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Hyphozyma lignicola sp. nov., a yeast-like hyphomycete from black galls and cankers of trembling aspen (*Populus tremuloides*)

LEONARD J. HUTCHISON,[1] LYNNE SIGLER[2] AND YASUYUKI HIRATSUKA[3]

[1] *Department of Forest Science, University of Alberta, Edmonton, Alberta, Canada T6G 2H1*

[2] *University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, University of Alberta, Edmonton, Alberta, Canada T6G 2E1*

[3] *Northern Forestry Centre, Forestry Canada, 5320–122nd Street, Edmonton, Alberta, Canada, T6H 3S5*

Hyphozyma lignicola sp. nov. is a yeast-like hyphomycete isolated from black galls and cankers on trunks of *Populus tremuloides* and is distributed in Alberta and British Columbia, Canada. It differs from previously described species of *Hyphozyma* by its pattern of assimilation of carbon compounds, by its colonies which darken to brown on some media, production of diffusible orange brown pigments, and by its specific association with cankers and tumour-like stem deformities on trembling aspen. A revised key to the known species of *Hyphozyma* is provided.

Surveys of microfungi from trunks of living and recently harvested trembling aspen (*Populus tremuloides* Michx.) revealed several isolates of a yeast-like hyphomycete specifically associated with cankers and tumour-like stem deformities (Fig. 1). These latter structures are referred to variously in the literature as either black galls (Hiratsuka *et al.*, 1990) or burls (Ostry *et al.*, 1989) and are considered to be of unknown origin. Fungal isolates grew initially as pinkish white or pale orange colonies consisting of yeast-like cells having prominent budding scars, then formed hyphae which produced conidia from inconspicuous intercalary phialides. Depending on the growth medium, colonies often developed reddish brown or dark brown pigmentation. These characteristics suggested a relationship to the anamorph-genus *Hyphozyma* de Hoog & M. T. Sm. (1981, 1986), the species of which possess pink or orange colonies, an initial yeast-like stage and repetitive production of conidia from reduced conidiogenous cells. Although formation of darkly pigmented colonies might have excluded our taxon from *Hyphozyma*, its similarity to *H. roseonigra* de Hoog & M. T. Sm., another species with dark pigmentation, warranted inclusion here rather than in black yeast genera such as *Exophiala* or *Wangiella* (de Hoog, 1977). Although species of *Hyphozyma* are not easily distinguished from each other by microscopic morphology, both colony characteristics and physiological features have been shown to be useful in differentiating *H. lignicola* sp. nov. from the four species and two varieties known at present (de Hoog & Smith, 1981, 1986; Sigler, 1990).

MATERIALS AND METHODS

Growth studies and physiological tests on H. lignicola

Cultures were isolated onto acidified malt extract agar (20 g malt extract, 15 g agar, 1 l water; 10 ml lactic acid (25%) added after autoclaving) from areas either from within the interior of black galls and cankers of aspen or from beneath the surface of the bark of these structures. For comparative purposes, all isolates were inoculated onto four different agar media: (1) modified 2% malt extract agar (MEA) (20 g malt extract, 1 g yeast extract, 15 g agar, 1 l water), (2) potato dextrose agar (PDA) (BBL or Difco), (3) modified Leonian's agar (MLA) (Malloch, 1981), and (4) corn meal agar (CMA) (BBL). Agar plates were inoculated in triplicate with single inoculum plugs (4 mm), sealed with parafilm and incubated upside down in the dark for 30 d at 25 °C. Colony diameters were measured at 30 d. Selected isolates were also examined on Pablum cereal agar (CER) (Padhye, Sekhon & Carmichael, 1973). Descriptions of colony morphology and colour were made using the terminology of Stalpers (1978) and Kornerup & Wanscher (1978) respectively. Microscopic observations were made using tease preparations from the various agar media and mounted in Melzer's reagent (Malloch, 1981), from slide culture preparations using CER and mounted in polyvinyl alcohol [1·66 g polyvinyl alcohol (Sigma), 10 ml distilled water, 10 ml lactic acid, 1 ml glycerine], and on PDA in Dalmau culture (van der Walt & Yarrow, 1984).

A physiological profile was determined for each isolate. The ability to ferment 7 carbohydrates (glucose, galactose, lactose, sucrose, maltose, raffinose and trehalose) was assessed using the Wickerham technique (van der Walt & Yarrow, 1984). Tubes were examined over a period of 14 d at 25°. The assimilation of 21 carbohydrates (D-glucose, glycerol, 2-keto-D-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, sorbitol, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, cellobiose, lactose, maltose, sucrose, trehalose, melezitose, raffinose, dulcitol and palatinose) was assessed by

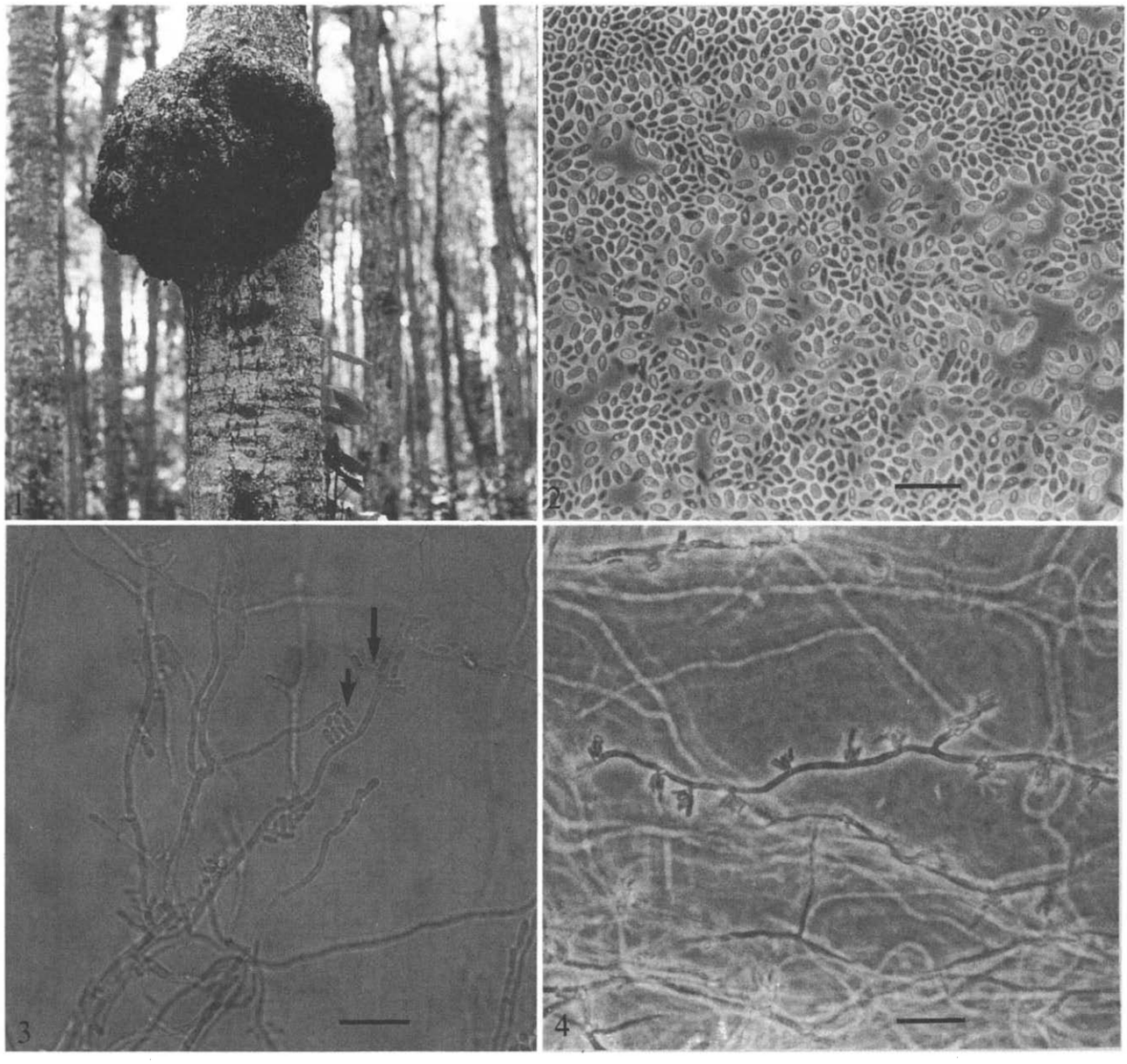


Fig. 1. Black gall on trunk of *Populus tremuloides*, in Alberta, Canada. **Figs 2–4.** Microscopic morphology of *Hyphozyma lignicola* (scale = 20 μm). **Fig. 2.** Yeast-like cells of various shapes (UAMH 7002). **Fig. 3.** Hyphae, budding cells (note arrow) and narrow conidia produced from inconspicuous pegs or from short lateral branches (note arrow) (UAMH 7228 grown in Dalmau conditions on PDA). **Fig. 4.** Hyphae and narrow conidia produced from inconspicuous pegs (tease mount from UAMH 7120 on CMA).

examining growth on these substrates as a sole carbon source employing the API 20C clinical yeast system (API Laboratory Products Ltd.) and the Auto Microbic System-Yeast Biochemical Cards (AMS–YBC) (Vitek Systems Inc.). Manufacturers' instructions were followed except that the API strips were incubated at 25° instead of 30° and read daily for 7 d rather than 3 d, whereas the YBC cards were incubated at 30° and read daily for 6 d instead of 4 d. Readings which were negative after 3 d but positive by day 6 or 7 were recorded as a delayed positive. Assimilation of D-ribose, L-sorbose, L-rhamnose and D-mannitol, not included in either commercial system, and melibiose and erythritol which were missing from the API 20C system was assessed by examining growth on each carbohydrate separately incorporated into agar containing Bacto yeast nitrogen base (Difco) following the methodology of van der Walt & Yarrow (1984). Nitrate assimilation was assessed by examining growth on agar containing potassium nitrate and Bacto yeast carbon base (Difco) following the methodology of van der Walt & Yarrow (1984) while growth tolerances to benomyl (2 μg ml^{-1}) and cycloheximide (100 μg ml^{-1}) were assessed following the methodology of Hutchison (1990) except that MEA was employed instead of PDA. Hydrolysis of urea to ammonia (urease activity) was tested using Christensen's urea agar (van der Walt & Yarrow, 1984), with tubes examined for urease activity daily for 21 d. Growth tolerance at 37° was assessed on MEA. All physiological tests were repeated.

Additional* Hyphozyma *species examined for comparison. Growth studies and selected tests were conducted on

Table 1. Physiological responses and growth rates of *Hyphozyma lignicola* (Hl), *H. roseonigra* (Hr), *H. sanguinea* (Hs), *H. variabilis* var. *variabilis* (Hvv), *H. variabilis* var. *odora* (Hvo) and the *Hyphozyma* synanamorph of *Eleutheromyces subulatus* (Es)

	Hl[1] 8 strains	Hr CBS 514.83	Hs CBS 406.52	Hvv CBS 523.79	Hvv UAMH 6050	Hvv[2] 5 strains	Hvo CBS 328.80	Es[3] 4 strains
Assimilation tests using API 20C & AMS–YBC								
D-glucose	+	+	+	+	+	+	+	+
glycerol	−[6]	−[6]	−[6]	−[8]	−[8]	v	−[8]	−
2-keto-D-gluconate	+/+D	+	+D[7]	−[8]	−	*	−[8]	+
L-arabinose	+[7]	+[7]	+	−	−	−	−	−
D-xylose	+	+	+	+	+	+	+	v
Adonitol (ribitol)	+D	−	+D[8]	−	−	−	−	+
Xylitol	+	+D[7]	+D	−	−	*	−	v
D-galactose	+	+	+	+D	+	+	−[8]	v
Inositol	−[8]	−	+D	−	−	−	−	v
Sorbitol (glucitol)	+Dw	−	+D	−	+	−	−	v
α-methyl-D-glucoside	−	−	−	−	−	−	−	−
N-acetyl-D-glucosamine	+/+D	+D[7]	−	−	+	*	−	v
Cellobiose	v	+	+D[7]	+	+	+	+	+
Lactose	−	+D	+D[8]	−	−	v	−[8]	−
Maltose	v	+	+D	+D	−	v	−	v
Sucrose	+	+	+	−	−	−	−	+
Trehalose	+	+D	+	+D	+	v	+	+
Melezitose	+	+	+	−	−	−	−	−
Raffinose	v	+	−	−	−	−	−	v
Dulcitol (galacitol)[4]	v	−	−	−	−	−	−	*
Palatinose[4]	v	+D	+D	−	−	*	−	*
Assimilation tests using methodology of van der Walt & Yarrow (1984)								
D-ribose	+D	+	−	+	−	v	−	*
L-sorbose	−	−	−	−	−	v	−	*
L-rhamnose	+/+D	+	+D	+	−	−	−	*
Melibiose[5]	+D[7]	+[7]	−	+D[7]	−	−	−	−
Erythritol[5]	+	+	+	+[7]	−	−	−	+
D-mannitol	+	+	+	+	+D	v	−	*
Additional tests								
Potassium nitrate[5]	+	+	+	+	+	+	+	+
Urea[5]	+	+	+	+	+	*	+	+/v
Cycloheximide (100 μg ml^{-1})	−	−	−	−	−	*	−	−/v
Benomyl (2 μg ml^{-1})	−	*	−	−	−	*	−	−
37°	v	*	+	−	+	+	+	−
MEA[9]	12–19(–30)	18–20	18	(41–)56–57	*	*	66–68	*
MLA[9]	14–25(–34)	14–20	20–22	65–68	*	*	68–70	*
PDA[9]	15–20(–23)	20–23	18–19	60–64	*	*	55–59	*
CMA[9]	25–31	10–14	17–18	55–58	*	*	60–61	*

[1] Only 5 strains tested by AMS–YBC; [2] from de Hoog & Smith (1981); [3] from Sigler (1990); [4] tested with AMS–YBS but not API 20C; [5] also tested by AMS–YBC; [6] results positive with AMS–YBC; [7] results negative with AMS–YBC; [8] results variable with AMS–YBC; [9] colony diameters (mm) measured at 30 d at 25°; * = data not available; + = growth positive after 3 d; − = growth negative after 7 d; +D = growth negative after 3 d but positive after 7 d; +Dw = growth negative after 3 d but weakly positive after 7 d; v = variable response. Urease test after 21 d.

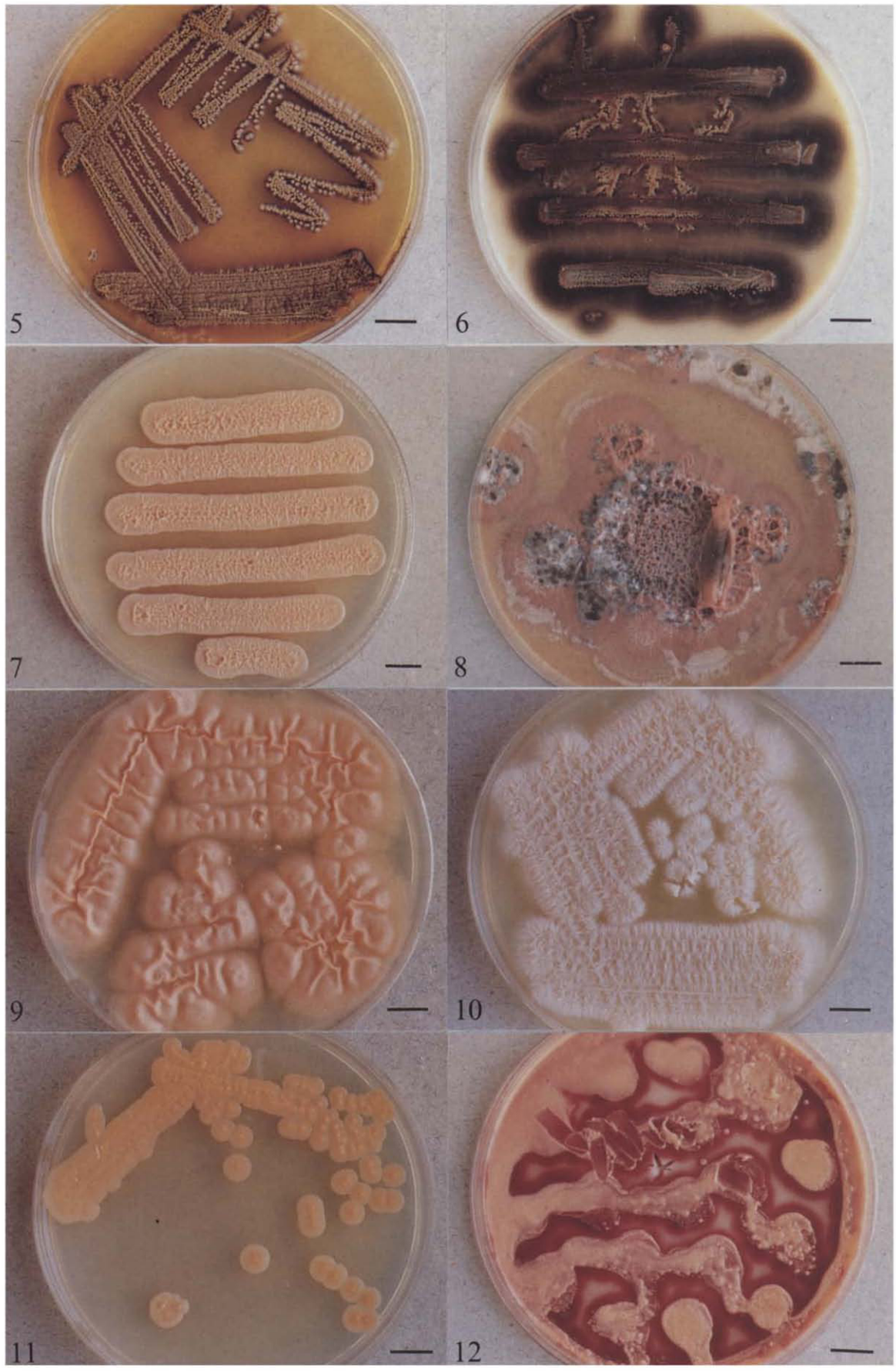
the following isolates: *H. roseonigra* (UAMH 7240 = CBS 514.83: type culture), from soil, M. I. Farbood, New Jersey, U.S.A.; *H. sanguinea* (C. Ramírez ex C. Ramírez) de Hoog & M. T. Sm. (UAMH 6100 = CBS 406.52: type culture), from tannin of *Quercus* and *Castanea*, Lyon, France, May 1947; *H. variabilis* var. *odora* de Hoog & M. T. Sm. (UAMH 6099 = CBS 328.80: type culture), from margarine, Vlaardingen, The Netherlands, 1980; *H. variabilis* var. *variabilis* de Hoog & M. T. Sm. (UAMH 6101 = CBS 523.79: type culture), from cold storage minced meat, D. A. A. Mossel, Utrecht, The Netherlands; UAMH 6050, from water of St Lawrence River, K. Millar, Quebec City, Canada, July 1987.

TAXONOMY

Hyphozyma lignicola L. J. Hutchison, Sigler & Y. Hirats., sp. nov. (Figs 2–6)

Etym.: *lignicola* (L.) – dweller on wood

Coloniae in agaro malto (2%) in duobus modis crescentes: primo ad diametro 12–19 mm post 30 dies in temperatura 25° attingentes, mucidae, adpressae, lacunosae vel cerebriformes in centro, ambitibus circularibus vel irregularibus, vinaceae vel lateritiae, a tergo sepiaceae vel brunneo-vinosae, pigmento senato leviter in agaro diffluenti; secundo ad diametro 20–30 mm post 30 dies in temperatura 25° attingentes, adpressae, leviter lacunosae vel leviter cerebriformes in centro, circularibus, fulvae vel vinaceae in centro, ad marginem roseo-



Figs 5–12. For captions see opposite.

albae, a tergo senatae vel brunneo-vinosae in centro, aurantiacae vel miniatae ad marginem, sine pigmento in agaro diffluenti. *Hyphae* hyalinae, (1–)1·5–3 μm latae. *Conidiophora* absunt. *Cellulae conidiogenae* enteroblasticae, phialidicae, intercalares in hyphis aut interdum in ramis curtis lateralibus, inconspicuae, < 1 μm latae, 0·5–1·0 μm altae. *Conidia* hyalina, laevia, unicellularia, cylindrica vel allantoidea vel oblonga, (2·5–)3–5(–8) × (0·5–)1(–1·5) μm, raro 2 μm lata. *Cellulae gemmantes* variae respectu forma, subglobosae vel ovales vel reniformes, (2–)3–7(–9) × (1–)2–4(–6) μm, hyalinae sed parum pigmentiferescentes in culturis vetustioribus. Gemmatio plerumque unipolaris (raro bipolaris), gemmis secondariis cicatrices in primariis saepe relinquentibus.

Holotypus: UAMH 7002; cultura exsiccata isolata e galla nigra in trunco *Populi tremuloides* Michx, versus meridiem a Hinton, Alberta, Canada, 28 September 1990 a C. Myrholm.

Two types of *colonies* on MEA. Type 1 attaining a diameter of 12–19 mm in 30 d at 25°; slimy, appressed, lacunose to cerebriform in centre; circular to irregular in outline; margin even; greyish red (7B6–8B4) to brownish red (8C7) sometimes becoming reddish brown (8D7), surrounded by greyish red (8B3), reverse dark brown (8F8–9F8) to violet brown (10F8) sometimes surrounded by reddish brown (9D8); with slight orange brown pigment diffusing into agar. Type 2 attaining a diameter of 20–30 mm in 30 d at 25°; appressed, slightly lacunose to slightly cerebriform in centre; circular to slightly irregularly circular in outline; margin even; greyish orange (6B4–6B6) to brownish orange (6C4) often greyish red (7B3–7B6), surrounded by pinkish white (7A2–8A2) at margin, reverse brownish orange (6C7–7C8) to dark brown (9F8) to violet brown (10F8) in centre surrounded by orange (6B7–6B8) to brownish orange (6C8); no pigment diffusing into agar. Colonies on MLA of two types, similar in morphology, colour, growth rate and pigment production to those present on MEA. Colonies on PDA similar to colony morphology and colour to those grown on MEA (type 1), growth 15–20 (–23) mm in 30 d at 25°, dark brown (8F6–9F8) at margin, slight orange brown pigment diffusing into agar. Colonies on CMA attaining a diameter of 25–31 mm in 30 d at 25°, appressed; circular, hyaline to yellowish white (1A2) rarely orange white (5A2); no diffusion of pigment into agar.

Hyphae straight, even, thin-walled, hyaline, (1–)1·5–3 μm wide, but in yeasty cultures hyphae sometimes becoming slightly torulose. *Conidiophores* absent. *Conidiogenous cells* inconspicuous, usually < 1 μm wide and 0·5–1 μm high when observable, phialidic, intercalary on hyphae but sometimes intercalary on short lateral branches. *Conidia* produced in basipetal manner and often remaining in a cluster at base of the conidiogenous cell, hyaline, smooth-walled, one-celled, cylindrical to allantoid to oblong, (2·5–)3–5(–8) × (0·5–)1(–1·5) μm, rarely 2 μm wide. *Yeast-like cells* of various shapes, subglobose to oval to obovoid to obclavate to elliptical, rarely allantoid to reniform, (2–)3–7(–9) × (1–)2–4(–6) μm, smooth-walled, one-celled, hyaline but becoming very slightly pigmented in mass in older cultures, mostly unipolar budding present (rarely bipolar budding) with secondary yeast-like cells often leaving behind scar on primary yeast-like cell. Yeast-like cells absent on CMA but present on MEA, PDA and MLA. Fermentative ability absent with glucose, galactose, lactose, sucrose, maltose, raffinose and trehalose. Physiological growth responses are given in Table 1.

Holotype: (dried culture) UAMH 7002, isolated from black gall on trunk of *Populus tremuloides* Michx., South of Hinton, Alberta, Canada, 28 September 1990, isolated by C. Myrholm.

The holotype specimen, the ex-type culture and other strains are maintained in the University of Alberta Microfungus Collection and Herbarium (UAHM); living strains are also held in the culture collection of the Northern Forestry Centre (NOF). An ex-type culture and an isotype specimen (dried colony) are deposited at the Canadian Collection of Fungus Cultures and the National Mycological Herbarium (DAOM 194284). The ex-type culture is also deposited at Centraalbureau voor Schimmelcultures (CBS 325.93).

Cultures examined: Canada: Alberta: South of Hinton, all isolated from black galls on trunks of *P. tremuloides*, 28.ix.1990, C. Myrholm, UAMH 7002 (NOF 1570), UAMH 7119 (NOF 1572), UAMH 7120 (NOF 1574), UAMH 7228 (NOF 1571), UAMH 7229 (NOF 1573), UAMH 7230 (NOF 1575); near Blue Ridge, vii.1989, P. Crane, UAMH 7121 (NOF 1831); British Columbia: nr Fort Nelson, isolated from canker on trunk of *P. tremuloides*. 22.vii.1992, P. Crane, UAMH 7231 (NOF 2015).

DISCUSSION

Hyphozyma lignicola, in common with all known species in this genus, produces abundant conidia from inconspicuous intercalary phialides, possesses yeast-like cells with prominent budding scars remaining from the production of daughter cells, assimilates nitrate, produces ammonia from urea, and lacks the ability to ferment various carbohydrates. However, the combination of conidia size and shape, colonial pigmentation, production of diffusible pigments, a unique physiological profile (Table 1), and substrate specificity easily distinguishes it from the other four species already known.

Colonies of *H. lignicola* (Figs 5–6) are distinguished by their development, usually within 7 d, of dark brown pigmentation at the margin (especially on MEA, PDA, CER), a dark brown reverse, and by production of diffusible orange brown (on MEA and PDA) or dark brown (on CER) pigments. Colonies of *H. roseonigra* de Hoog & M. T. Sm. (Figs 7–8) also develop dark pigmentation in older cultures, being most pronounced on media promoting hyphal development such as CER or cherry decoction agar (de Hoog & Smith, 1986). Young colonies are smooth or cerebriform and yeast-like (on PDA and MEA) (Fig. 7), but by 30 d patches of white to brownish grey

Figs 5–12. Colonial morphologies of *Hyphozyma* species at 25° (scale = 1 cm). **Fig. 5.** *H. lignicola* on PDA after 23 d (UAMH 7119). **Fig. 6.** *H. lignicola* on CER after 31 d (UAMH 7002). **Fig. 7.** *H. roseonigra* on PDA after 8 d (CBS 514.83 = UAMH 7240). **Fig. 8.** *H. roseonigra* on CER after 40 d (same strain). **Fig. 9.** *H. variabilis* var. *variabilis* on PDA after 24 d (CBS 523.79 = UAMH 6101). **Fig. 10.** *H. variabilis* var. *odora* on PDA after 23 d (CBS 328.80 = UAMH 6099). **Fig. 11.** *H. sanguinea* on PDA after 23 d (CBS 406.52 = UAMH 6100). **Fig. 12.** *H. sanguinea* on CER after 40 d (same strain).

hyphae develop (Fig. 8). The reverse is light orange if colonies are yeast-like or pale brown if hyphal. No diffusible pigments are produced. In contrast, colonies of *H. sanguinea* are distinctive for their formation of a dark red diffusible pigment which is pronounced on CER (Fig. 12), but lacking on PDA (Fig. 11). No diffusible pigments are produced by strains of either variety of *H. variabilis*. Moreover, colonies of these two varieties (Figs 9–10) are pale pinkish white or pale to light orange and never develop dark pigmentation. In contrast to all other species, young colonies of the *Hyphozyma* synanamorph of *Eleutheromyces subulatus* (Fr.) Fuckel (not shown) are mucoid or runny on PDA, initially yellowish white, but develop distinctive intense reddish orange pigmentation and patches of aerial mycelium within 2–4 wk. They also have a strong fragrant odour. Older cultures develop pycnidia of the *Eleutheromyces* state on some media (Sigler, 1990).

Physiological characters also allowed for the separation of species, provided the methodology was consistent. Where methodology was dissimilar however, as with the case in using different brands of carbon assimilation test kits for yeast identification (AMS–YBC *vs* API 20C), physiological data in a few cases gave conflicting results. For example, isolates of all taxa tested were negative for assimilation of glycerol by the API 20C method, but results were positive or variable in the AMS–YBC method. However, the latter results are in agreement with the published observations of de Hoog & Smith (1981, 1986) for *H. sanguinea, H. roseonigra, H. variabilis* var. *variabilis* and *H. variabilis* var. *odora*. Using an auxanographic method (van der Walt & Yarrow, 1984), we found that *H. lignicola, H. roseonigra* and *H. variabilis* var. *variabilis* assimilated melibiose but results were consistently negative by the AMS–YBC method. De Hoog & Smith (1981, 1986) reported a positive result for *H. roseonigra* but a negative result for *H. variabilis* var. *variabilis*. A few of our other results also differed from those previously published: (1) *H. roseonigra* tested negative for adonitol (ribitol), sorbitol (glucitol), α-methyl-D-glucoside and L-sorbose rather than positive, (2) *H. sanguinea* tested negative for α-methyl-D-glucoside, dulcitol (galactitol), D-ribose and L-sorbose rather than positive, and (3) *H. variabilis* var. *odora* failed to utilize sorbitol (glucitol). In their key to species, de Hoog & Smith (1986) separated var. *odora* from var. *variabilis* by its growth with sorbitol (D-glucitol) and its winey odour. Our results suggest that the separation into two varieties is not well supported by physiological characteristics. In our key, we have treated them as a single taxon.

Concurrent with our taxonomic studies on the fungal flora of black galls of trembling aspen, isolates are being investigated for secondary metabolites, especially ones exhibiting antifungal activity against the major decay pathogen of aspen, *Phellinus tremulus* (Bond artsev & Borissov). Ayer & Miao (personal communication) showed that the ex-type culture (UAMH 7002) of *H. lignicola* produced five trehalose esters when grown in liquid culture. Although none of these is known to have antifungal activity to *P. tremulus*, trehalose esters are rarely known from fungi.

***Key to the described species of* Hyphozyma (*adapted from de Hoog & Smith, 1986*)**

1. Colonies initially mucoid to runny on PDA; with a strong fragrant odour; pycnidia developing in culture (after one month on some media) . . . . . *Hyphozyma* synanamorph of *Eleutheromyces subulatus*
1. Colonies initially pasty; odour lacking or wine-like; pycnidia not developing in culture 2
   2. Colonies on some media (CER, cherry decoction agar) developing a dark red diffusible pigment; growth present with inositol . . . . . *H. sanguinea*
   2. Colonies without a dark red diffusible pigment; growth absent with inositol 3
3. Colonies developing some brown pigmentation; young conidia produced from hyphae, less than 2 μm wide; growth present with sucrose and melezitose 4
3. Colonies remaining yellowish white, pale or pinkish orange; young conidia greater than 2·5 μm wide; growth absent with sucrose and melezitose . . . . . *H. variabilis*
   4. Colonies developing patches of white or brownish grey mycelium by 30 d; no diffusible pigments; growth present with lactose; from soil . . . . . *H. roseonigra*
   4. Colonies developing brown pigmentation at margin by 7 d on PDA & MEA; diffusible orange brown pigment produced; growth absent with lactose; from black galls and cankers on *Populus tremuloides* . . . . . *H. lignicola*

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