Scutellospora rubra, a new arbuscular mycorrhizal species from Brazil

S. L. STÜRMER AND J. B. MORTON

Division of Plant and Soil Sciences, 401 Brooks Hall, West Virginia University, Morgantown, WV 26506-6057, U.S.A.

Scutellospora rubra is a new arbuscular mycorrhizal species trapped in a pot culture of rhizosphere soil from *Eucalyptus dunnii*. Spores are dark red-brown, 140–220 µm diam., and closely resemble those of *S. heterogama* in size, colour, and subcellular organization, but the outer layer of the spore wall is smooth rather than ornamented. Resolution of such species-level variation to properties of the spore wall is consistent with that measured among all other species of arbuscular mycorrhizal fungi studied to date.

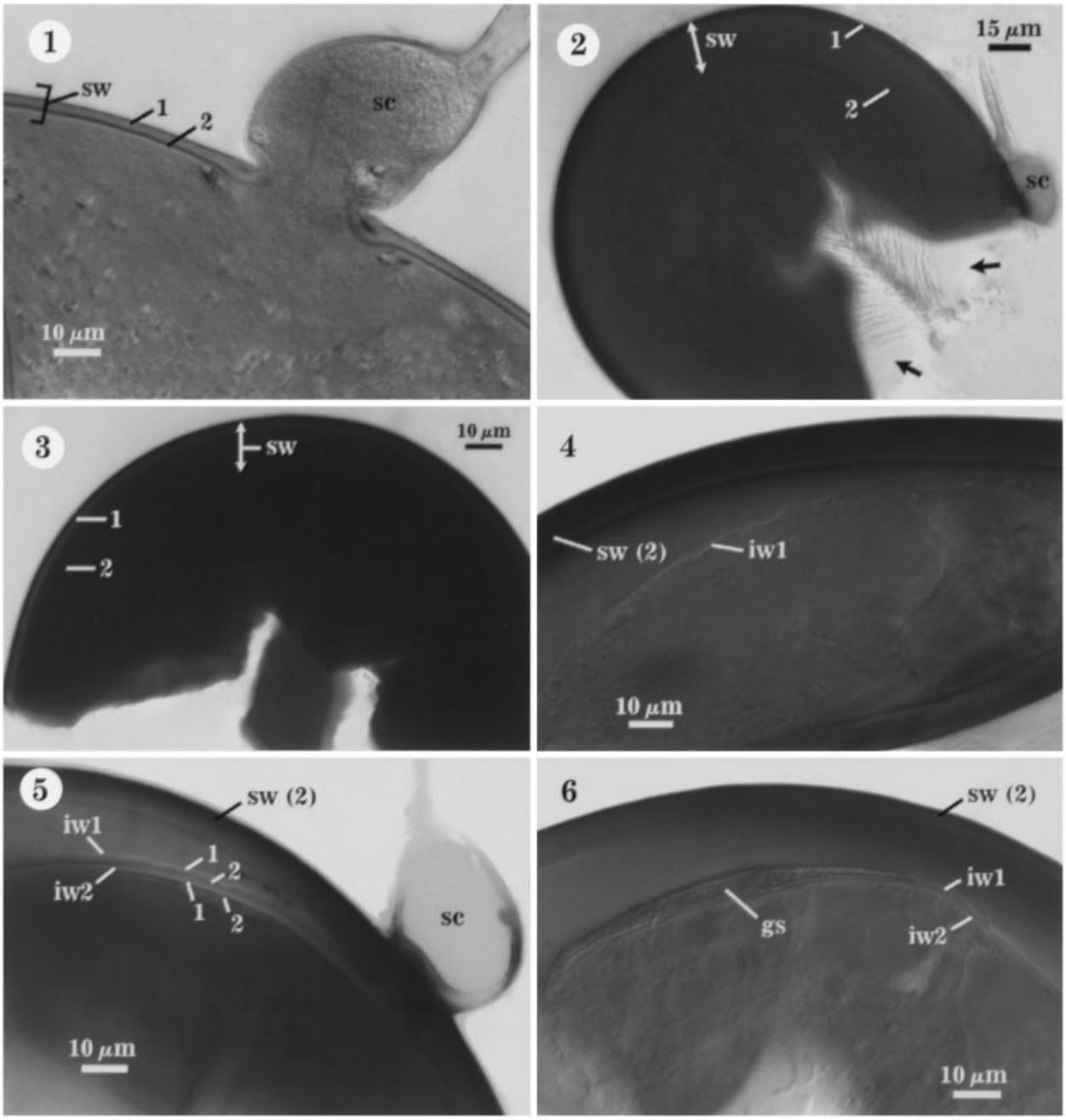
During a study on the dynamics of fungal colonization by AMF and ectomycorrhizal species in *Eucalyptus* plantations in south Brazil (Oliveira, Schmidt & Bellei, 1997), a new species of *Scutellospora* was found in a stand of *E. dunnii* Maiden previously cropped with soybean. A pot culture consisting solely of this fungus was established using *Sorghum* as host and then deposited in the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) as accession BR211. This species is described as *Scutellospora rubra* sp. nov. based on ontogenesis of subcellular spore structure, morphology of mature spores, and morphology of the mycorrhizae. Terminology of taxonomic characters in spores of *S. rubra* is based on developmental patterns in *Scutellospora* elucidated by Franke & Morton (1994) and Morton (1995).

MATERIALS AND METHODS

Inoculum production and spore extraction. Fresh spores were obtained by reculturing *S. rubra* in pot culture using standard protocols of INVAM (Morton, Bentivenga & Wheeler, 1993). Briefly, inoculum of the INVAM accession BR211, consisting of chopped roots, soil, hyphal fragments and spores, was diluted 1:10 (v/v) with a potting medium consisting of a sandy loam soil premixed 1:2 (v/v) with quartzite sand (final pH = 6·2). This mixture was placed in a 15 cm diam. plastic pot and overseeded with sudangrass [*Sorghum sudanense* (Piper) Staph]. Plants were grown for 4 mo in a growth room under fluorescent lights with a photon flux density of 245 µmol m^{-2} s^{-1} at pot level, a 14 h photoperiod, and ambient temperature ranging from 21 to 28 °C.

Spores were extracted from soil by wet-sieving using two nested sieves with 500 µm and 45 µm openings. Content retained on the 45 µm sieve was placed in a 50 ml tube containing a sucrose gradient of 20 % and 60 % and centrifuged at 900 ***g*** for 2 min. The supernatant was poured into a smaller 45 µm sieve, washed with tap water for 1–2 min and transferred to a Petri dish. Spores and auxiliary cells were collected using a Pasteur pipette extruded to a fine tip. Spores were mounted in polyvinyl alcohol lacto-glycerol (PVLG) and PVLG mixed with Melzer's reagent (1:1, v/v) to observe and measure spore subcellular structures. Mature spores were also mounted in water as recommended by Spain (1990). Colour of whole spore was determined by comparison with INVAM colour chart (available from the authors) illuminated with the same light source; colour is described with a name and a formula based on the proportion of cyan/magenta/yellow/black. Slides of spores were incubated in a convection oven at 65° for 24–48 h and stored as permanent vouchers in INVAM. Selected images of spores were captured by a Sony CCD video camera on a Nikon Eclipse E600 Microscope and printed using a Tektronic Phaser 450 dye-sublimation printer. Colour versions of the images in Figs 1–16 can be viewed on INVAM's world wide web site at http://invam.caf.wvu.edu/myc_info/taxonomy/gigasporaceae/scutellospora/rubra/rubra/htm

Spore development. Whole inoculum of INVAM accession *S. rubra* BR211 was diluted 1:10 (v/v) in a potting medium (see above) and the mixture placed in ten 4 × 21 cm cone-tainers™ (Stuewe and Sons, Inc., Corvallis, OR). Five to seven surface sterilized seeds of sudangrass were placed in each cone-tainer. Plants were grown in the same growth room conditions described above. Spores and auxiliary cells were extracted from at least one cone-tainer between 4 and 8 wk. Another pot technique was used to obtain spores in different stages of differentiation. A cone-tainer containing inoculum of *S. rubra* was seeded with sudangrass and grown for 12 wk. After this period, the content of the cone-tainer was placed within a nylon mesh sleeve with 100 µm openings and transplanted in the middle of a 15 cm diam. pot. Growth medium was then placed around the sleeve and seeded with sudangrass. A soil core (*ca* 30 ml) was sampled from the



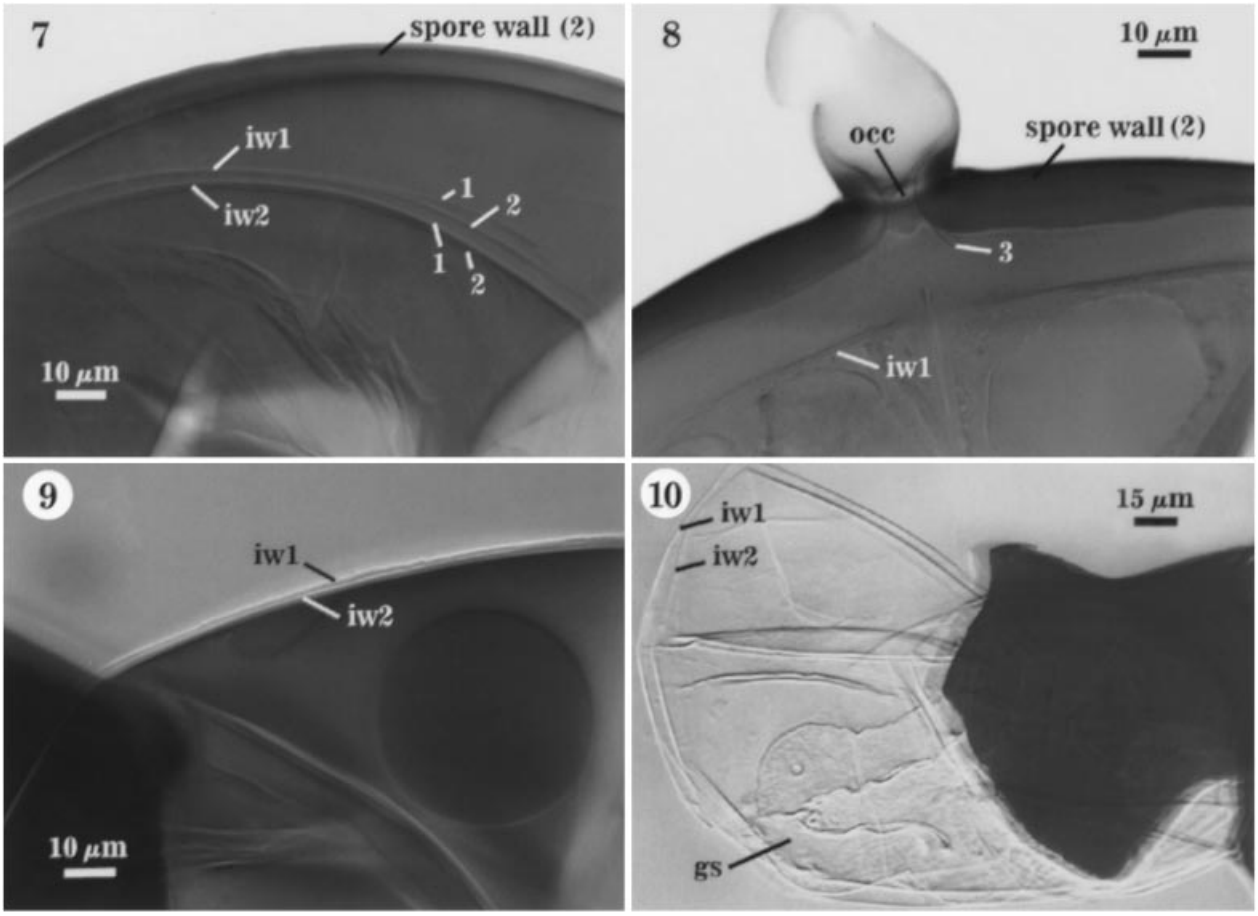
Figs 1–6. Sequence in the differentiation of spores of *Scutellospora rubra* BR211. Voucher number in parentheses indicates slide from which each photo was obtained. Spores are mounted in PVLG unless stated otherwise. **Fig. 1.** Most juvenile spore (stage 1) with the spore wall consisting of two thin layers (1 and 2) (S2961). **Fig. 2.** Stage 2 spore in which layer 2 of the spore wall has thickened and becomes plastic so that it expands (arrows) with applied pressure (S2964). **Fig. 3.** Stage 2 spore in Melzer's reagent, with layer 2 of the spore wall staining dark reddish black (S2964). **Fig. 4.** Synthesis of the first flexible inner wall (iw1) (stage 3) after the spore wall has become more rigid and completed differentiation (S2973). **Fig. 5.** Synthesis of a second flexible inner wall (iw2) delimits stage 4; each inner wall now with two resolvable layers (1 and 2) (S2974). **Fig. 6.** Synthesis of a germination shield (gs) between iw1 and iw2 defines stage 5 (S2974).

region outside the mesh at weekly intervals between four and eight weeks after plant emergence. Spores were extracted from the soil as described above.

Mycorrhizal development. Characteristics of fungal colonization of *S. rubra* were studied in different hosts. Whole inoculum was diluted 1:10 (v/v) with sterile potting medium (see above) and placed in cone-tainers. Each cone-tainer was seeded with *Zea mays* L., *Trifolium pratense* L., *Glycine max* (L.) Merr., *Festuca arundinacea* Schreb., or *Sorghum sudanense*. Roots were sampled after 6–8 wk, stained according to Koske & Gemma (1989), and mounted in PVLG.

RESULTS

Spore development. Spore development of *S. rubra* could be separated into five discrete stages based on origin of subcellular structures and their relation to each other during ontogenesis. In stage 1, the spore wall has two layers. The outer layer (1)



Figs 7–10. Mature spores of *Scutellospora rubra* BR211. Voucher number in parentheses indicates slide from which each photo was obtained. Spores are mounted in PVLG unless stated otherwise. **Fig. 7.** Broken spore in Melzer's reagent showing the laminate layer (layer 2) of the spore wall and both layers (1 and 2) of each flexible inner wall (iw1 and iw2) (S2966). **Fig. 8.** Region of sporogenous cell attachment, showing a thick occlusion (occ) with remnants of a thin flexible layer of the spore wall (3) still attached (S2974). **Fig. 9.** Crushed spore in Melzer's reagent with both flexible inner walls (iw1 and iw2) adherent and thus easily misinterpreted as one wall with four layers (S2974). **Fig. 10.** Intact germination shield (gs), which is positioned between iw1 and iw2 (S3699).

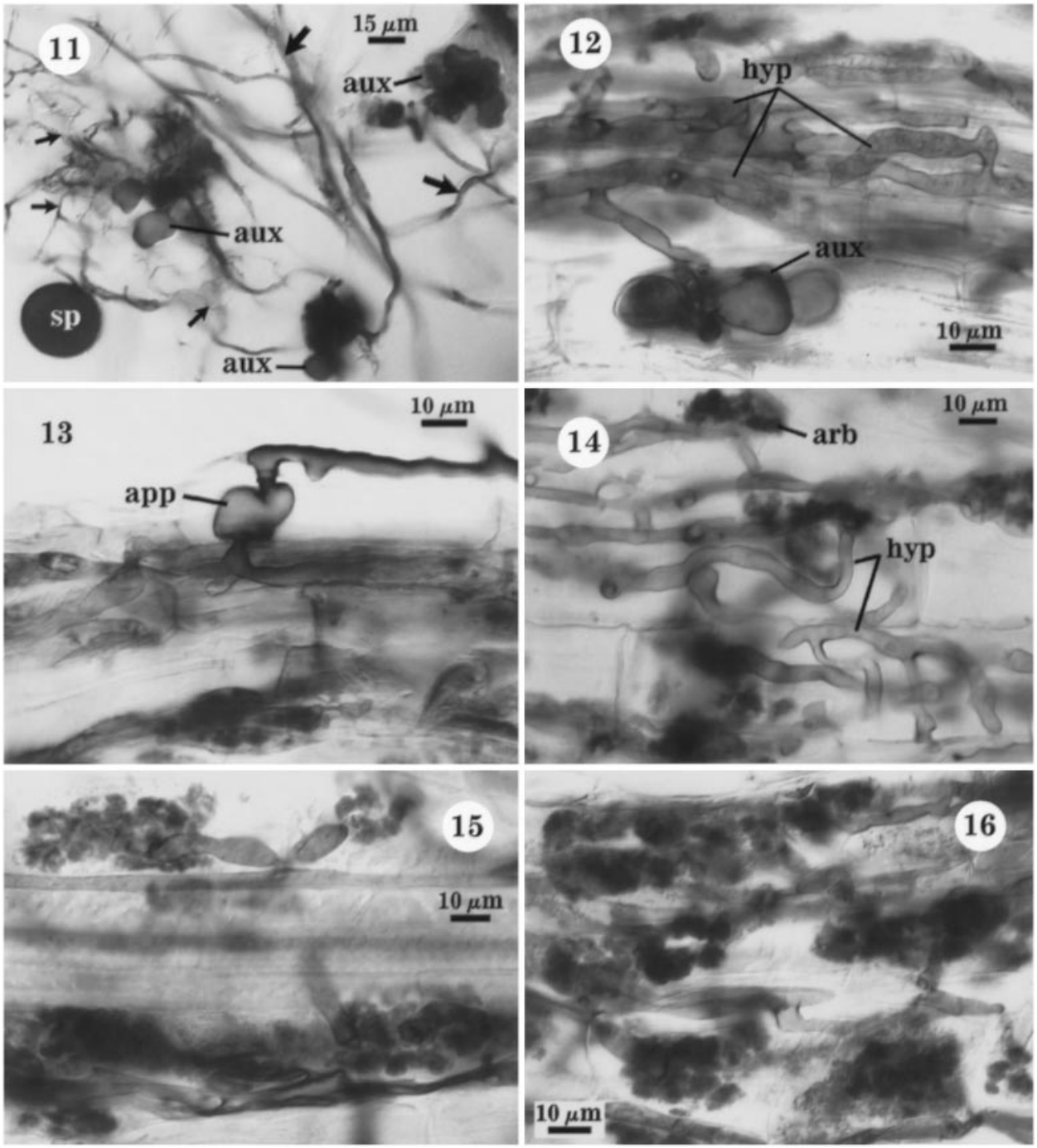
is 0·9–1·8 μm thick (mean = 1·4 μm) and the inner layer (2) is 0·3–0·9 μm thick (mean = 0·6 μm) (Fig. 1). All spores are pale cream (0/20/60/0) with contents densely opaque. In stage 2, morphological transformations occur only in the second layer as more sublayers are added. The laminae are plastic (amorphous) and expand as much as 48 μm in thickness with applied pressure (Fig. 2); also staining dark red in Melzer's reagent (Fig. 3). All subsequent stages arise after termination of spore wall synthesis, so changes are not reflected in morphology of intact spores under a stereomicroscope. Stage 3 involves differentiation of the first flexible hyaline inner wall (iw1), in which each of its two layers form sequentially (Fig. 4). Stage 4 is distinguished by synthesis of a second flexible inner wall (iw2), with each of its two layers forming sequentially (Fig. 5). Synthesis of germination shield marks stage 5 and the end of internal spore differentiation (Fig. 6).

Scutellospora rubra Stürmer & J. B. Morton, sp. nov. (Figs 1–16)

Etym.: Latin, *rubra*, referring to the dark reddish colour of mature spores under reflected light.

Sporae singillatim in cellula sporogena productae, obscure aurantio-brunneae vel atrorubrae, globosae vel subglobosae, 140–220 μm diam., pariete sporo et parietibus interioribus, flexilibus, duobus. Paries sporus stratis duobus: stratum exterius laeve, 1·2–1·8 μm crassum, obscure brunneo-rubrum; stratum interius laminare, plasticum, 3·5–13 μm crassum, obscure brunneo-rubrum. Paries flexilis, interior, primus stratis duobus, hyalinis, adhaerentibus, utrumque stratum 0·6–1·3 μm crassum. Paries flexilis, interior, secundus similiter stratis duobus, hyalinis adhaerentibus, stratum exterius 0·5–0·6 crassum et stratum interius 0·6–1·3 μm crassum, in solutione Melzeri perpallide roseolescens.

Spores formed singly in the soil, terminally or subterminally on a bulbuous suspensor cell; dark orange-brown (0/60/100/0) to dark red-brown (20/80/100/0); mostly globose to subglobose, 140–220 μm (mean 180 μm) in size. *Spore wall* composed of two phenotypically distinct layers (1 and 2). The outer layer (1) is red-brown, smooth, 1·2–1·8 μm thick, tightly adherent to layer 2 (Fig. 7); boundaries are very difficult to detect in mature spores except when stained with Melzer's reagent. Layer 2 a dark red-brown, of finely adherent laminae (sublayers), highly plastic in PVLG and thus 3·5–12 μm thick, depending on amount of pressure applied when breaking a spore. In water, the intact spore wall is 3·5–7·5 μm thick. The second layer of the spore wall stains dark red-black in Melzer's reagent (20/80/70/10) (Fig. 3). A very thin flexible layer,



Figs 11–16. Mycorrhizal structures formed by *Scutellospora rubra* BR211 in 6 wk old maize roots stained with 0·05% trypan blue and mounted in PVLG. Voucher number in parentheses indicates slide from which each photo was obtained. **Fig. 11.** Extraradical auxiliary cells (aux), together with thicker dark brown (large arrows) and thinner hyaline (small arrows) extraradical hyphae and a juvenile spore (sp) (S3321). **Fig. 12.** Knobby intraradical hyphae (hyp) and a cluster of auxiliary cells (aux) within a root cortical cell (S3321). **Fig. 13.** Appressorium (app) formed on the surface of the root from a thick knobby extraradical hypha (S3321). **Fig. 14.** Typical coiled intraradical hyphae (hyp) with smaller arbuscules (arb) (S3321). **Fig. 15.** Arbuscule showing the thick trunk and rapidly narrowing of the branching hyphal tips (S3321). **Fig. 16.** Typical dense distribution of arbuscules (S3321).

0·6 μm thick, is present, but it usually is detected only from remnants at its point of attachment to the spore wall in the region of the occlusion plug (Fig. 8).

Inner wall 1 is 0·6–1·1 μm thick and consists of two hyaline adherent layers of equal thickness (Fig. 7). It forms an 'endospore' that is completely separate from the spore wall. Inner wall 2 consists of two adherent hyaline layers; the outer one is 0·5–0·6 μm thick and the inner one is 0·6–1·3 μm thick (Fig. 7). The inner layer differentially produces a pale pink (0/20/20/0 to 0/40/20/0) reaction in Melzer's reagent. In some spores, both inner flexible walls group together after breakage, so they appear as one wall with four layers (Fig. 9). Germination shield consistently violin-shaped, slightly lobed, 60–68 × 82–109 μm, with pale yellow-brown (0/5/20/0)

boundaries; always forming between iw1 and iw2 (Fig. 10). Sporogenous cells concolorous with the spore wall, 18–35 µm broad; the wall consisting of two layers 1·2–3·0 µm thick (thickest near the base of the spore) that are continuous with layers of the spore wall (Fig. 8). The inner surface of the inner wall layer sometimes warty in older spores. Auxiliary cells dark yellow-brown (0/20/80/0), 15–28 µm broad, smooth or with flattened knobby projections; forming in clusters of 5–11 cells generally borne on a coiled external hypha (Fig. 11) or more rarely inside root cortical cells (Fig. 12).

Mycorrhiza properties: extraradical hyphae were of two morphotypes, one thinner and hyaline (0·8–1·6 µm) and the other thicker (2·4–4·7 µm) and yellow-brown (0/40/100/0) (Fig. 11). The thicker hyphae were most abundant near entry points. Within roots, intraradical hyphae vary considerably in thickness (1·6–6·3 µm) as a result of irregular swellings (Figs 12–14) and grow parallel to the root axis or form loose to tight coils (Figs 12, 14). Coiled hyphae were most abundant near entry points, but also are distributed throughout a mycorrhiza. Hyphal entry into roots often occurred through root hairs, but also at root surfaces where appressoria were evident (Fig. 13). Arbuscules were of both the Arum and Paris type (Smith & Read, 1997), although the latter were most abundant. Most arbuscules consisted of a swollen trunk, single or branched, 1·6–7·9 µm thick, and many fine terminal hyphal branches that stained darkly (Figs 15–16).

Distribution and habitat: known from a sample collected from a 8 mo old stand of *Eucalyptus dunnii* plantation in the state of Paraná, Brazil (INVAM accession BR211). The site had been cropped with soybean and in 1991 was turned into an industrial plantation of *Eucalyptus*. The site is within the domains of the forest dominated by *Araucaria angustifolia* (Bertolini) Kuntze. Soil was an oxisol derived from a latosol. Soil chemistry properties are: pH = 5·4, P = 11 ppm; K = 25 ppm; Al = 0·4 meq 100 g^{-1} soil; organic matter = 2·7 %. Also known from a site grown with oats during the dry season and soybean during the rainy season in Distrito Federal, Brazil (INVAM accession BR101). This site is within the domains of the Cerrado ecosystem. Soil was a dark red latosol. Soil chemistry properties of this site are: pH = 5·8, P = 5 ppm, K = 60 ppm, Al = 0·07 meq 100 g^{-1} soil.

Mycorrhizal association: found in the rhizosphere of *Eucalyptus dunni* in the field. Forming arbuscular mycorrhizae in pot culture with *Zea mays*, *Trifolium pratense*, *Glycine max*, *Festuca arundinacea*, and *Sorghum sudanense*.

Collection sites: Brazil – Paraná state – São Mateus do Sul, plantation site Laginski at the company Rigesa, Celulose, Papel e Embalagens Ltda. Distrito Federal – Planaltina, experimental field of CPAC-EMBRAPA.

Collection examined Holotype – Brazil, Paraná, São Mateus do Sul, S. L. Stürmer, 20 Sep. 1991. Spores from a single culture originated from a soil sampled under *Eucalyptus dunnii*. HOLOTYPE: deposited at Oregon State Collection (OSC) and consists of spores on microscopic slides in PVLG and PVLG mixed with Melzer's reagent, mycorrhizal roots on slides, and mature and immature spores and auxiliary cells preserved in 0·05 % sodium azide. ISOTYPE: deposited at Farlow Herbarium (FH). The cultotype of *S. rubra* is accession BR211 at INVAM.

DISCUSSION

Spore development of *S. rubra* follows a linear pattern of synthesis of spore wall, two flexible inner walls, germination shield identical to that measured in other *Scutellospora* species (Franke & Morton, 1994; Morton, 1995). Both *S. rubra* and *S. heterogama* Koske & C. Walker have unique transitory changes in synthesis of the spore wall. The laminate layer (2) initially differentiates with highly plastic properties expressed phenotypically by highly variable thickness in PVLG with applied pressure and by a dark red-purple reaction in Melzer's reagent. It then becomes rigid with resolvable thin and adherent sublayers (or laminae). This process is not identical in these two species. A discrete developmental stage can be defined for *S. heterogama* because the transition from an 'amorphous' to rigid laminate layer is accompanied by a change in spore colour from white to dark red-brown (Franke & Morton, 1994). In spores of *S. rubra*, changes in structure of the laminate layer are less dramatic (diminished plasticity) and pigmentation is acquired earlier so that spores change little in colour.

Mature spores of *S. rubra* also most closely resemble those of *S. heterogama*, with intergradation of shape, colour, and size. Both produce globose to subglobose spores ranging in colour from dark orange brown (0/60/100/0) to dark red brown (20/80/100/0). Spores of *S. heterogama* isolates range in mean size of a population, 160–180 µm (Franke & Morton, 1994) while that of *S. rubra* BR211 is 180 µm. If more isolates of *S. rubra* are found, we expect they would fall within the same size range as *S. heterogama*. Subcellular organization of the two species also is identical, with three layers in the spore wall, two flexible inner walls each consisting of two thin hyaline adherent layers, and the inner layer of iw2 staining pink in Melzer's reagent. The thin flexible layer (3) of the spore wall reported here for *S. rubra* is not reported in descriptions of *S. heterogama*, but reexamination of voucher slides indicate its presence in some spores (Morton, unpublished).

The distinctions between *S. rubra* and *S. heterogama* occur exclusively in properties of layers of the spore wall. The outer layer of the spore wall (1) of *S. rubra* is smooth whereas it has numerous rounded warts on the surface of *S. heterogama* spores. Without ornamentation, spores of *S. rubra* lack the white patchy coating often seen on freshly-extracted spores of *S. heterogama*. The laminate layer looks similar in mature spores, but the differentiation process early in development differs in pigmentation development and magnitude of changes in plasticity (see above). Differences between these two species do not appear extensive, but they are of the same degree and in the same spore structure (spore wall) as measured in all other species of *Gigaspora* (Bentivenga & Morton, 1995) and *Scutellospora* (Franke & Morton, 1994; Morton, 1995). Species-level variation also is restricted to spore wall properties in *Glomus* (Stürmer & Morton, 1997) and *Acaulospora* (Stürmer & Morton, unpublished).

Under the stereomicroscope, spores of *S. rubra* resemble those of *S. erythropa* Koske & C. Walker in colour and bright reflective surface, but *S. erythropa* spores are larger (mean 240 µm diam. in INVAM reference isolate MA453B) and more oblong. Subcellular structure of *S. erythropa* spores is

quite distinctive from that of *S. rubra*, with three flexible bilayered inner walls instead of two. *S. rubra* also can be confounded with spores of *S. hawaiiensis* Koske & Gemma as the darker spores of the latter can range to dark-orange brown. Spores of *S. hawaiiensis* are, however, larger (mean 240 µm diam.) and the innermost layer (2) of the second flexible inner wall (iw2) stains dark red-purple in Melzer's reagent.

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