

New species, combinations, host associations and location records of fungi associated with hemp (*Cannabis sativa*)

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Micropeltopsis cannabis sp. nov. and *Orbilia luteola* (Roum.) comb. nov. are proposed. New *Cannabis* host associations include binucleate *Rhizoctonia* spp., *Curvularia cymbopogonis*, *Sphaerotheca macularis*, *Glomus mosseae*, and *Pestalotiopsis* sp. The geographic ranges of *Pseudoperonospora cannabina*, *Septoria neocannabina* and *Fusarium graminearum* are expanded.

A 12-yr study of fungi associated with *Cannabis* has revealed that many taxa cited in the *Cannabis* literature are based on misdeterminations or synonyms of species with wide host ranges (McPartland, 1992, 1994*b*, 1995*b*). New species have been discovered – *Phomopsis ganjae* (McPartland, 1983), *Schiffnerula cannabis* (McPartland & Hughes, 1994*a*), *Fusicoccum marconii*, *Leptodothiorella marconii* (McPartland, 1994*c*), and *Ascochyta arcuata* (McPartland, 1994*d*). Taxonomic changes include *Phoma cannabis* (L. A. Kirchn.) McPartl. (McPartland, 1994*d*) and *Septoria neocannabina* McPartl. (McPartland, 1995*a*). New host associations include *Botryosphaeria obtusa*, *Lasiodiplodia theobromae* (McPartland, 1994*c*), *Colletotrichum dematium*, *Diaporthe arctii*, *Leptosphaeria acuta* and *Leptosphaerulina trifolii* (McPartland, 1995*b*).

In this final report of the 12-yr study, a new species is described, a combination made, five new *Cannabis* associations described, and three *Cannabis* pathogens described from new locations.

MATERIALS AND METHODS

Methods for downloading taxonomic literature and locating type materials are described previously (McPartland, 1994*b*). Common names of diseases follow those adopted by the American Phytopathological Society (McPartland, 1991).

Fruiting structures from stems and leaves were removed from host tissue, rehydrated in 3% KOH and either hand-sectioned with a razor blade or teased apart with needles under a dissecting microscope at 4×. Fungal tissue was stained with either cotton blue in lactic acid, phloxine in water or acid fuchsin in lactic acid. Root tissues with mycorrhizas were fixed in 4% glutaraldehyde (in 0.1 M cacodylate buffer), dehydrated in a graded ethanol series, infused with paraffin, sectioned longitudinally at 20 µm thickness with a sliding microtome, and stained with either periodic acid–Schiff reagent

(Nemec, 1981) or trypan blue (Phillips & Hayman, 1970). Curved spores were measured along their circumference and widths measured at their widest part. Measurements are listed as a range; for ample material ($n \geq 50$), mean measurements are given with extremes in brackets.

Root tissues with *Rhizoctonia* were washed and plated on 1.5% water agar amended with streptomycin and tetracycline. Isolates were transferred to potato dextrose agar (PDA), stained with DAPI (Martin, 1987) and Safranin O (Yamamoto & Uchida, 1982) to determine nuclear condition of hyphal cells. Isolates were assigned to a specific anastomosis group (AG) by pairing with a tester strain representing each binucleate *Rhizoctonia* AG (Burpee *et al.*, 1980; Ogoshi *et al.*, 1983) following the procedure of Kronland & Stanghellini (1988).

Genomic DNA was extracted from each isolate following a modification of the procedure described by Cubeta *et al.* (1991). Briefly, lyophilized, buffered mycelia were extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1 v/v), treated with RNAase (1 mg ml⁻¹, DNase free, Sigma), extracted twice in 250 µl of chloroform–isoamyl alcohol (24:1), adjusted to 3 M sodium acetate (pH 5.0) and precipitated for 24 h in 2 vol. of 95% EtOH at –20°. The genomic DNA pellet was washed with 80% EtOH, dried under vacuum, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na₂EDTA). Extracted DNA was purified by electrophoresis in a 0.8% low melting agarose gel (Sea Plaque, FMC) in TBE buffer (100 mM Tris-HCl, 20 mM Na₂EDTA, 100 mM Boric acid). *Escherichia coli* (strain HB101) DNA was used to estimate the concentration of DNA for each isolate.

Genomic DNA was amplified with two oligonucleotide primers (LROR = 5'-ACCCGCTGAAGTAAAGC-3' and LR7 = 5'-TACTACCACCAAGATCT-3'). LROR and LR7 are homologous to a region in 25S ribosomal DNA (rDNA) from base position 17 to 1448. PCR reactions were conducted with

Amplitaq DNA polymerase (U.S. Biochemicals, Cleveland, OH) in 50 µl volumes. Thirty PCR cycles were conducted on an automated thermocycler (Perkin-Elmer-Cetus, Norwalk, CT). The following protocol was used; 1 min denaturation at 94°, annealing at 50° for 45 s, 50–72° gradual increase for 1 min and primer extension at 72° for 1 min. To avoid possible contamination, PCR experiments were conducted in accordance with the stringent procedures described by White *et al.* (1990). Also, tubes without DNA template were included in each experiment (negative control). After amplification, a 3 µl aliquot from each sample was subjected to electrophoresis. Lambda DNA digested with *EcoR* I/*Hind* III was used to determine size of PCR products. Gels were stained with ethidium bromide and photographed over a uv transilluminator to record results.

Amplified PCR products were extracted in chloroform/isoamyl alcohol (24:1), precipitated in 3 M Na acetate and 95% EtOH at –20° for 24 h, washed with 80% EtOH, dried under vacuum and resuspended in TE buffer. For restriction analysis, each PCR product was divided into equal aliquots and digested with either *Hha* I, *Hpa* II, *Sau*3A I, or *Taq* I (Promega). After digestion, samples were subjected to electrophoresis in a 4% agarose gel (NuSieve, FMC Bioproducts). ×174 DNA digested with *Hae* III was used as a molecular weight standard to determine size of restriction fragments. Gels were stained with ethidium bromide and photographed as described above.

RESULTS AND DISCUSSION

Micropeltopsis cannabis McPartl., sp. nov. (Figs 1–3)

Ascomata catathecioida, 45–130 µm diam., complanata, ampulliformia, 25–46 µm alta, Brunnea vel nigra, ostiolata, margine integro; paries superior ex radialiter dispositis cellulis quadratis, peridia textura prismatica; paries basilaris similiter sed pallidior. *Ostiolum* centrale, elevatum, compositum e parvis cellulis et coronam gerens setarum divergentium. *Setae* rectae, subulatae, crassitunicatae, non-septatae, laeves, brunneae, 12–22 µm longae. *Asci* bitunicati, ovoidei ad obclavati, 4–8-spori, 21–40 × 4–9 µm. *Ascospores* hyalinae, guttulate, ellipsoideae, septo unico in medio quaeque indutae, nonsetulatae, 11–12 × 2.5–3.0 µm. Paraphyses non visae.

In caulibus emortuis *Cannabis sativae*. Holotypus: France: Villernur, autumnus 1880 (*Fungi Gallici exsiccati*, Centuria 27, No. 1671), BPI No. 802622 A et B.

Ascomata catathecioid, 45–130 µm diam., flattened ampulliform, 25–46 µm high, dark brown to black, ostiolate, margin entire; upper layer composed of radially arranged quadrangular cells, peridium *textura prismatica*; basal layer of similar construction to upper layer but paler. *Ostiole* central, raised, composed of small cells and bearing a crown of divergent setae forming an inverted cone of the ostiole. *Setae* straight, subulate, thick-walled, non-septate, smooth, dark brown, 12–22 µm long. *Asci* bitunicate, ovoid to obclavate, 4–8-spored, 21–40 × 4–9 µm. *Ascospores* hyaline, guttulate, ellipsoid, with a single median septum, nonsetulate, 11–12 × 2.5–3.0 µm.

This fungus occurs on the BPI exsiccatus of *Calloria luteola* Roumeguère. It does not appear on BR or CUP exsiccati. *M. cannabis* resembles *M. palustris* (J. P. Ellis) Spooner & P. M.

Kirk, a parasite of Diatrypaceae on dead culms of *Phalaris arundinacea* L. It differs by the smaller ascomata, asci and ascospores, and *M. cannabis* ascospores lack setulae. If the lack of setulae weighs as a genus-delimiting character (as questioned by Spooner & Kirk, 1990), then this species belongs in *Chaetothyriopsis*.

Orbilina luteola (Roum.) McPartl., comb. nov. (Fig. 1)

≡ *Calloria luteola* Roum., *Rev. Myc.* 3(12):7 (1881).

Apothecia superficial on stems, sessile, waxy translucent yellow-orange when hydrated, margin entire and round to ellipsoidal, up to 0.5 mm diam. and 100 µm thick. *Excipulum* consists of hyaline thin-walled *textura globulosa*. *Asci* small, cylindrical, 8-spored, 26.0 × 4.5 µm. *Paraphyses* hyaline, filiform, slightly enlarged at the apex. *Ascospores* hyaline, single-celled, fusiform, indistinctly guttulate 6.5 × 1.5 µm.

Collections examined: Holotype. France: Villernur, leg: H. Garonne, det: Roumeguère (*Fungi Gallici exsiccati*, Centuria 27, No. 1671), stems of *Cannabis sativa* L., fibre variety – automme 1880 (BR). Isotypes examined: BPI no. 802622A, CUP.

Only Dr Garonne has found this fungus on *Cannabis*, but he found enough to distribute between 60 and 100 specimens in Roumeguère's *Fungi Gallici exsiccati*. Most *Orbilina* spp. occur on wood or herbaceous stems, as saprotroph. Benny, Samuelson & Kimbrough (1978) observed pockets of blue-green algae in *O. luteorubellas* (Nyl.) P. Karst. They propose transferring this association to the lichens. No cyanobacterial cells were found in *O. luteola*.

Binucleate *Rhizoctonia* spp.

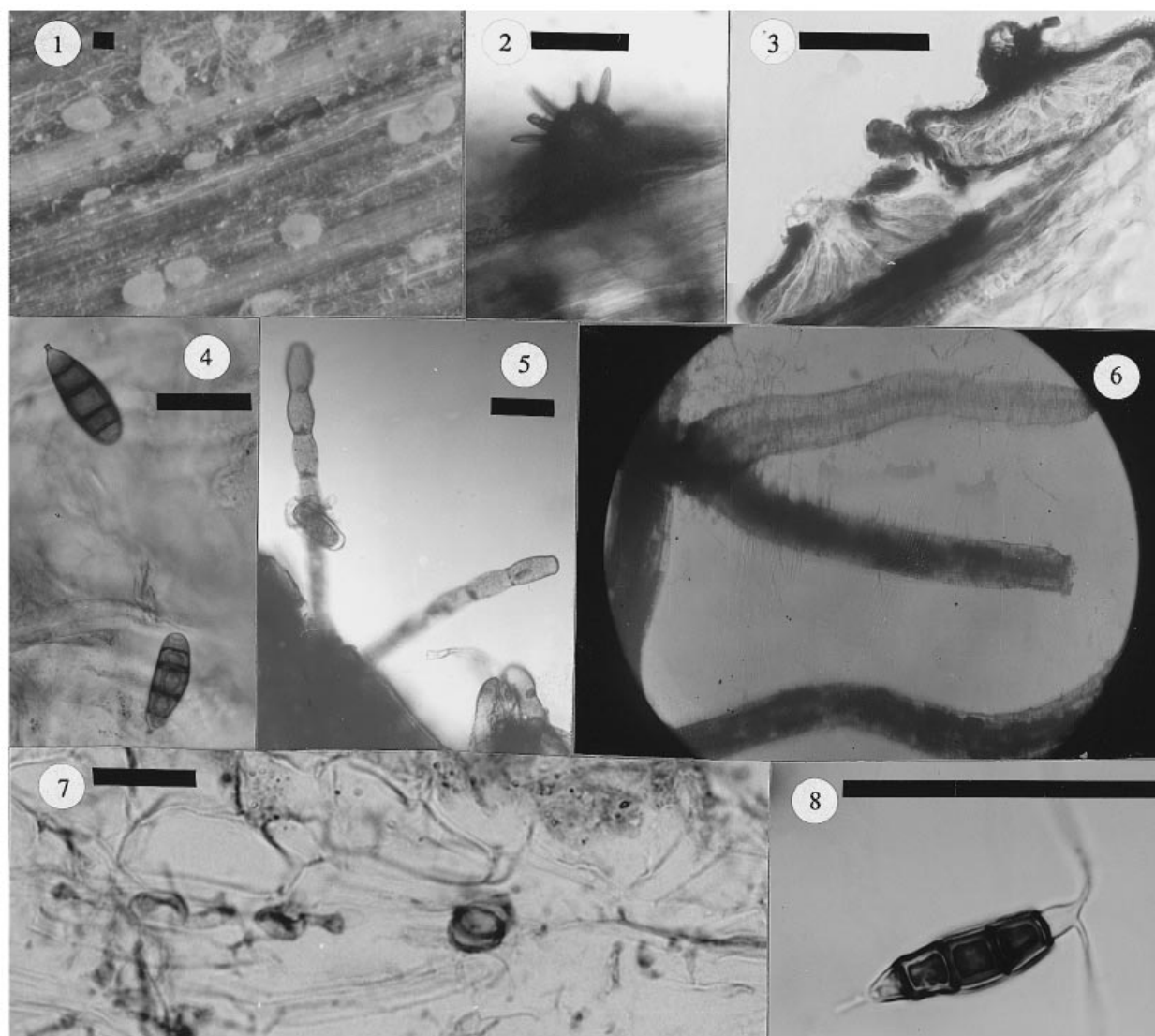
Hyphae without clamp connections, at first colourless, but rapidly becoming brown; branches form geometrically at 45° or 90° angles from parent hyphae, constricted slightly at the branching point: a septum always forms near the base of the branch, 4–7 µm diam.

Collection examined: Holland: Amsterdam, leg: McPartland, on roots of *C. sativa* L., euphoriant variety – 5.1993 (BPI no. 802758, ATCC culture no. 96145).

Staining with DAPI and Safranin O demonstrated a binuclear hyphal condition. This isolate fused (followed by death of hyphal cells at the fusion point) only with binucleate *Rhizoctonia* spp. anastomosis group G (AGG) tester strain of Ogoshi *et al.* (1983).

After PCR amplification of rDNA, a 1.4 kB product was obtained. No PCR products were obtained in any of the controls. After digestion of amplified rDNA with four different restriction endonucleases, the restriction phenotype of the *Cannabis* isolate was identical to the AGG tester strain and possessed all restriction fragments described by Cubeta *et al.* (1991).

Binucleate *Rhizoctonia* spp. are morphologically similar to *Rhizoctonia solani* J. G. Kühn, but have thinner hyphae (4–7 µm), and usually only possess two nuclei per hyphal cell. Unlike *R. solani*, binucleate *Rhizoctonia* spp. often produce



Figs 1–8. Photomicrographs of fungi associated with *Cannabis*. Scale bars, 38 μ m. **Fig. 1.** Cauliculous habit of *Orbilia luteola* (large white apothecia) and *Micropeltopsis cannabis* (small black ascocarps). **Fig. 2.** *M. cannabis*, exterior aspect of ascocarp with setae. **Fig. 3.** *M. cannabis*, sectioned ascocarp with asci. **Fig. 4.** *Curvularia cymbopogonis* conidia. **Fig. 5.** *Sphaerotheca macularis* conidiophores and conidia. **Fig. 6.** Photomicrograph by E. G. Arzberger *ca* 1925, labelled 'Endotrophic mycorrhiza on *Cannabis sativa*', unknown magnification. **Fig. 7.** *Glomus mosseae*, intercellular hyphae, tight hyphal coils, and H-connections. **Fig. 8.** *Pestalotiopsis* sp., conidium.

Ceratobasidium teleomorphs. In this study, we were unable to produce the teleomorph of our strain of binucleate *Rhizoctonia*.

Curvularia cymbopogonis (C. W. Dodge) J. W. Groves & Skolko, *Canadian Journal of Research* **23**:96 (1945)

(Fig. 4)

\equiv *Helminthosporium cymbopogonis* [as *cymbogoni*] C. W. Dodge, *Annals of the Missouri Botanical Garden* **29**: 139.

Teleomorph: *Cochliobolus cymbopogonis* J. A. Hall & Sivan., *Transactions of the British Mycological Society* **59**:315 (1972).

Conidiophores simple, septate, brown, up to 300 μ m long. *Conidia* acropleurogenous, smooth, straight or curved, clavate to ellipsoidal, obconical at the base with a protuberant hilum, 4 (sometimes 3) septate, middle cells dark brown with end cells paler, averaging 40–50 \times 12–15 μ m (slightly smaller than cited by Sivanesan, 1984).

Collection examined: Nepal: 2 km north of Pokhara, leg: McPartland, on seeds of *Cannabis indica* Lam., euphoriant variety – 10-1986 (BPI no. 802117).

Non-germinating seeds began sporulating with *C. cymbopogonis* when placed in a humidity chamber. The fungus was isolated on potato dextrose agar. Other seeds (from a fibre variety of *C. sativa* in Illinois) fulfilled Koch's postulates.

Babu *et al.* (1977) cite another *Curvularia* sp. on *Cannabis*, *C. lunata* (Wakker) Boedijn. Litzenberger, Farr & Lip (1963) describe a '*Curvularia* sp.' causing leaf spots on Cambodian *Cannabis*. Although they claim representative specimens were deposited at BPI, none was located.

C. cymbopogonis occurs on dicotyledons, monocotyledons and gymnosperms around the world. The fungus often causes seed and seedling blights, but also arises in leaf spots. The homothallic pseudothecia have only been seen in culture.

Sphaerotheca macularis (Wallr.: Fr.) Lind, *Danish Fungi*: 160 (1913) (Fig. 5)

≡ *Erysiphe macularis* (Wallr.) Fr., *Systema Mycologium* 3: 237 (1829).

= *Sphaerotheca humuli* (DC.) Burrill, *Bulletin of the Illinois State Laboratory of Natural History* 2: 400 (1887)

Anamorph: *Oidium* sp.

Superficial hyphae flexuous, branched, with inconspicuous appressoria, cell diam. 4–7 µm, length (37–) 64·5 (–80) µm. *Conidiophores* upright, simple, hyaline, 50–100 µm high. *Conidia* produced in chains, hyaline (turning brown with age), containing fibrosin bodies (which disappear with age), ovate to barrel-shaped, single-celled, averaging 30·2 × 14·0 µm.

Collection examined. U.S.A.: Illinois, Champaign, leg: Sebastian, det: McPartland, on leaves of *C. sativa*, euphoriant variety – 8.1981 (BPI no. 802123).

The anamorph of this powdery mildew was previously described (McPartland, 1983). No teleomorph developed. Recent attention has focused on the taxonomic significance of erysiphaceous anamorphs. 'Imperfect keys' for powdery mildews have gained a measure of reliability. Using a key by Boesewinkel (1980), the *Oidium* sp. is easily identified as *S. macularis*.

S. macularis occurs worldwide and commonly parasitizes hops, *Humulus lupulus* L., a member of the Cannabidaceae (Miller, Weiss & O'Brien, 1960). Doidge *et al.* (1953) report an unidentified *Oidium* sp. attacking *Cannabis* in South Africa, and Hirata (1986) notes *Oidium* sp. on Russian and Italian hemp.

Glomus mosseae (T. H. Nicolson & Gerd.) Gerd. & Trappe, *Mycological Memoirs* 5:40 (1974) (Fig. 7)

External hyphae grow in septate, stolon-like strands along surfaces of roots and extend into soil. External hyphae contiguous with internal hyphae, via penetration points in endodermis. *Internal hyphae* in cortex grow longitudinally in roots, rarely radially or circumferentially, inter- and intracellular, septate, narrower than external hyphae, but always > 1 µm diam., uncommonly producing H-connections, hyphal loops, tight hyphal coils and vesicles; neither arbuscules nor sporulating structures seen.

Collection examined: U.S.A.: Illinois, Hanna City, leg: McPartland, from roots of *Cannabis sativa*, fibre variety – 9.1985 (BPI).

Feeder roots from a naturalized stand of hemp were harvested, sectioned and stained. They revealed mycorrhizal hyphae, but no sporulating structures. Abbott & Robson (1978) devised a key to VA mycorrhizal fungi based on infection morphology, in the absence of sporulating structures. The characteristics described above are typical for *Glomus* spp., and closely resemble *G. mosseae* (Carling & Brown, 1982).

Mosse (1961) produced an artificial mycorrhizal relationship in *C. sativa*, utilizing 'an *Endogone* species' (prior to 1974, all VA mycorrhizas were labelled *Endogone* sp.). Her description of infection morphology matches that of the Illinois mycorrhizal fungus: longitudinally oriented intercellular hyphae, tight hyphal coils, H-connections, and no arbuscules. Mosse

(1961) goes on to describe spore morphology. Her *Endogone* sp. spores resemble those of *G. mosseae*, according to Trappe's (1982) synoptic key. Mosse's *Cannabis* fungus also colonized apple, clover, onion, strawberry and tomato; *G. mosseae* occurs on these hosts (Gerdemann & Trappe, 1974).

Evidence of VA mycorrhizal association with *C. sativa* proceeds Mosse's study. Archives at BPI contain a glass-plate negative labelled 'Endotrophic mycorrhiza on *Cannabis sativa*'. The undated photomicrograph (of unknown magnification) was taken by E. G. Arzberger, who conducted his research in the 1920s and 1930s. It is reproduced in Fig. 6.

Pestalotiopsis sp. (Fig. 8)

Acervuli epiphyllous, circular to oval in outline, dark brown, up to 280–480 µm diam. *Conidiophores* cylindrical, septate, occasionally branched, hyaline, up to 10 µm in length. *Conidiogenous cells* holoblastic, annellidic, hyaline, cylindrical. *Conidia* fusiform, 4-septate, smooth, averaging 25 × 5·6 µm; basal cell hyaline, with a simple hyaline appendage averaging 6·6 µm in length; three median cells umber to olivaceous brown, thick-walled, slightly collapsed between the septa; apical cell hyaline, conical, with appendages. *Appendages* (setulae) tubular, flexuous, with three or less commonly two branches, averaging 17·1 µm in length.

Collection examined: Nepal: 2 km north of Pokhara, leg: McPartland, on leaves and stems of *Cannabis indica* euphoriant variety – 10.1986 (BPI no. 802681).

Using keys in Guba (1961), the fungus can be placed in section Quinqueloculatae, Non-spathulatae, Vesicolorae, Umbrae-Olivea. It could be any one of 40 fungi in this section. Of the species described by Nag Raj (1993) this *Pestalotiopsis* sp. resembles *Pestalotiopsis karstenii* (Sacc. & Syd.) Steyaert, except *P. karstenii* lacks basal appendages. Paulsen (unpublished 1971 report, Kansas State University) isolated a *Pestalotia* sp. from a naturalized stand of fibre-variety *Cannabis* near Lawrence, but the fungus was not described and no voucher specimens retained.

Expanded geographic ranges

Pseudoperonospora cannabina (G. H. Otth) Curzi is one of two fungi causing downy mildew on *Cannabis*. A distribution map published by the Commonwealth Mycological Institute (1971) includes Europe, Kazakhstan, Pakistan, India and Japan. Waterhouse & Brothers (1981) expand the range to other parts of the former U.S.S.R. and China. A collection of *P. cannabina* on wild hemp in Illinois (Hanna City) expands the fungus range to the Western Hemisphere.

Septoria neocannabina has previously been reported in New York (Peck, 1884). The taxon *Septoria cannabis* var. *microspora* Briosi & Cavara is identical to *S. neocannabina* (McPartland, 1995a). This synonymy expands the range of *S. neocannabina* to the Eastern Hemisphere.

The anamorph of *Gibberella zeae* (Schwein.: Fr.) Petch, *Fusarium graminearum* Schwabe, has previously been described from hemp in Germany (Wollenweber & Reinking, 1935) and Romania (Ceapiou, 1958). A collection of *F. graminearum* collected from wild hemp near Hanna City, Illinois, expands

the geographic range on this host to the Western Hemisphere. The Illinois strain is homothallic and readily produces perithecia. A culture is deposited at the Fusarium Research Center (accession number R-8965), The Pennsylvania State University.

Curators of the following herbaria are acknowledged for lending collections: BPI, BR, CUP, FH, MICH and PAV. P. Nelson at the Fusarium Research Center (State College, PA, U.S.A.) confirmed the identification of *G. zeae*. This work was partly supported by a grant from Hortapharm, B.V., The Netherlands.

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