad diametrum 25 mm crescentes. Coloniae in agaro farina avenacea confecto planae, siccae, 20 mm radio post 10 dies, subinde ad diametrum 80 mm crescentes. In agaro extractis *Solani tuberosi* et *Dauci carotae* confecto hyphae expandentes rectae vel modice undulatae, diametro constante, ramis primariis 3–4 μm latis. Conidia ex ostiolis 0.5–1.5 μm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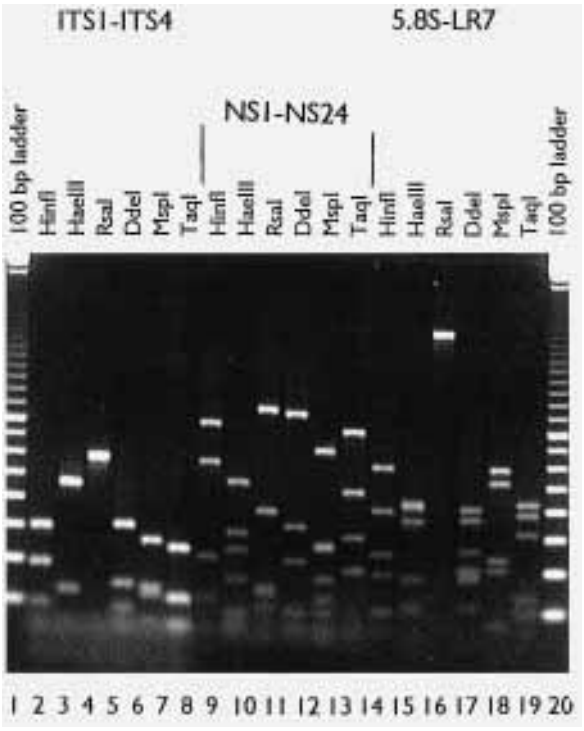

Figure 8. Digestion patterns of amplicons ITS1–ITS4 (lanes 2–7), NS1–NS24 (lanes 8–13) and 5.8S-LR7 (lanes 14–19) of *Hormonema schizolunatum*, CBS 707.95. Lanes 1 and 20 are 100 bp ladder markers.

protrudentibus (singulis vel binis in quaque cellula) successione basipetali oriunda, neque collare neque annellationem relinquentia. Conidia primum hyalina, levia et tenuitunicata, falcata vel musiformia, 8–18 × 1.8–4 μm, deinde inflata, olivascentia et magis crassitunicata et asperata, nonnumquam uno vel compluribus septis transversalibus divisa. Qualitates physiologicae in Tabulis 1 et 2 compositae.

Holotypus exsiccatus culturae CBS 707.95 ex folio *Salviae canariensis* L. var. *candidissima* Bolle prope Barranco de Arguineguín in insula Gran Canaria isolatae, in Herb. CBS praeservatus.

Cultural characteristics. Colonies (CBS 707.95) on MEA4 initially moist, whitish to pale olivaceous-grey, flat, later becoming dry, smooth, strongly lobed or cerebriform, black at the centre with smooth, dirty white marginal zone, with numerous smooth, jet black, glistening sectors at the margin; expansion growth 10 mm/10 d, ultimate diam after 2 months 25 mm. Colonies on OA flat, dry, pale olivaceous, finely zonate and with olivaceous-black sectors, partly remaining pale pink, with a narrow, subhyaline marginal zone; expansion growth 20 mm/10 d, ultimate diam after 2 months 80 mm. Colonies on PCA thin, glassy to dirty white; expansion growth 8 mm/10 d.

Morphology. On PCA hyphae are prevalent; expanding hyphae straight or somewhat undulate, evenly wide throughout, main branches 3–4 μm wide, with few lateral branches 2–3 μm wide; hyphae irregularly septate (12–20 μm), without dichotomous branching, later schizolytic disarticulation at some septa. On MEA4 hyphae are poorly developed, showing indeterminate development with local production of chlamydospore-like cells. Conidia on MEA4 and PCA produced repetitively from butts which protrude 0.5–1.5 μm (1 or 2 per hyphal cell) without visible collarettes or annellations (Figure 1). On OA strongly branched, coralloid hyphal systems (Figure 3B) present. Conidia initially hyaline, smooth- and thin-walled, sickle- or banana-shaped to lunate, 8–18 × 1.8–4 μm, soon inflating, becoming olivaceous-green and firm- and often somewhat rough-walled, sometimes developing one, later several transverse septa (Figure 2A).

Type strain: CBS 707.95, ex leaf of *Salvia canariensis* var. *candidissima*, Barranco de Arguineguín, Gran Canaria, Spain; dried holotype maintained at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Physiology. Results of nutritionally physiological and tolerance tests according to the classical method are listed in Tables 1 and 2. Of the additional sources tested, butylamine and putrescine were not assimilated. Xylan was readily utilized, but other polysaccharides such as pectin, polygalacturonic acid and carboxymethyl cellulose were not. Of the benzene compounds tested, only 3-hydroxybenzoate and 3-hydroxycinnamate were assimilated by strain CBS 706.95, not by 707.95. Phenol, hydroquinone, phloroglucinol, pyrogallol, orcinol, gallate, vanillate, ferulate and 4-hydroxyphenylacetate were not assimilated.

Ultrastructure. Cell walls were electron-transparent, with in the outermost wall layer of electron-dense granules, probably melanin-like pigments (Figure 7). The innermost layer was melanin-free. Septa were double and allocated to the outer wall with a triangular space. Central septal pores were simple and septa were attenuated towards the pore canal; Woronin bodies were not observed.

Cell wall composition. The gas chromatograms of the two strains were identical (results not shown). Galactose was found to be the predominant monosaccharide; small peaks representing mannose were also detected. Both strains displayed two additional peaks in gas chromatograms, probably representing arabinitol and mannitol.

Molecular data. Restriction patterns of amplicons of both 18S and ITS of CBS 707.95 are shown in Figure 8. Patterns of CBS 706.95 were strictly identical (results not shown).

Discussion

Colonies were initially pale and moist, and finally became black and tough, as is typically observed in *Hormonema* and *Aureobasidium* species. The appearance of colonies was drastically affected by the medium used: colonies on OA were flat and widely expanding, while those on MEA4 were restricted and strongly cerebriform. At the margin, sterile sectors were abundantly produced. Growth of strain CBS 706.95 was poor on most media; cultures were strongly lobed and remained pale over long periods. Possibly a mutant with decreased sporulation was concerned. On OA it produced sterile bodies, strongly resembling immature pycnidia. On MEA4 expanding hyphae of CBS 706.95 were irregularly inflated, often with dichotomous or trichotomous branching, and locally with densely spaced septa (Figure 2B). Budding was abundant on MEA4; a tough film or crust was formed covering the surface which could easily be lifted from the thin submerged mycelium. Conidia were orange section-shaped, often somewhat wider than those of CBS 707.95, 10–16 × 3–5 μm (Figure 2B). Frequently more than 2 conidiogenous butts per hyphal cell were present. Liberated cells budded repetitively at their poles or laterally, often enlarged and became 1(-3)-septate, either remaining hyaline or converting into thick-walled, melanized, chlamydospore-like cell clumps (Figure 6).

Unlike many other ascomycetous and basidiomycetous yeasts (Middelhoven et al. 1985), the aliphatic amines butylamine and putrescine were not assimilated as sole carbon source by *H. schizolunatum*. Xylan from birch wood was readily utilized, but other polysaccharides, such as pectin, polygalacturonic acid, carboxymethyl cellulose, were not (Table 2). Phenol, hydroquinone, phloroglucinol, orcinol, vanillate, ferulate and 4-hydroxyphenylacetate supported growth of the herpotrichiellaceous black yeast *Exophiala jeanselmei* (Langeron) McGinnis & Padhye (Middelhoven 1993), but were not assimilated by *H. schizolunatum*. Four strains of *Hormonema dematioides* assimilated hydroquinone, 3-hydroxybenzoic acid, 4-hydroxyphenylacetate, 3-hydroxycinnamate and gallate (W.J. Middelhoven, unpublished results). In comparison to *E. jeanselmei*, *Hormonema dematioides* and *H. schizolunatum* displayed little activity to benzene compounds.

A relationship of *H. lunatum* to dothideaceous anamorphs was supposed on the basis of the following characters. Terminal cells were sometimes dichotomous or trichotomously branched (Figure 3A) and showed indeterminate thallus maturation (Takeo & De Hoog 1991). Woronin bodies were absent from simple septal pores (Figure 7); this has thus far been noted in purported anamorphs of Dothideaceae only (Mittag 1994; Figueras et al. 1995; De Hoog et al. 1996). In addition, urease was present, 10% NaCl was tolerated but the strains were sensitive to cycloheximide (compare De Hoog & Yurlova 1994). Budding cells were abundantly present. Hyphal as well as budding cells easily converted to isodiametric development, and extracellular DNAse was produced (De Hoog, unpublished results).

Lloyd (1970, 1972) found the cell walls of the dothideaceous black yeast *Hortaea werneckii* (Horta) Nishimura & Miyaji to contain peptido-galactomannan complexes. This matches with the preponderance of galactose in our cell hydrolyzates. In many other ascomycetes glucose is one of the predominant peaks (Weijman & De Hoog 1985). Species of the *Aureobasidium/Hormonema* complex produce massive amounts of extracellular polysaccharides, mainly the glucose-polymer pullulan (Seviour et al. 1992). Ethanol-precipitated extracellular polysaccharides in *Aureobasidium* yield only glucose under the experimental conditions used in the present study (H.J. Roeijmans, unpublished results).

Small subunit ribosomal digest patterns could not be matched with those of any member of Herpotrichiellaceae (Uijthof & De Hoog 1995). ITS RFLP patterns were also different from those of meristematic black yeasts (Wollenzien et al. 1996). However, similarities were found with 5.8S-LR7 amplicon RsaI and MspI digests of species of the *Aureobasidium/Hormonema* complex (Yurlova et al. 1995; N.A. Yurlova, pers. comm.). With RsaI, the 1750 bp amplicon was undigested in *H. schizolunatum, H. merioides, H. prunorum* and *A. pullulans*. The MspI digestion revealed four

bands in *H. schizolunatum* (Figure 8), which were not found in any of the other species of the complex.

Conidia were produced from non-elongating annellated zones, located either on undifferentiated hyphae (Figure 1) or on budding cells. No synchronous production of conidia from a single cell was observed. Therefore the fungus was classified in the dothideaceous anamorph genus *Hormonema* Lagerberg & Melin (De Hoog & Yurlova 1994). The nearest *Hormonema* species is *H. merioides* Funk et al. (1985). Both species are unable to assimilate lactose, *myo*-inositol, lysine, creatine and creatinine. *Hormonema merioides* differs from *H. schizolunatum* by the absence of *meso*-erythritol assimilation and by ellipsoidal conidia. Several species of *Aureobasidium* Viala & Boyer in the sense of Hermanides-Nijhof (1977), i.e. comprising cultural states of *Kabatiella* Bubák, have similar, curved conidia, but differ by strictly synchronous conidium production. *Hormonema schizolunatum* might be a cultural state of a species of *Kabatina* R. Schneider & v. Arx, a genus of leaf-inhabiting, sporodochial fungi with percurrent conidiogenesis. However, all taxa described to date are pathogens on conifers or deciduous trees with ellipsoidal rather than lunate conidia (Schneider & Von Arx 1966; Butin & Schneider 1976; Butin & Pehl 1993). Alternatively, it might be linked to *Pringsheimia* Schulz. and similar dothideaceous genera, e.g. to *P. smilacis* E. Müller, which also has curved conidia. The species tested to date are physiologically different (De Hoog & Yurlova 1994).

The lunate, sometimes septate conidia are reminiscent of the genus *Cyphellophora* de Vries (De Hoog & Guarro 1995), but this genus contains monomorphic species without budding cells and has phialidic collarettes. *Microdochium* Sydow (Samuels & Hallett 1983) is excluded because it generally lacks budding cells and has an unpigmented thallus; *Idriella* Nelson & Wilhelm, listed by Von Arx (1981) under *Microdochium*, has erect, brown conidiophores. *Plectosporium* Palm et al. has discrete adelophialides with collarettes (Palm et al. 1995). In addition, these genera are known to be anamorphs of quite unrelated Ascomycetes, such as *Ramphoria* Niessl (Trichosphaeriaceae; Müller & Samuels 1982) *Monographella* Petrak (Amphisphaeriaceae; Samuels et al. 1987), *Hymenoscyphus* (Helotiaceae; Kimbrough & Atkinson 1972) and *Plectosphaerella* Klebahn (Sordariaceae; Uecker 1993).

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