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Anchoring the species *Rhizophagus intraradices* (formerly *Glomus intraradices*)

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Abstract: The nomenclatural type material of *Rhizophagus intraradices* (basionym *Glomus intraradices*) was originally described from a trap pot culture established with root fragments, subcultures of which later became registered in the INVAM culture collection as FL 208. Subcultures of FL 208 (designated as strain ATT 4) and a new strain, independently isolated from the type location (ATT 1102), were established as both pot cultures with soil-like substrate and *in vitro* root organ culture. Long-term sampling of these cultures shows spores of the species to have considerable morphological plasticity, not described in the original description. Size, shape and other features of the spores were much more variable than indicated in the protologue. Phylogenetic analyses confirmed earlier published evidence that sequences from all *R. intraradices* cultures formed a monophyletic clade, well separated from, and not representing a sister clade to, *R. irregularis*. Moreover, new phylogenetic analyses show that *Rhizoglomus venetianum* and *R. irregularis* are synonymous. The morphological characters used to separate these species exemplify the difficulties in species recognition due to the high phenotypic plasticity in the genus *Rhizophagus*. *Rhizophagus intraradices* is morphologically re-described, an epitype is designated from a single-spore isolate derived from ATT 4, and *R. venetianum* is synonymised with *R. irregularis*.

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INTRODUCTION

Glomus intraradices, described from a citrus plantation in Florida (Schenck & Smith 1982), is an arbuscular mycorrhizal fungus (AMF) that predominantly forms its spores intraradically. After *Glomus* was shown to be well separated at the generic level from this species, *G. intraradices* was renamed *Rhizophagus intraradices* (Schüβler & Walker 2010) following previous use of the genus name for AMF forming their spores in roots (Butler 1939, Gerdemann & Trappe 1974). Sieverding *et al.* (2014) proposed that *Rhizophagus* should be replaced with *Rhizoglomus*, but Walker *et al*. (2017) challenged this and proposed that the generic name *Rhizophagus* should be conserved, but with a change of type species to *R. intraradices*. We follow recommendation 14A.1 of the International Code of Nomenclature for algae, fungi, and plants (ICNafp) (Turland *et al.* 2018) by retaining 'existing usage'. Consequently, the current name, *Rhizophagus intraradices*, will be used throughout this work except where additional clarity will be gained by specifically using previous names.

From examination of published literature and DNA sequence databases, *R. intraradices* would seem to be common and widespread throughout the world, and organisms named *Glomus intraradices* have been used very extensively in mycorrhiza research. On 30 October 2021, a search for the species in the University of Western Australia's library (https://onesearch.library.uwa.edu.au) produced 5 739 peer reviewed references to *Glomus intraradices*, 1 470 to *Rhizophagus intraradices*, and 176 to *Rhizoglomus intraradices*, though in some publications, more than one of these names occur. For many of those published works it is impossible to verify the identity of the fungi used, and in most molecular ecological studies the species has been ascribed to *G. intraradices*, now classified in the genus *Rhizophagus* (Schüßler & Walker 2010). However, several distinct species have been confounded in most of these studies and erroneously named (Stockinger *et al.* 2009). In particular, the fungus formerly known as '*G. intraradices* DAOM197198', a widely used 'model organism' and the first genome-sequenced arbuscular mycorrhizal fungus (AMF) (Martin *et al.* 2008), was later determined to be *R. irregularis* (synonym *G. irregulare*), not *R. intraradices* (Stockinger *et al*. 2009, Sokolski *et al.* 2010). *Rhizophagus intraradices* cultures identifiable through molecular

[a]Part of the Belgian Coordinated Collections of Microorganisms (BCCM).

sequencing seem to have rarely been collected or isolated since its original description by Schenck & Smith (1982).

Molecular community studies, identifying the fungus based on species-resolving molecular characterisation, have shown the presence of *R. intraradices* in *Zea mays* in Belgium (Alaux *et al.* 2021), *Capsicum annuum* var. *glabriusculum*, *Glycine max* and *Citrus sinensis* in Mexico (Senés-Guerrero *et al*. 2020), *Triticum aestivum* from Switzerland and composite root samples from Ecuador (Schlaeppi *et al.* 2016) but, to date, it appears to have been established in pure culture only from citrus plantations (Schenck & Smith 1982, this study) and the Konza prairie, Kansas, USA (INVAM culture KS906).

The morphological descriptions of some species in the genus *Rhizophagus* overlap considerably (*e.g*., *R. irregularis* (Błaskowski *et al*. 2008), *R. custos* (Cano *et al*. 2009) *R. prolifer* (Declerk *et al.* 2000), *R. venetianum* (Turrini *et al.* 2018), *R. aggregatum* and *R. intraradices* (Schenck & Smith 1982)) and it is very difficult or impossible to distinguish them from spores collected from field soils. Difficulties in interpreting type species, their descriptions and the possibility of cryptic speciation in the genus *Rhizophagus* present serious problems for interpreting and assigning species names with confidence. The paucity of molecular data for accurately identified species in the phylum *Glomeromycota* is a further barrier to interpreting species of AMF, as discussed by Stefani *et al.* (2020).

Glomeromycotan fungi presently cannot be maintained in axenic culture, and are normally grown in pot culture (PC) with a suitable host plant, or monoxenically in root organ culture (ROC) or with tissue-cultured plants or disinfested seedlings on a gel-based substrate in sealed systems (Vestberg & Uosukainen 1992, Fortin *et al.* 2002, Lalaymia & Declerck 2020). *Rhizophagus intraradices* and its close relatives can be established by these methods, so we compared its spore morphology in both PC and ROC from type material, ex-type cultures (including a single-spore isolate), and a new isolate established from samples taken from the original type location approximately 30 yr after the species was first collected. Samples of subcultures of different ages and with different host plants were used to define the taxonomical molecular and morphological characteristics of the species.

Based on spore characteristics and phylogenetic data, the aims of this study were:

- to re-describe the fungus *Rhizophagus intraradices* (synonym *Glomus intraradices*) from an ex-type culture,
- to compare ex-type culture material with a new isolate established from the type locality approximately three decades after the original type was collected,
- to define an epitype from a single spore isolate derived from the original ex-type culture,
- to compare the phenotypic plasticity of spores formed in ROC and PC on different plant hosts, providing a detailed description of *R. intraradices* spore variation.

MATERIALS AND METHODS

Specimens

For convenience, we follow Seifert & Rossman (2010), by referring to type-descendant cultures as 'ex-type'. The nomenclatural code (ICNafp) (Turland *et al.* 2018) recommends, but does not mandate (Recommendation 8B.2), the use of this term for cultures derived from type material that were "… permanently preserved in a metabolically inactive state". The parental cultures of such 'type-descendent cultures' are not metabolically inactive and may have changed or have been contaminated over time. Nevertheless, the term is easily understood and acts as a useful shorthand. The term as applied to *R. intraradices* refers to many culturing generations over almost four decades, most of which lack detailed published records.

The holotype of *R. intraradices* was borrowed from the herbarium at Oregon State University (OSC 40255). Some of the spores from this collection (preserved in lactophenol) were washed in water and placed in a Petri dish of water for initial observations. Some of these were then transferred to microscope slides for observation through the compound microscope.

Cultures

The original 'type culture' appears not to have been given an identifier, but an ex-type culture was designated culture FL 208 upon incorporation in the INVAM culture collection. A sample of substrate, containing roots and spores, was obtained from INVAM, and established in PC and ROC. Cultures were catalogued with an attempt (ATT) number and subculture number (Walker & Vestberg 1998), the former being the unique identifier of the first attempt at establishing a culture, and the latter indicating the particular subculture (Fig. S1). Initial culture attempts are always "number-0" and all subsequent culture attempts derived from it are automatically given their unique subculture number. The original trap culture, established by S. Nemec, at the United States Department of Agriculture, Agricultural Research Service, Orlando, Florida was catalogued as ATT 4-0 and the 'type culture' was labelled ATT 4-1. The full history of subculturing from ATT 4-0 is unknown and Fig. S1 provides all the available information. Where data were not available, some attempt numbers (*e.g*. ATT 4-1 and 4-3) (Fig. S1-1) are 'notional entries' covering several subcultures. Subcultures were established from ATT 4-36 resulting in isolates from single propagules in both PC and ROC (Fig. S1-2). A sample of ATT 4-88 (of single-spore ancestry) was sent to M. Saito (Tohoku and Iwate Universities, Japan) and incorporated in the National Agriculture and Food Research Organisation, Japan (NARO) Genebank as MAFF 520088.

Three decades after the first isolation of ATT 4-0, a new sample collected by S. Nemec, from *Citrus* sp. at the type locality, was used to establish a closed soil-trap PC (ATT 1102-0) with *P. lanceolata* as host. A single spore from this culture was then used to establish a new isolate, ATT 1102-7 as a culture-line independent of the *R. intraradices* type culture and the resultant INVAM FL 208 culture (Fig. S1-3). Further PC and ROC subcultures with various host plants were established, allowing comparison of two independent cultures, established 30 years apart, from the type locality.

The database also controls the identifier given to samples and specimens therefrom, whether from field collections or from cultures. Each such sample receives its unique number, and consequently a culture may produce more than one voucher, *e.g.*, W 5413 and W 5501 from ATT 4-41 (Fig. S1-2) if sampled at different dates. Voucher numbers (usually applied to prepared microscope slides, but sometimes dried PC substrate containing roots and spores) were prefixed by 'W', thus, for example, the voucher from the notional ATT 4-36 (the INVAM culture from which material was supplied to us) is W 5128. Unless otherwise

stated, all vouchers are part of the C. Walker collection, lodged at the Herbarium of the Royal Botanic Garden Edinburgh (E) (see Index Herbariorum – http://sweetgum.nybg.org/science/ih/). Specimens from these collections and cultures were examined by light microscopy to determine morphological characteristics.

Specimen extraction

For PC, extraradical spores and root fragments containing spores were extracted from the substrate by suspending a sample (approx. 30 mL) in a beaker of water, agitating vigorously with a spatula, stirring to produce a vortex, and decanting through a 53 µm sieve after approx. 10 s of settling ('swirling and decanting'). The resultant sievings were then backwashed into 6-cm-diam Petri dishes for observation under a dissecting microscope with reflected light. The spores and roots from ROC were retrieved with forceps and washed in water to remove any remaining gel before being similarly transferred to a dish of water. Spores were handled with finely sharpened flexible stork bill tweezers (http://vomm.com, item 113 SA, Solingen, Germany) that facilitate the handling of individual spores without causing physical damage.

Phylogenetic analyses

The extended barcode for AMF (Stockinger *et al.* 2010) was used as DNA marker for molecular phylogenetics, consisting of the 3' region small subunit rRNA gene (SSU), the ITS region including the 5.8S rRNA gene, and a 5' region of the large subunit rRNA gene (LSU), usually described as SSU-ITS-LSU fragment or sequence, amplified with AMF-specific primers SSUmCf and LSUmBr (Krüger *et al.* 2009). To improve robustness and resolution of deeper branches, individual SSU-ITS-LSU sequence variants (~1.5 kb) from *R. intraradices*, if available, were concatenated with a SSU consensus sequence (~1.8 kb) of the same isolate (Krüger *et al.* 2012). An analysis excluding this SSU as 'anchor' was consistent and resulted in the same clades, but partly with lower bootstrap support (not shown). Sequences of the highly variable ITS1 and ITS2 regions were excluded from the analyses, because they show a very high intraspecific variability of up to 15 % for *Rhizophagus* species (Stockinger *et al.* 2010), making unambiguous alignment difficult.

For *R. irregularis* DAOM197198, phylogenetic trees including short sequences had been already published (Stockinger *et al.* 2009); here, only near-full-length SSU-ITS-LSU sequences allowing good phylogenetic resolution were used. PCR primer binding sites were excluded from all analyses. Sequences of the closely related genus *Sclerocystis* were used as the outgroup.

A maximum likelihood phylogenetic analysis was computed using the raxmlGUI v. 2.0 (Edler *et al*. 2020). The analysis, based on an alignment of 158 sequences with a length of 2 739 base pairs was computed with RAxML v. 8 (Stamatakis 2014) with 1 000 bootstraps. The GTRGAMMAI substitution model was selected as the best substitution model, using modeltest as implemented in RAxML v. 8.

Morphological analysis

Where possible, specimens were separated into extraradical or intraradical spores which were measured separately. Observations on specimens were made following the established methods, initially, by reflected light, under a dissecting microscope at magnifications of up to 50×, followed by detailed examination of individual spores mounted on microscope slides in polyvinyl alcohol lacto-glycerol with (PVLG-M) or without (PVLG) the addition of Melzer's reagent (Walker *et al.* 1993, Walker & Vestberg 1998).

Images were recorded digitally with a Canon EOS D30, 5D, 60D or 6D camera mounted on a phototube with 80 mm, 5× or 10× projective lens. Spore colour was established by comparing the specimens in a dish of water (BPI watchglass - https://catalog.ndsglass.com/viewitems/all-categories-new-products/bpi-watch-glasses) under a Leica MZ8 microscope with the Royal Botanic Garden Edinburgh (RBGE) colour chart (Anon 1969), the Munsell® Soil colour chart (Anon 1990) or the Methuen Book of Colour (Kornerup & Wanscher 1978). On occasions when it was not possible to make comparisons with a chart, a vernacular colour name was given. Charts were illuminated by the third arm of the split fibre optic illumination source as described in Walker *et al.* (1993) to match colours. The RBGE colours are indicated by a name with a number [*e.g.*, ochre (9)] representing the colour chip on the chart. Munsell numbers are in standard notation (*e.g.*, 10YR 5/8, strong brown) representing the hue, value and chroma and a standard colour name. Methuen colours are designated with the plate number and colour patch number with the associated general colour name (*e.g.*, 5F8, brown).

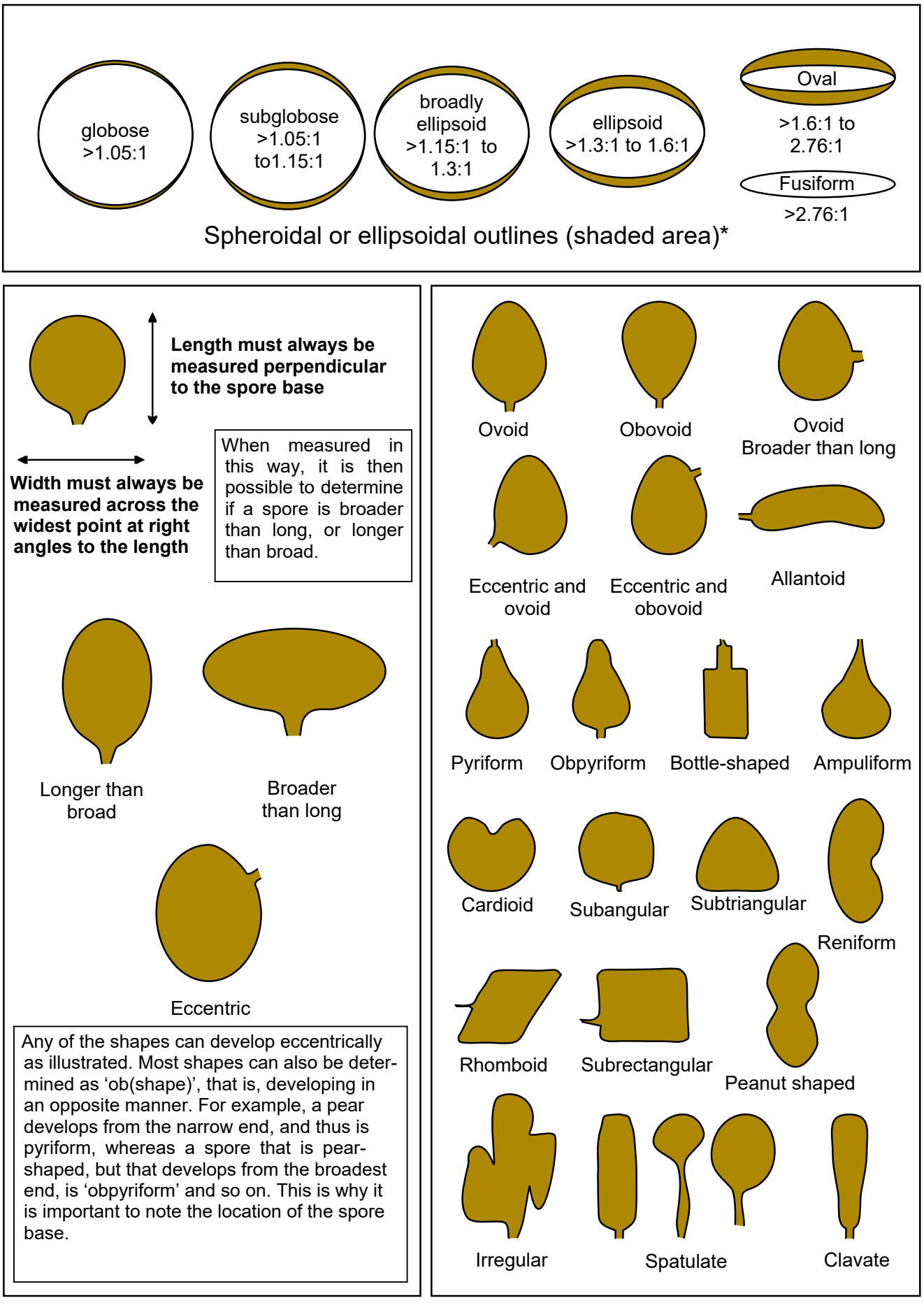
Spore dimensions were measured by means of a calibrated eyepiece graticule. Most were measured with a graticule division size of 1.6 µm, but those larger than about 160 µm had to be measured at a lower magnification, with graticule divisions of 2 or 2.5 µm. Consequently, though most measurements are accurate to within 0.8 µm, overall accuracy should be assumed to be ± 1.25 µm. Measurements were always length by width, the length being taken as the longest dimension perpendicular to the point of development from the subtending hypha, and the width at right angles to this, hence many specimens are 'broader than long'. Guidance on spore measurements and shape determination is summarised in Fig. 1. Because the size and shape of irregular spores are so variable, these were treated separately.

Statistical analyses

Measurements of spores from the type material and subsequent subcultures (ATT 4) and from the new strain (ATT 1102) were analysed statistically (Tables 1, S1). Not every culture produced both intraradical and extraradical spores. From the type culture material (ATT 4-1), 52 extraradical spores and 100 intraradical spores were measured. All were from a PC of unknown age. From the subsequent 9 PCs sampled, 675 extraradical, and 461 intraradical spores were measured from 10 vouchers aged between 95 and 1 789 d from inoculation. There were seven ROCs from culture line ATT 4, one of which was sampled on two different dates. Together, these produced 654 extraradical spores, but only two within root tissue.

ATT 1102 was sampled from three PCs, one of which was sampled on two different occasions, resulting in 350 extraradical spores and 300 intraradical spores. Three ROCs were sampled resulting in 245 extraradical spores and no intraradical specimens.

Twenty-nine different spore shapes were identified (Table 2) and compared by strain (ATT 4 vs ATT 1102), spore position (extraradical or intraradical), culture type (PC or ROC) (Table 1) and shape of spores (Table 2) The effect of host plant on the main spore shape was also examined (Tables 3, 4).



*Adapted partly from Kirk *et al*. (2010)

Fig. 1. Guidance for measuring spores of glomeromycotan fungi, including standard spheroid descriptions (adapted from Kirk *et al*. 2010) and some common different shape outlines.

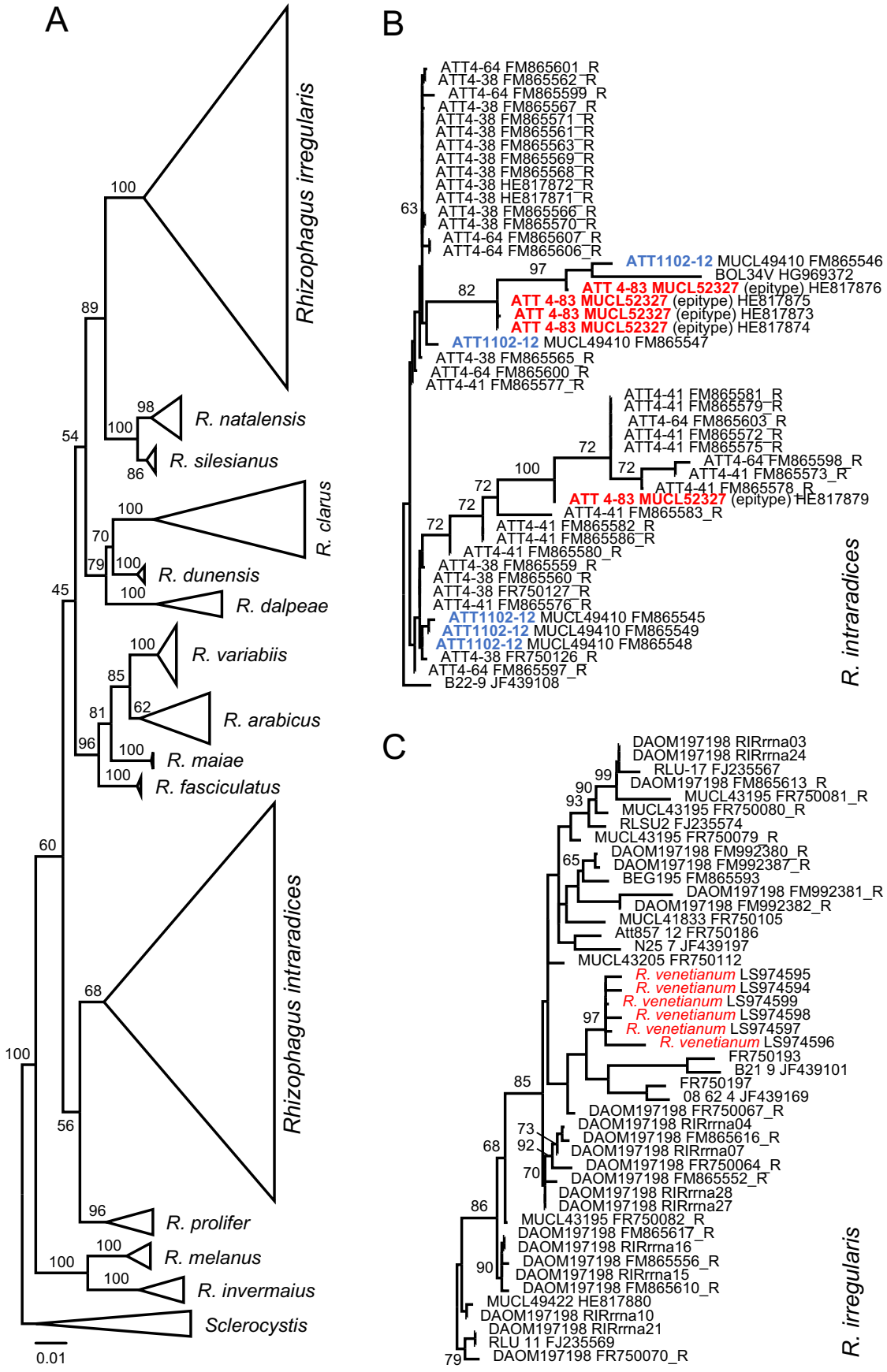


Fig. 2. Phylogenetic maximum likelihood tree of *Rhizophagus* species and isolates ATT 4 (FL 208) and ATT 1102 of *R. intraradices*. For the completely resolved and annotated tree see Fig. S2. **A.** Characterised *Rhizophagus* species, with *Sclerocystis* as outgroup. Size of triangles represent the sequence numbers (vertically) and distances (horizontally). **B.** Details of *R. intraradices*, showing that descendants (ATT 4-38, ATT 4-41, ATT 4-64) of the ex-type culture FL 208, including (red typeface) the culture from which the epitype was taken (MUCL 52327 = ATT 4-83) and (blue typeface) the strain (MUCL 49410 = ATT 1102-12) newly isolated from the type locality cluster in the same monophyletic clade. **C.** Details of *R. irregularis*, showing that "*Rhizoglomus venetianum*" (red typeface) represents one subtype of the DNA sequence variants of *R. irregularis*; sequence variants annotated 'RIRrrna##' are from a genome sequencing project (Maeda *et al.* 2018).

Table 1. Spore lengths and widths (µm) of *Rhizophagus intraradices* strains (ATT 4 and ATT 1102 and both combined), for pot cultures (PC) and root organ cultures (ROC) by culture type and spore position (intra- or extraradical). For each strain, means were compared to each other. Means with the same letters are not significantly different (P ≤ 0.05). To aid comparison, the protologue measurements (Schenck & Smith 1982) and our own measurements from the holotype material (ATT 4-1) are shown separately.

				Spore length (µm)					Spore width (µm)				
Strain	**Culture type**	**Spore position**	**n**	**min**	**median**	**max**	**mean ± SD**	**CV %**	**min**	**median**	**max**	**mean ± SD**	**CV %**
ATT 4	PC	intraradical	561	18	82	234	84 ± 29 b	34	16	72	152	74 ± 27 b	37
	PC	extraradical	727	29	99	224	98 ± 27 a	28	29	96	165	96 ± 26 a	27
	ROC	extraradical	654	30	97	182	98 ± 24 a	24	30	96	178	96 ± 25 a	26
	PC & ROC	extraradical	1 381	29	98	224	98 ± 26 a	26	29	96	178	96 ± 26 a	27
	PC & ROC	intraradical	561	18	82	234	84 ± 29 b	34	16	72	152	74 ± 27 b	37
	PC & ROC	intra- & extraradical	1 942	18	94	234	94 ± 27	29	16	91	178	90 ± 28	31
ATT 1102	PC	intraradical	300	18	93	218	99 ± 37 a	37	25	80	202	85 ± 31 c	36
	PC	extraradical	350	26	88	383	92 ± 39 b	42	26	88	398	92 ± 40 b	43
	ROC	extraradical	245	48	96	147	96 ± 18 a	19	46	94	146	95 ± 18 a	19
	PC & ROC	extraradical	595	26	91	383	94 ± 32 a	34	26	91	398	93 ± 33 a	35
	PC & ROC	intraradical	300	18	93	218	99 ± 37 a	37	25	80	202	85 ± 31 b	36
	PC & ROC	intra- & extraradical	895	18	93	383	95 ± 34	35	25	88	398	90 ± 32	36
Combined	PC	intraradical	861	18	85	234	89 ± 32 a	36	16	75	202	77 ± 29 a	38
	PC	extraradical	1 077	26	96	383	96 ± 32 b	33	26	94	398	95 ± 31 b	33
	ROC	extraradical	899	30	96	182	97 ± 22 b	23	30	96	178	96 ± 23 b	24
	PC & ROC	extraradical	1 976	26	96	383	96 ± 28 a	29	26	95	398	95 ± 28 a	29
	PC & ROC	intraradical	861	18	85	234	89 ± 32 b	36	16	75	202	77 ± 29 b	38
	PC & ROC	intra- & extraradical	2 837	18	93	383	94 ± 29	31	16	90	398	90 ± 29	32
Isotype	PC	extraradical	52	42	103	224	104 ± 30 a	29	42	98	154	99 ± 23 a	23
	PC	intraradical	100	29	71	186	79 ± 31 b	39	18	61	147	66 ± 29 b	44
	PC	intra- & extraradical	152	29	86	224	87 ± 33	38	18	79	154	77 ± 31	40
protologue	PC	intraradical	n/a	40.5	n/a	191.5	n/a	n/a	93	n/a	131	n/a	n/a

All statistical analyses were conducted in R (R Core team, 2017) with a significance level of p ≤ 0.05. Normality of distribution and homogeneity of variance were checked for spore dimension data, followed, where appropriate, by ANOVA for specific factors (*e.g.*, to determine if number of attempts or type of culture had an impact on the spore dimensions). Significant ANOVA (p ≤ 0.05) tests were followed by a post-hoc Tukey's test for comparisons among means (p ≤ 0.05). The spore shape data were analysed with Chi square tests (p ≤ 0.05) in relation to attempt number, spore position, type of culture, and host plant used for subcultures.

RESULTS

Molecular analysis

Rhizophagus intraradices sequences, including all culture lines studied here (Figs 2, S2), form a monophyletic clade at the species level, separated from other species in the genus. The species is more closely related to *R. prolifer* than to *R. irregularis* (Fig. 2A). Sequences from ATT 1102-12 (the new strain from the type locality) are scattered within this clade (Fig. 2B).

During the studies, it became evident that *Rhizoglomus venetianum* (Turrini *et al.* 2018), was described based on a biased sequence selection. Its molecular phylogenetic position was therefore re-analysed. The analysis showed that the published sequences are phylogenetically embedded within the *R. irregularis* clade (Fig. 2C).

Morphology

Both ATT 4 and ATT 1102 produced spores externally in the substrate and within the root cortex or bursting through the roots (Figs 3A, B, 4A, B, 5G, H, 6G, H, 10B, G, H). Spore morphology, including length, width, shape (Figs 7, 8), colour and characteristics of the subtending hypha (Fig. 9) were much more variable among the ex-type cultures (ATT 4) and the new strain (ATT 1102) than in the type material.

Extraradical spores occurred singly, in loose clusters, in dense clusters (fascicles) in the substrate, loosely or densely around roots, in voids such as empty seed coats (Fig. 10A) or insect and mite integuments, and occasionally in mats on surfaces of soil components such as decaying leaves, but not all from any particular sample. They were similar in both PC and ROC (Figs 5, 6), except for differences in production of irregular spores, although in the latter they were usually much less darkly coloured. Intraradical spore production varied from none or few (particularly in ROC) through occasional individual spores in cortical cells (Fig. 4F), to roots crammed full of spores (Figs 3A, 4A), often bursting through the epidermis (Figs 5C, 6C). Most irregularly-shaped spores (Fig. 8) were identified as having come from root tissue.

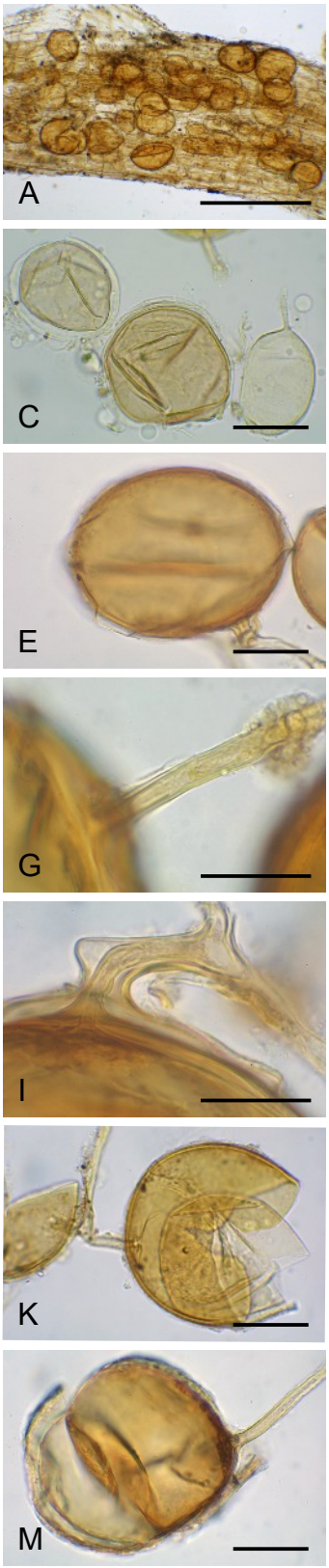


Fig. 3. Micromorphology of the holotype specimen of *Rhizophagus intraradices* (OSC 40255, 5 May 1981). **A.** Intraradical spores. **B.** With extra-radical spores singly and in clusters. **C.** Thin-walled spores extruded from a crushed root. **D.** Globose spore with subtending hypha (SH) detached close to the spore. **E.** Broadly ellipsoid spore. **F.** 'Pip-shaped' spore. **G.** Parallel-sided SH. **H.** Recurved SH. **I.** Recurved angular SH with lateral protrusion. **J.** SH with tubaeform flare. **K.** Crushed spore showing separation of wall components. **L.** Outer wall with thickening by bacterial colonies. **M.** Wall components separating on crushing. **N.** Bacterial colonies giving an impression of ornamentation. Scale bars: A = 250 µm; B = 1 mm; C–F, K–M = 50 µm; G–J, N = 25 µm.

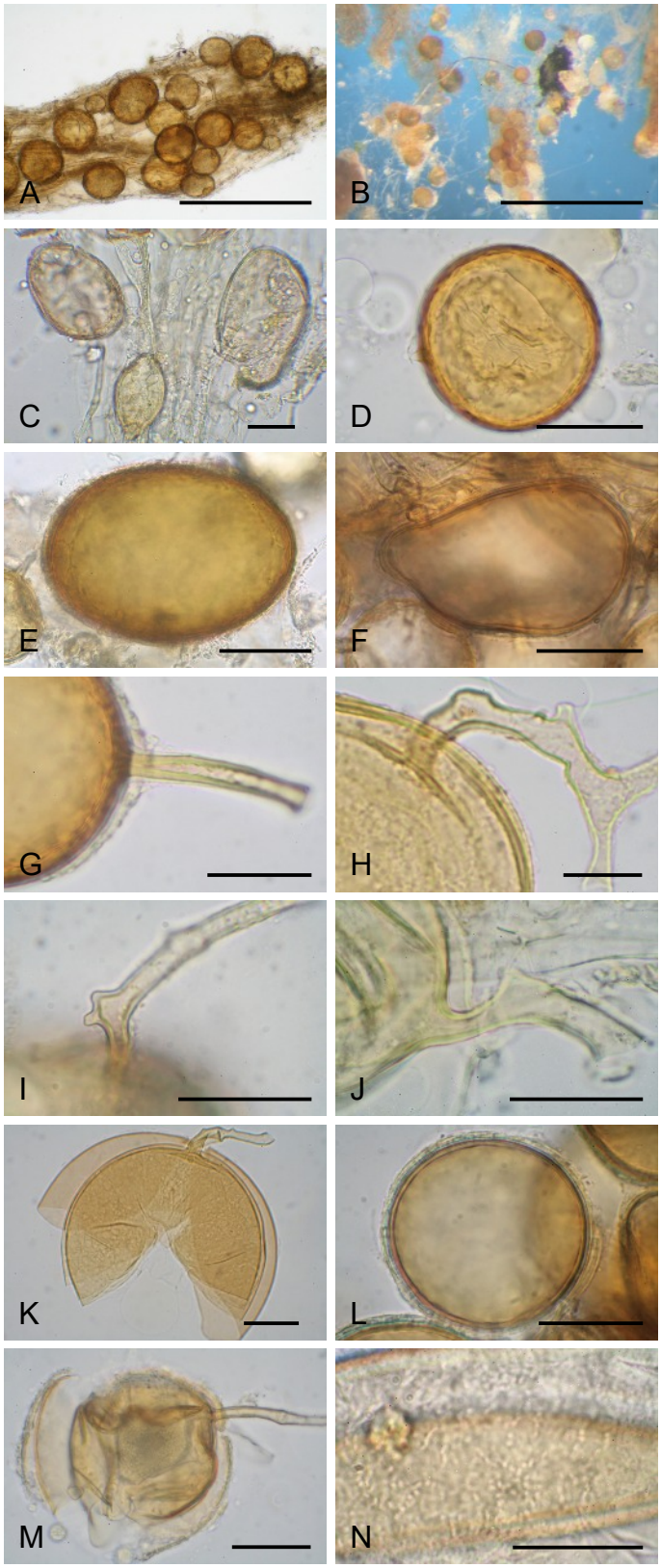


Fig. 4. *Rhizophagus intraradices* strain re-isolated from type locality in 1974. **A.** Intraradical spores. **B.** With extra-radical spores singly and in clusters. **C.** Thin-walled spores extruded from a crushed root. **D.** Globose spore with subtending hypha (SH) detached close to the spore. **E.** Broadly ellipsoid spore. **F.** 'Pip-shaped' spore. **G.** Parallel-sided SH. H. Recurved SH. **I.** Recurved angular SH with lateral protrusion. **J.** SH with tubaeform flare. **K.** Crushed spore showing separation of wall components. **L.** Outer wall with thickening by bacterial colonies. **M.** Wall components separating on crushing. **N.** Bacterial colonies giving an impression of ornamentation. Scale bars: A = 250 µm; B = 1 mm; C–M = 50 µm; N = 25 µm.

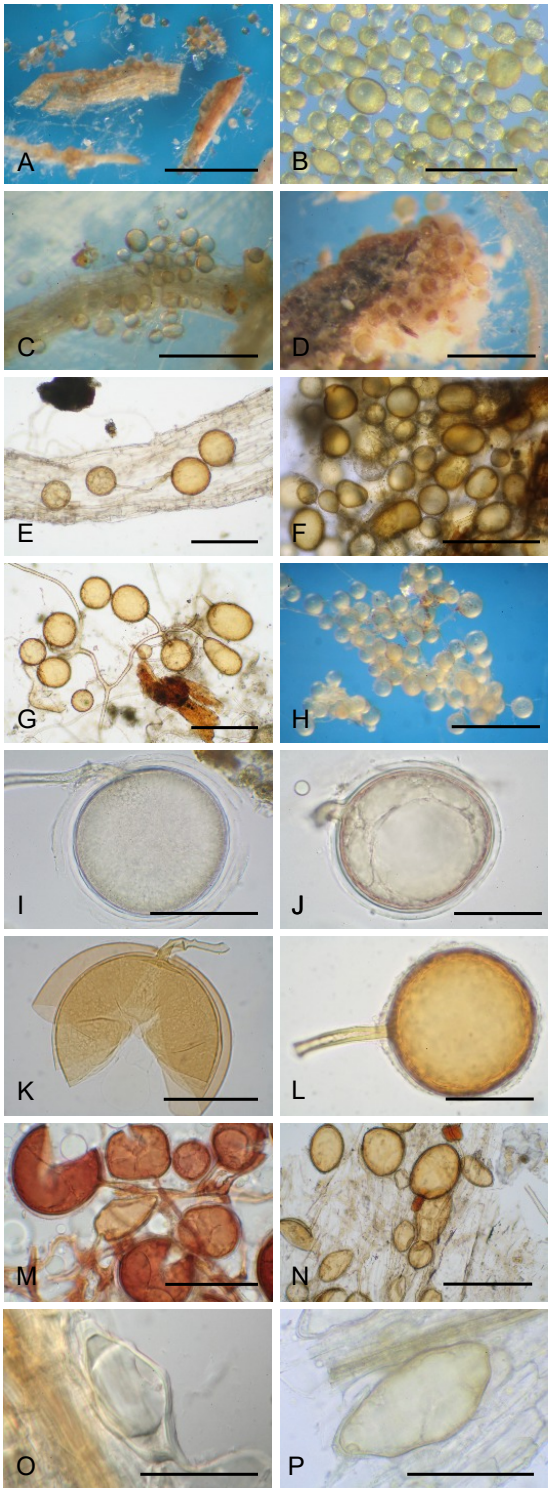


Fig. 5. *Rhizophagus intraradices* from pot cultures. **A.** Intra- and extra-radical spores. **B.** Extracted spores that were formed singly. **C.** Spores bursting through the root cortex and epidermis. **D.** Aggregation (fascicle) of pale extraradical spores. **E.** Spores formed in the root cortex. **F.** Fascicle of darkly coloured spores. **G.** Loose cluster of extraradical spores. **H.** Small, dense clusters of spores. **I.** Almost colourless young spore with expanding outermost wall component. **J.** Older spore developing pigmentation and with colourless outer component. **K.** Crushed spore showing wall components 2 & 3, having lost the evanescent outermost component. **L.** Darkly coloured spore retaining colourless outer component. **M.** Cluster of young spores with red reaction to Melzer's reagent. **N.** Cluster of old spores lacking a reaction to Melzer's reagent. **O.** Intraradical spore showing cap-like distal thickening of wall component 2. **P.** Misshapen (irregular) intraradical spore. Scale bars: A = 1 mm; B–D, H = 500 µm; E, G = 150 µm; F = 250 µm; I, K, M, P = 100 µm; N = 200 µm; J, L, O = 50 µm.

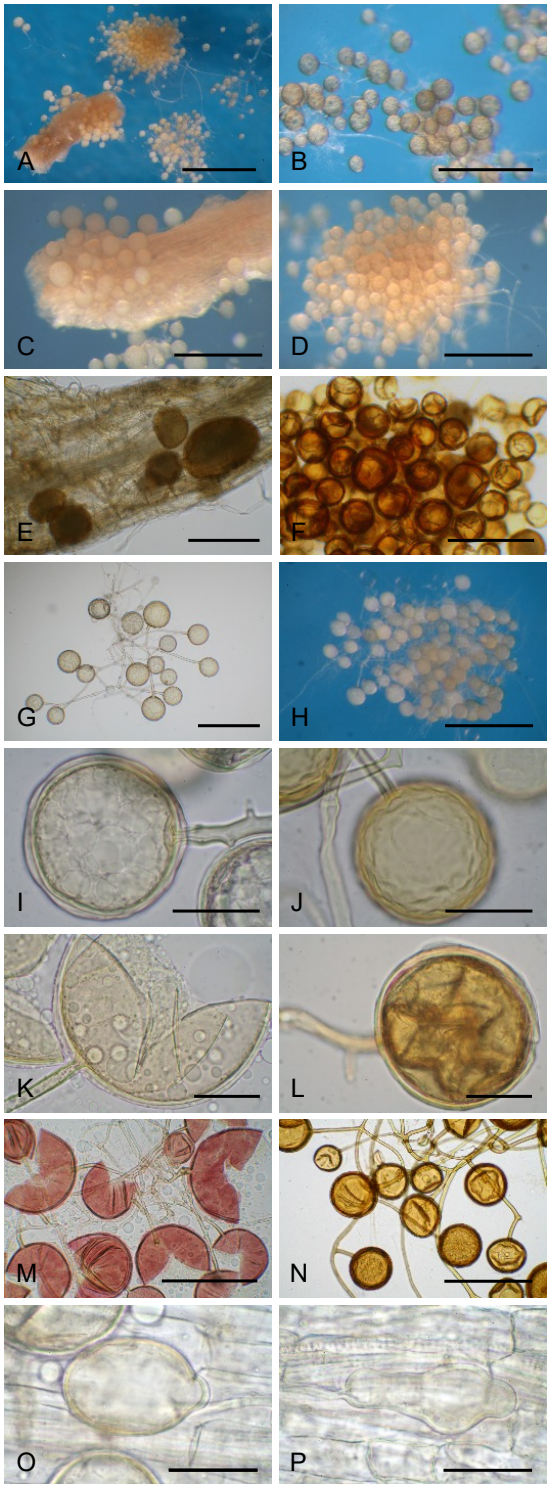


Fig. 6. *Rhizophagus intraradices* from root organ cultures. **A.** Intra- and extra-radical spores. **B.** Extracted spores that were formed singly. **C.** Spores bursting through the root cortex and epidermis. **D.** Aggregation (fascicle) of pale extraradical spores. **E.** Spores formed in the root cortex. **F.** Fascicle of darkly coloured spores. **G.** Loose cluster of extraradical spores. **H.** Small, dense clusters of spores. **I.** Almost colourless young spore with expanding outermost wall component. **J.** Older spore developing pigmentation and with colourless outer component. **K.** Crushed spore showing wall components 2 & 3, having lost the evanescent outermost component. **L.** Darkly coloured spore retaining colourless outer component. **M.** Cluster of young spores with red reaction to Melzer's reagent. **N.** Cluster of old spores lacking a reaction to Melzer's reagent. **O.** Intraradical spore showing cap-like distal thickening of wall component 2. **P.** Misshapen (irregular) intraradical spore. Scale bars: A = 1 mm; B–D, H = 500 µm; E = 150 µm; F, G = 250 µm; I–L, O, P = 50 µm; M, N = 200 µm.

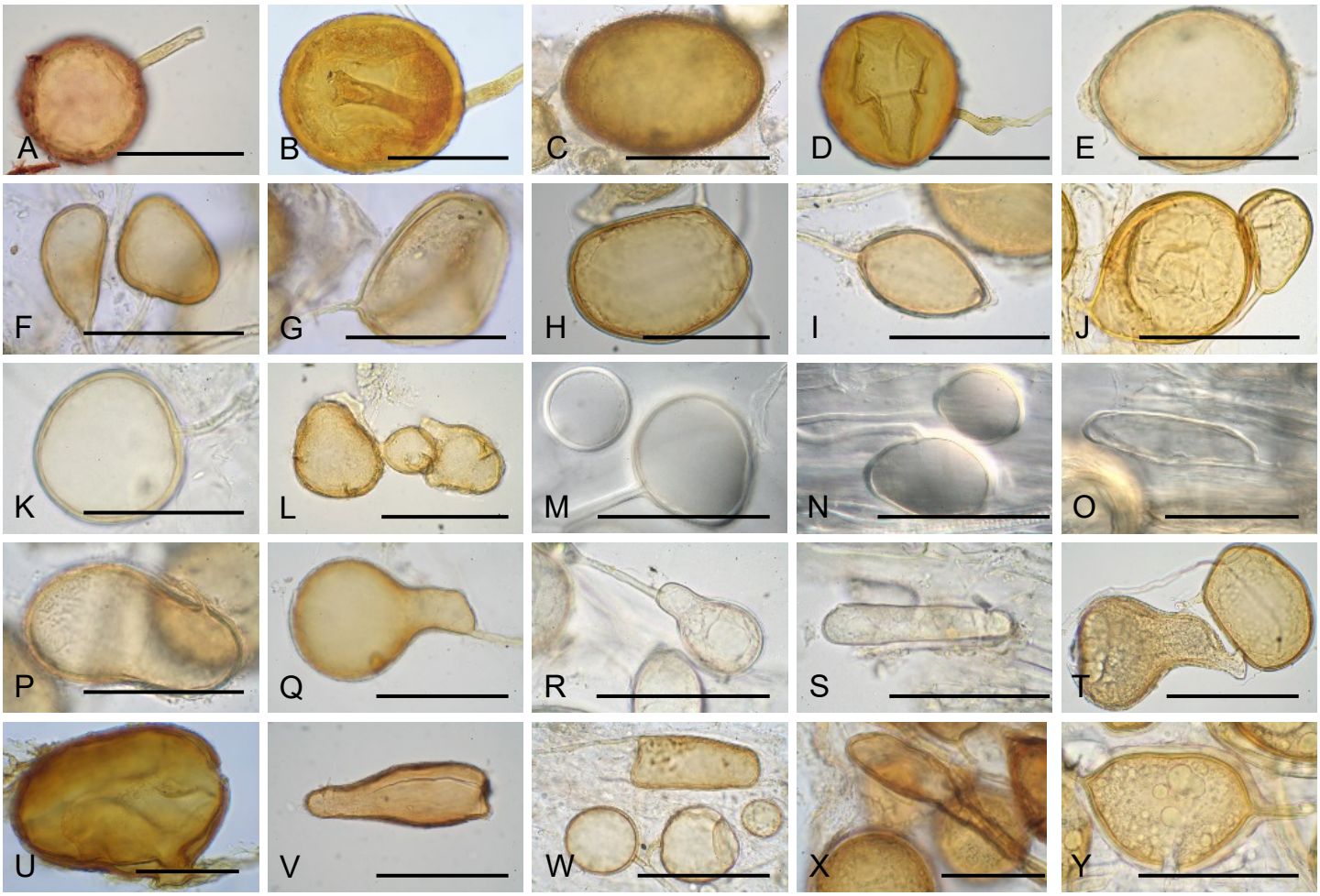


Fig. 7. Some examples of the many sizes, colours and shapes of spores of *Rhizophagus intraradices* (basionym *Glomus intraradices*). Scale bars: A–N, P, R, S, U, Y = 100 µm; O = 50 µm; Q, T, V, W, X = 150 µm.

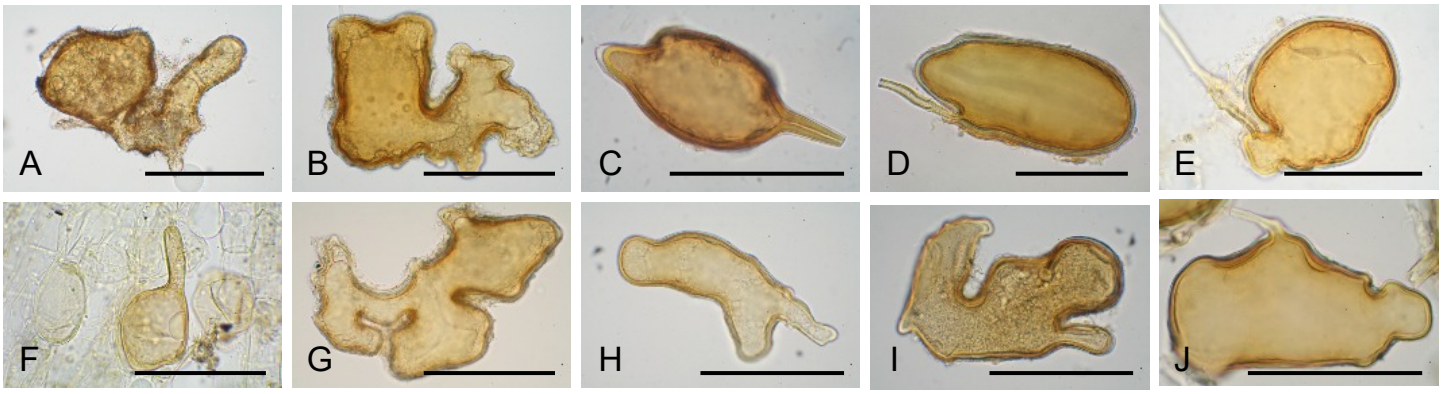


Fig. 8. Examples of the convoluted and irregular shapes found amongst pot cultures, but rarely in root organ cultures, of *Rhizophagus intraradices* spores. Scale bars: A = 250 µm; B, G = 200 µm; C–F, J = 100 µm; H–I = 150 µm.

Spore colour

The colour (Table S2, Figs 10, 11) of both extraradical and intraradical spores (both ATT 4 and ATT 1102), was very variable, ranging from colourless through shades of yellow to shades of brown. At first spores are thin-walled and very pale in colour (Fig. 11A), but as they develop, the laminated components thicken and darken, and gradually the overall spore colour changes through yellow (Fig. 11B) to yellowish brown, until they may appear quite dark brown (Fig. 11C). Most of the colour change occurs in the second (innermost) laminated component (Fig. 11D).

Spore shape

Combining data from both ATT 4 and ATT 1102 in PC and ROC, from 2 679 spores, the overall distribution of shapes varied considerably. The majority were globose (57.1 %) to subglobose (21.8 %). Other relatively common shapes included broadly ellipsoid (5.7 %), ellipsoid (3.7 %), oval (3.0 %) irregular (2.7 %) or ovoid (2. 4) specimens. Spores of 22 other shapes were observed, each with a frequency of < 2 % (Fig. 12). When comparing the shape of spores produced by ATT 4 and ATT 1102, the former had more different shapes (25:16) than the latter.

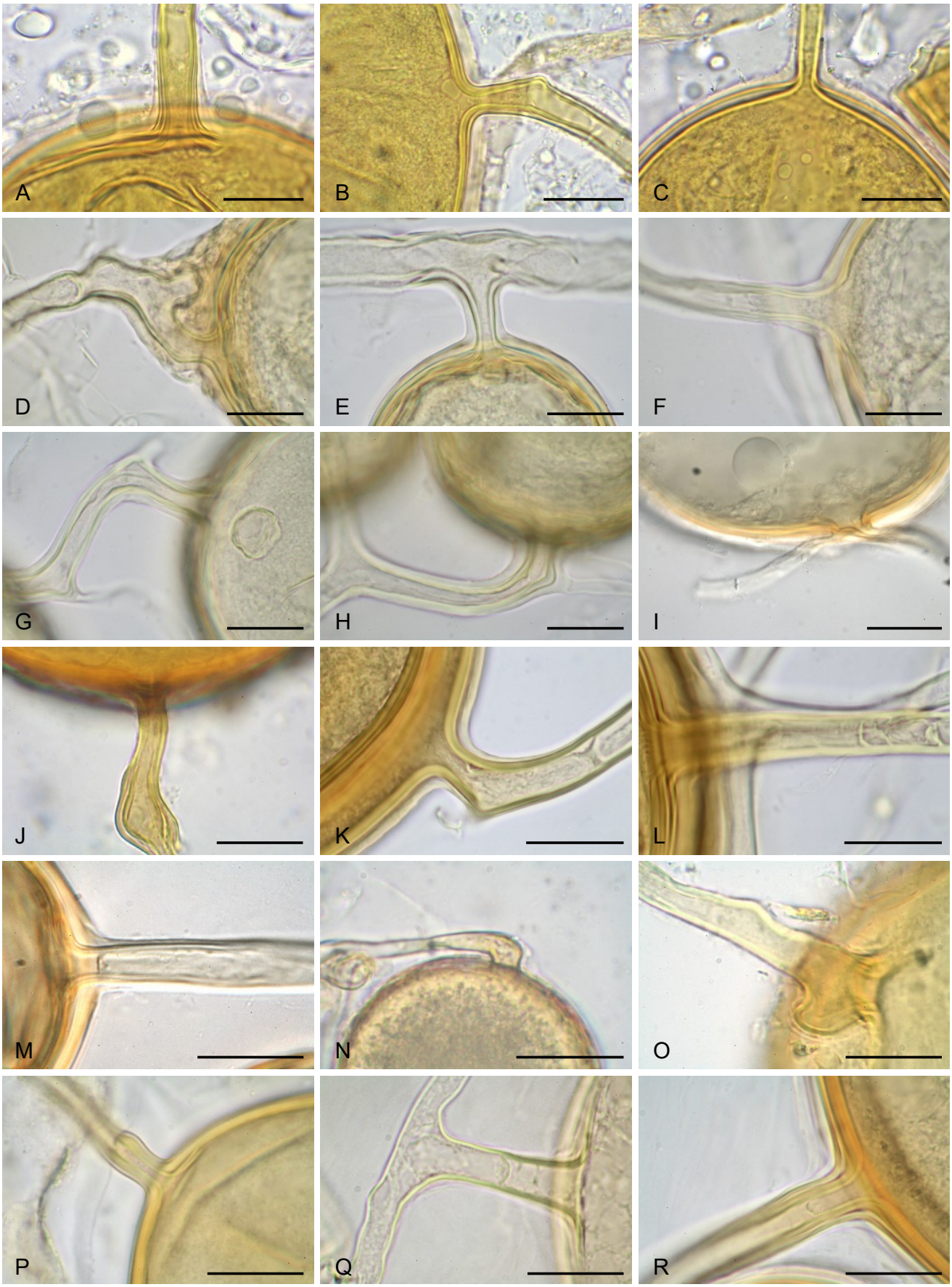


Fig. 9. Some of the variation among subtending hyphae of *Rhizophagus intraradices*. **A.** Parallel-sided, slightly flared proximally. **B.** Slightly recurved and narrowed at spore base. **C.** Gradually narrowing towards spore. **D.** Convoluted, branched. **E.** Short branch, narrowing towards spore. **F.** Funnel-shaped. **G.** Recurved, with tubaeform flare. **H.** Recurved, angular, flared. **I.** Laterally budded (lacking stalk). **J.** Swollen distally, tapering proximally. **K.** Recurved, angular with lateral peg. **L.** Flared, tapering slightly distally. **M.** Flared, tapering slightly proximally. **N.** Sharply recurved, expanded towards the spore base. **O.** Subangular, swollen and flared at the spore base. **P.** Thickened and constricted proximally. **Q.** Short branched, tubaeform. **R.** Tapering proximally, slightly flared, with septal occlusion. Scale bars = 20 µm.

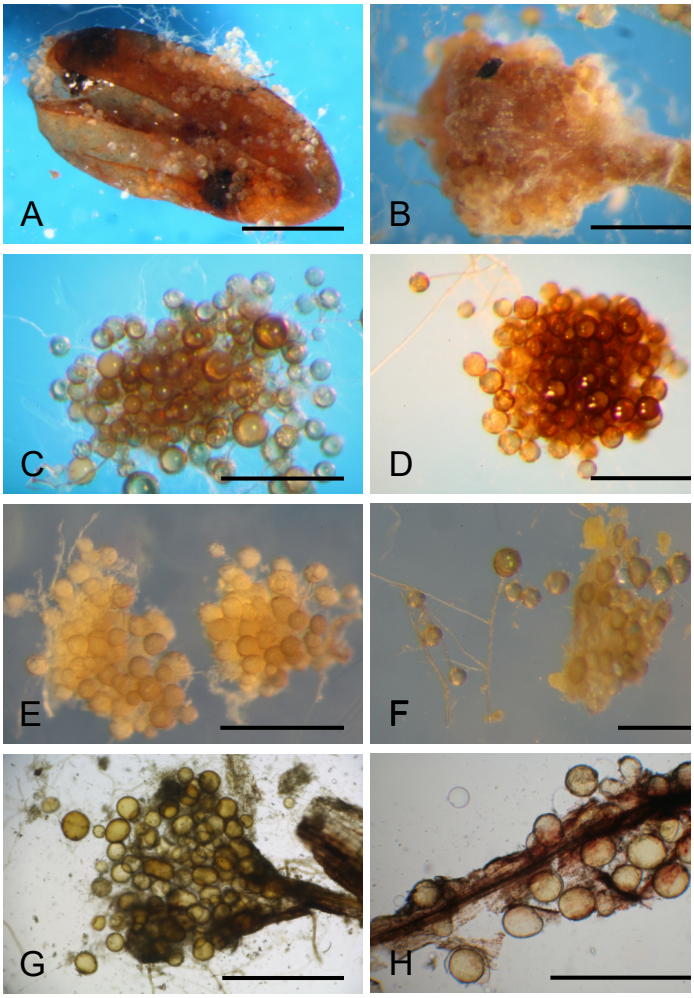


Fig. 10. *Rhizophagus intraradices* clusters of spores. **A.** Spores occupying an empty *Plantago lanceolata* seed. **B.** A dense cluster of spores amongst fine mycelium surrounding a root fragment. **C–F.** Spores in clusters of varying density showing colour variation. **G.** Cluster of spores bursting through the cortex of a decaying root. **H.** Spores clustering around the surface of a decaying root. Scale bars: A = 1 mm; B–H = 500 µm.

In ATT 4, only 1.66 % of spores were irregular, whereas for ATT 1102, the proportion was 5.19 % (Fig. 12). Moreover, this spore shape distribution is significantly different between ATT 4 and ATT 1102 at $p \leq 0.001$ (Table 2).

Several factors, predominantly spore position (whether extraradical or intraradical) (Fig. 13), culture type (Fig. 15) and host plant (Table 3, Fig. 16) significantly influence spore shape. For example, 91 % of extraradical spores were mostly globose (70 %) or subglobose (21 %), whereas 90 % of intraradical spores were globose (26 %), subglobose (23 %), ellipsoid (13 %), broadly ellipsoid (13 %), oval (9 %) or irregular (6 %) (Fig. 14).

Culture type had significant effect on spore shape with the distribution of shapes being significantly different ($p \leq 0.001$) between PC and ROC (Table 2). There were 8 different shapes from ROC (Fig. 15), mainly represented by globose (81 %), subglobose (15 %) and obovoid (2 %) spores, while spore shape in PC was much more variable, producing 27 shapes, mainly represented by globose (47 %), subglobose (25 %), broadly ellipsoid (6 %), ellipsoid (5 %), irregular (5 %), oval (4 %) and obovoid (3 %) spores. Similarly, spore shapes varied significantly among plant hosts (Fig. 16, Table 4), each host plant having its own specific spore shape distribution, except for *P. lanceolata* and *L. japonicus*.

Comparing the two lineages, cultures of ATT 4 and ATT 1102 produced predominantly globose (56.8 and 56.4 %) to subglobose spores (22.9 and 18.4 %). However, the remaining proportion of spores from the two cultures differed (Fig. 16). ATT 4 produced 21 different shapes of spores, including many irregular spores and some angular or asymmetrical in outline, whereas ATT 1102 had only 14, most of which were smooth in outline and bilaterally symmetrical.

For ATT 4, both the holotype collection and ex-type cultures, spores were produced both extra- and intra- radically (Fig. 13). The spores from the holotype were predominantly regular in shape (spheroid to ellipsoid), whereas much greater morphological variation occurred among the ex-type cultures (Figs 3, 7, 8). Although most were regular (globose, subglobose, broadly ellipsoid, ellipsoid or oval, fusiform, obovoid or ovoid), the range of shapes also encompassed bottle-shaped, pyriform, obpyriform, lacrimoid, rhomboid, reniform, subreniform, subcardioid, subtriangular, subangular, clavate, spatulate, lanceolate, flattened on one side through juxtaposition with other developing spores, or misshapen (irregular) (Figs 3–8). There were clear differences between the degree of shape variation among intraradical and extraradical spores. In particular, irregularly shaped spores were found predominantly in the PCs (both origins). However, ATT 4-84, a PC established from the ROC, ATT 4-88, yielded irregular intraradical spores, but no misshapen extraradical specimens.

For type and ex-type specimens (ATT 4) in PC, 27 (~5 %) of the intraradical spores and only 5 (~0.7 %) of the extraradical spores (and two base-unidentified) were irregular in shape. In ROC, there were two misshapen spores. For the new isolate (ATT 1102) in PC, 23 (9.2 %) intraradical spores and 22 (~8.6 %) extraradical spores were misshapen (irregular). All of these came from a subculture (ATT 1102-13) of (ATT 1102-7), a single spore isolate that had not produced misshapen spores. This strain did not produce irregular spores in ROC (extraradical spores only were formed), whereas in PC, there were differences among subcultures. For example, ATT 1102-7 (with *Plantago lanceolata*), the first generation of the single spore isolate, produced very large numbers of mainly extraradical spores (W 4655), whereas intraradical spores were sparse. Of the 100 extraradical and 50 intraradical spores examined, none was irregular, although two of the latter were somewhat flattened (asymmetrical) due to juxtaposition in the root. A later sample from the same pot (W 5576) produced predominantly intraradical spores which were abundant, often bursting through the roots, along with lower numbers of extraradical spores, and of the 97 spores examined, all were spheroid (including two obovoid and two ovoid intraradical spores). In contrast, a first-generation subculture from this, ATT 1102-13 (a PC with a mixture of *P. lanceolata, Allium schoenoprasum* and *Festuca ovina*) produced an abundance of both intraradical and extraradical spores (W 5580) with considerable variation in shape. This culture produced predominantly spheroid spores, but also a few pyriform, subpyriform, subangular, subreniform, and subtriangular spores, along with a high proportion of misshapen specimens (23 % extraradical and 16 % intraradical) (Fig. 6).

Other than the greater variation in spore shape, it was not possible morphologically to distinguish spores of ATT4 (ex-type origin) from those produced by ATT 1102 from the type locality.

Table 2. *Rhizophagus intraradices*: numbers of specimens of different spore shapes by strain ATT 4 (type and ex-type cultures), and ATT 1102 (new strain from type locality established some 30 years later): listed by ATT, spore position (extra- or intraradical) and nature of culture (pot culture or root organ culture). Data from repeated sampling over almost 40 yr (ATT 4) and 19 yr (ATT 1102).

Spore shape	Attempt number		Spore position		Type of culture	
	ATT 4	ATT 1102	extraradical	intraradical	PC	ROC
globose	1 097	435	1 288	188	729	725
subglobose	441	142	383	170	380	138
broadly ellipsoid	121	32	43	95	96	11
ellipsoid	81	37	18	96	79	2
oval	48	32	11	68	65	0
irregular	32	40	26	45	69	0
obovoid	38	26	43	18	42	19
ovoid	15	6	12	9	19	2
fusiform	16	0	0	15	13	0
subtriangular	9	3	5	6	11	0
subangular	6	6	4	6	10	0
flattened	2	6	0	8	8	0
pyriform	2	1	2	0	2	0
peanut-shaped	2	0	2	0	1	1
subcardioid	2	0	1	1	2	0
bottle-shaped	2	0	0	2	1	0
rhomboid	2	0	0	2	2	0
spatulate	0	2	0	2	2	0
flask-shaped	1	0	0	1	1	0
funneliform	1	0	0	1	1	0
clavate	1	0	0	1	1	0
balloon-shaped	1	0	1	0	0	1
lacrimoid	1	0	0	0	0	0
lanceolate	1	0	0	1	1	0
cardioid	0	1	0	1	1	0
pip-shaped	1	0	1	0	1	0
reniform	1	0	0	0	0	0
subpyriform	0	1	0	1	1	0
subreniform	0	1	1	0	1	0
TOTAL	1 930	771	1 847	737	1 545	899
Chi square test	X^2 = 51.843, df = 7, p-value ≤ 6.269e-09		X^2 = 652.0621, df = 7, p-value ≤ 2.2e-16		X^2 = 284.7482, df = 7, p-value ≤ 2.2e-16	

Table 3. *Rhizophagus intraradices*: differences in the number and proportion of spores of the main (spheroid and ellipsoid) shapes in relation to host plant indicating a possible host-induced effect.

Host plant	Culture type	globose		subglobose		broadly ellipsoid		ellipsoid	
		n	%	n	%	n	%	n	%
Cichorium intybus	ROC	423	78.6	104	2.4	10	1.6	1	0.2
Festuca ovina	PC	23	56.1	9	8.7	2	1.9	7	6.5
Lotus japonicus	PC	27	55.1	17	14.8	4	3.4	1	0.8
Paspalum notatum	PC	46	26.9	46	91.3	42	14.5	37	12.2
Plantago lanceolata	PC	640	58.0	323	12.3	79	6.7	62	5.2
Daucus carota	ROC	302	89.3	34	0.3	1	0.2	1	0.2

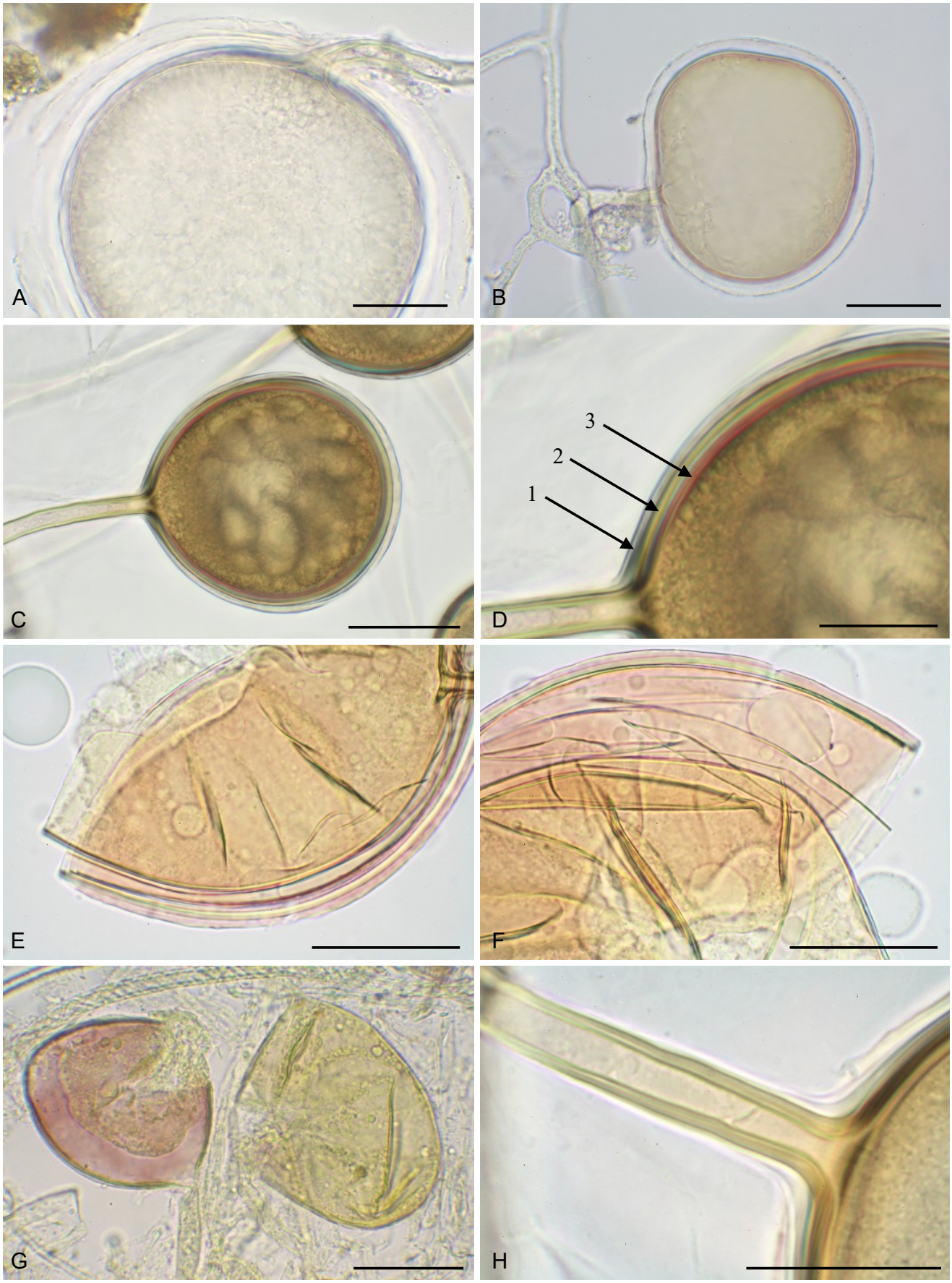


Fig. 11. Main spore characteristics of *Rhizophagus intraradices*. **A.** Immature spore from ROC showing expansion and apparent layering of the outer component. **B.** Semi-mature spore with outer component still intact and pigmentation of main structural wall. **C.** Mature spore from root organ culture (ROC). **D.** Detail of the wall structure of the spore in C, showing three wall components (1–3): 1, evanescent before degradation; 2, yellow, finely laminate pale yellow; 3, coarsely laminated dark brown. **E, F.** Crushed spores in polyvinyl alcohol lacto-glycerol with Melzer's reagent (PVLG-M) showing pale pink reaction of the evanescent wall component. **G.** Different reactions to PVLG-M; right no reaction, left outer component pink. **H.** Parallel-sided subtending hypha with slight tubaeform flare and proximal wall thickening. Scale bars: A, B, G = 50 µm; C, E, F = 100 µm; D, H = 25 µm.

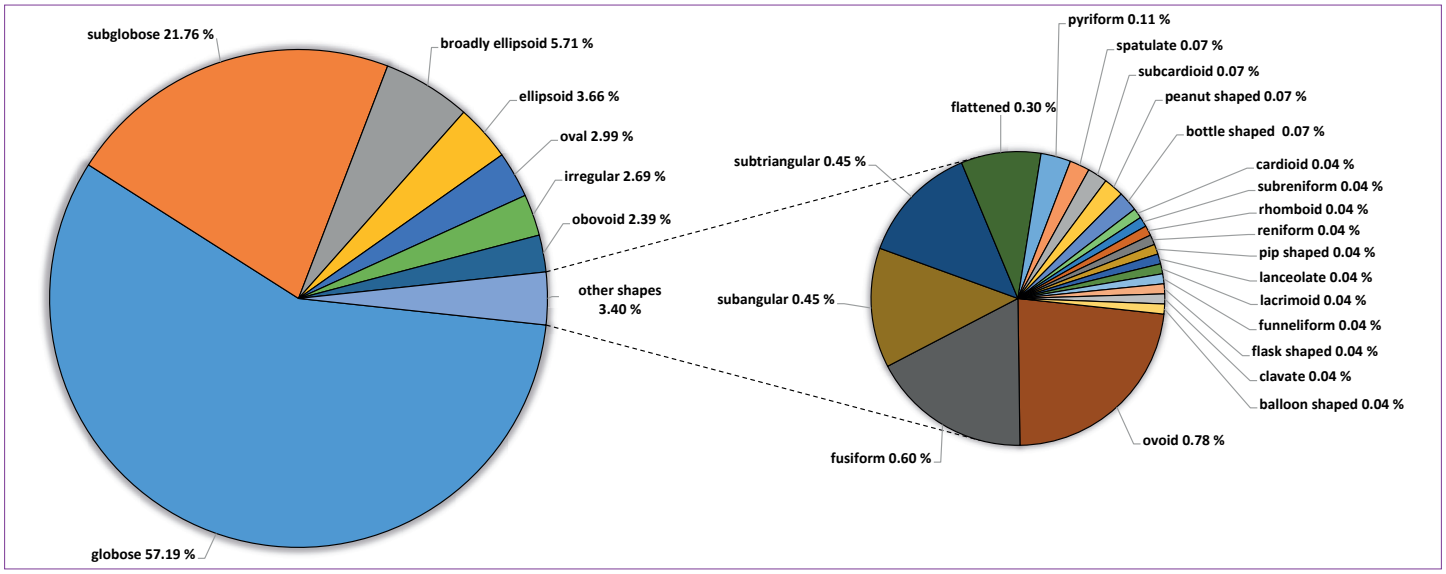


Fig. 12. *Rhizophagus intraradices*: distribution of spore shapes over all treatments (n = 2 673). The 'other shapes' section includes all those occurring with a frequency < 2 %.

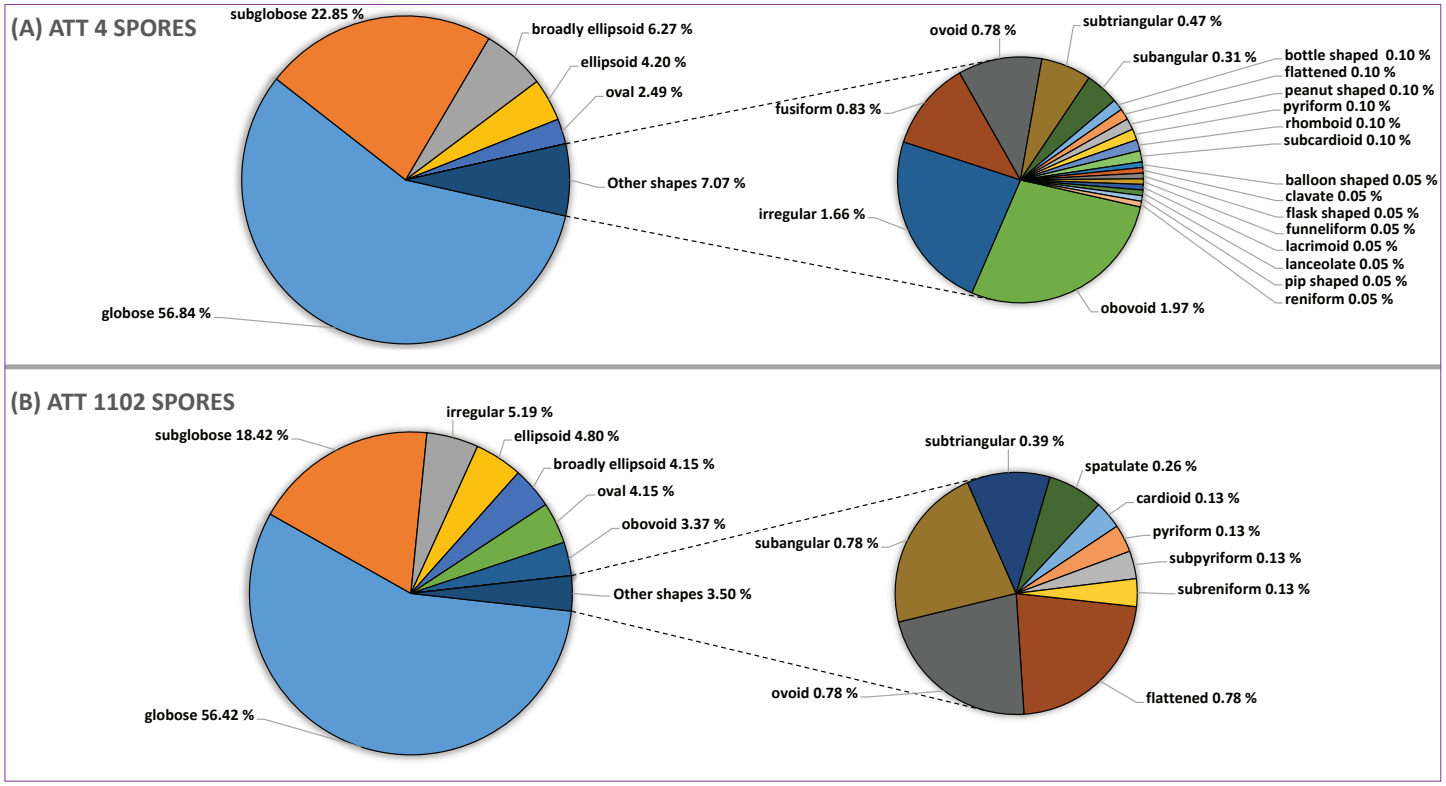


Fig. 13. *Rhizophagus intraradices*: distribution (percentage of measured spores) of the spore shapes between those from ATT 4 (Panel A) (n = 1 924) and ATT 1102 (Panel B) (n = 771). Other shapes include those occurring with a frequency < 2 %.

Table 4. *Rhizophagus intraradices*: matrix comparison of the spore shapes in relation to the host plant used for subcultures (two strains, ATT 4 and ATT 1102, combined). Results of Chi square tests are expressed with the X^2 value, and its significance level (*** when P ≤ 0.001; ** when P ≤ 0.01; * when P ≤ 0.05; NS when P > 0.05).

Host plant	*Cichorium intybus*	*Festuca ovina*	*Lotus japonicus*	*Paspalum notatum*	*Plantago lanceolata*	*Daucus carota*
Cichorium intybus						
Festuca ovina	X^2 = 190 ***					
Lotus japonicus	X^2 = 33 ***	X^2 = 20 **				
Paspalum notatum	X^2 = 306 ***	X^2 = 22 ***	X^2 = 28 ***			
Plantago lanceolata	X^2 = 149 ***	X^2 = 48 ***	X^2 = 8 NS	X^2 = 150 ***		
Daucus carota	X^2 = 20 **	X^2 = 133 ***	X^2 = 56 ***	X^2 = 263 ***	X^2 = 168 ***	

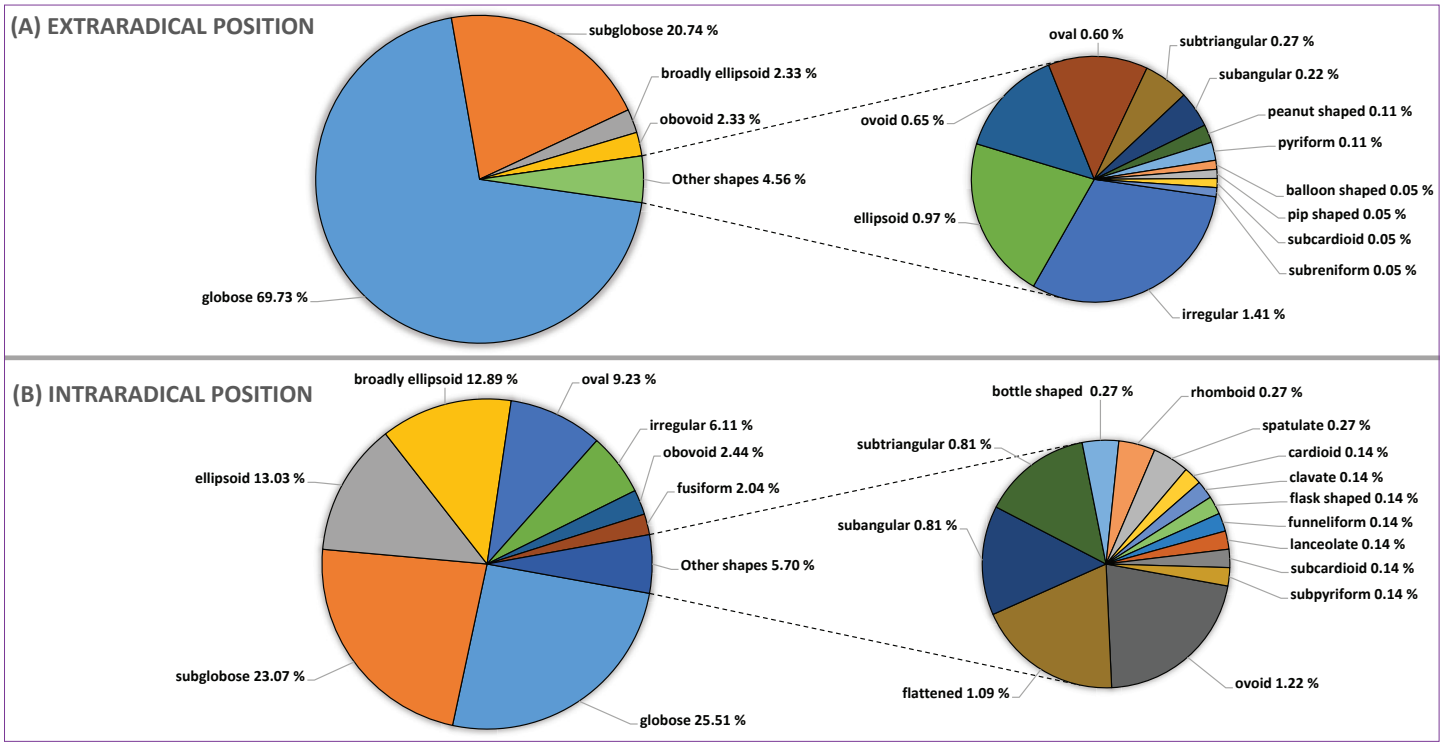


Fig. 14. *Rhizophagus intraradices*; distribution (percentage of measured spores) of the spore shapes between extraradical spores (Panel A) (n = 1 841) and intraradical spores (Panel B) (n = 737). Other shapes include those occurring with a frequency < 2 %.

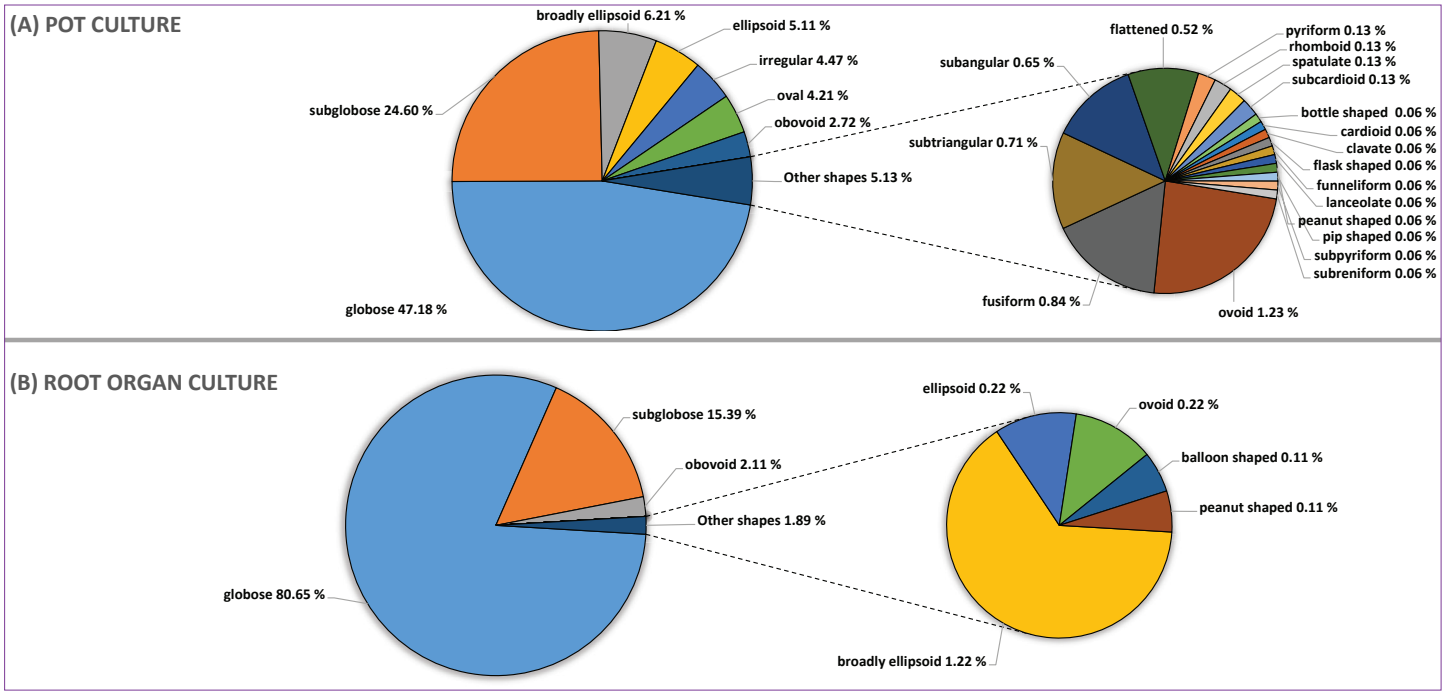


Fig. 15. *Rhizophagus intraradices*: distribution (percentage of measured spores) of the spore shapes between (Panel A) pot cultures (n = 1 559) and (Panel B) root organ cultures (n = 899). Other shapes include those occurring with a frequency < 2 %.

Subtending hypha

The spores develop centrally or eccentrically from a 'subtending hypha' (Figs 3, 4G–I, 9, 11H) that is very variable in size, shape, colour, and wall thickness. In some specimens, it is parallel-sided or tapered distally, with very little expansion at the spore base (Figs 9M, 10H), in others it is flared through expansion (tubaeform) proximally (Figs 3J, 9L–Q). In others it may be swollen asymmetrically (Fig. 9O), or constricted proximally (Fig. 9J). The hypha may be straight, parallel with the major axis of the spore (Fig. 11C), or it may be angled or recurved to varying degrees (Figs 3H, I, 4H, I, 6L, K) or otherwise distorted (*e.g.*, Fig. 9D, O). In most specimens, the spore is open-pored (*e.g.*, Fig. 9C), but it may be occluded by a thickened plug (Fig. 9K) or a proximal or distal septum (Fig. 9L, M, Q) formed from the innermost laminated component. The subtending hyphae can be colourless (*e.g.*, Fig. 7M, N), pale yellow (*e.g.*, Fig. 9H) or brownish yellow (*e.g.*, Fig. 9A, C, K).

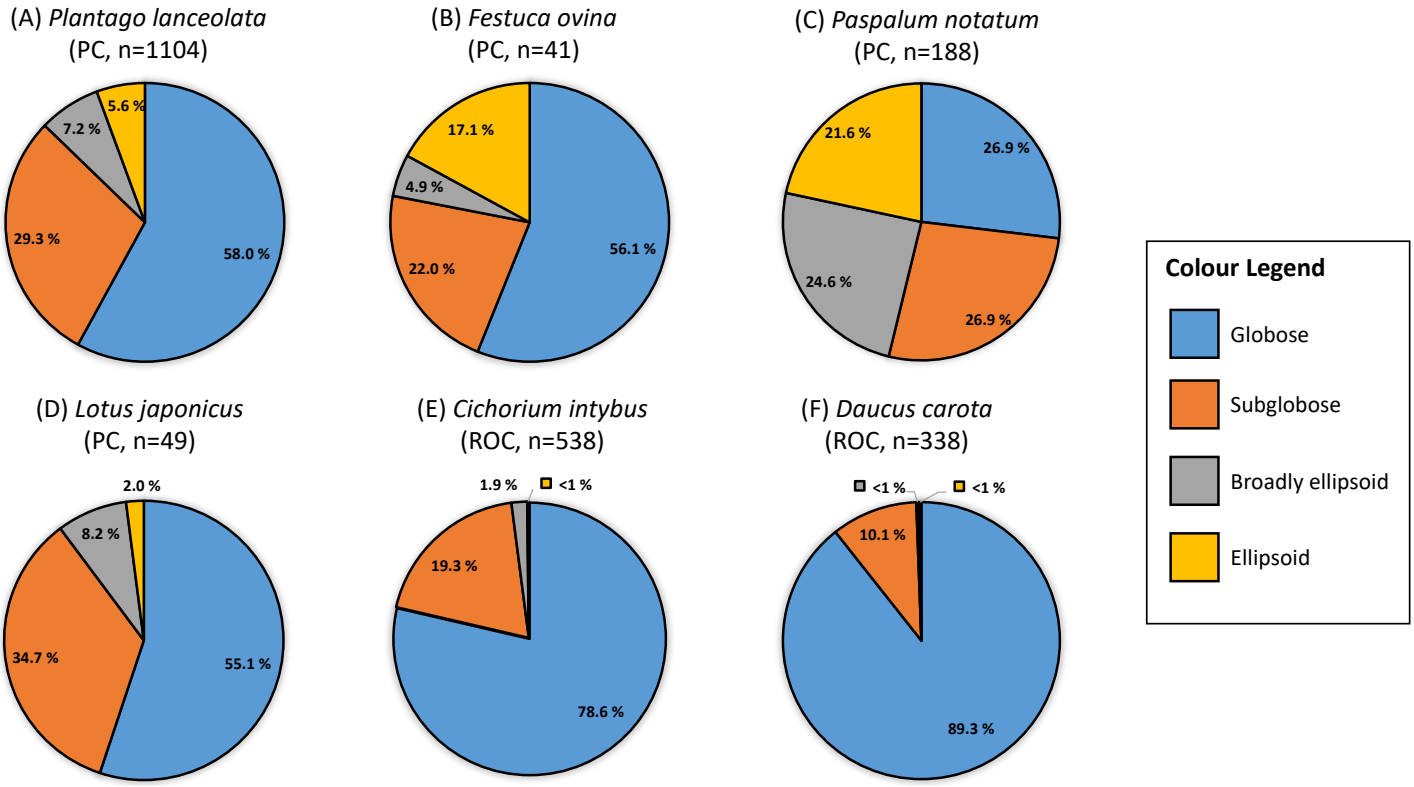


Fig. 16. *Rhizophagus intraradices* distribution of the four most abundant spore shapes (globose, subglobose, broadly ellipsoid, and ellipsoid) in relation to their host plant. For each panel, the culture method (Pot Culture, PC or Root Organ Culture, ROC) and the number of spores measured are given. Values are given as a percentage of the total spores measured. When a percentage is very low and the colour barely visible (*i.e.*, < 1 %), the colour legend is indicated next to the number.

Spore dimensions

It was difficult to decide which parameter to use for analysis of the data. Length, width, longest, widest, volume and the ratio of longest to widest measurement could all be analysed. Volume calculations necessarily assumed an isodiametric shortest dimension, but many spores appear somewhat pulvinate, and the irregular specimens also were much thinner than broad. Consequently, a two-dimensional representation of the longest and widest measurement was chosen. By almost any class variable, there were significant differences among individual samples (Table S1), but overall, the analysis of the two different culture lines (ATT 4 and ATT 1102) did not differ significantly. It should be noted, however, that the sample size and number of repetitions of samples (over time) were grossly different. There were probably too few irregular spores to make a good comparison, and the lack of significance may be the results of the low number of observations. Spores that could not be determined as either extraradical or intraradical were excluded from the measurements, as were those spores with the subtending hypha obscured or broken so that the spore base could not be identified.

For ATT 4 spores, 70 % were extraradical and 30 % were intraradical. For ATT 1102, 72 % were extraradical and 28 % were intraradical. For ATT 4-1, the type culture, measurements of 52 extraradical spores (42–224 × 42–154 µm, mean 104 × 99 µm) and 100 intraradical spores (29–186 × 18–147 µm, mean 79 × 66 µm) gave an overall size range of 29–224 × 18–154 µm, mean 87 × 77 µm. These data together with measurements from ex-type PCs gave dimensions of 29–224 × 29–165 µm, mean 98 × 96 µm for 727 extraradical spores and 18–234 × 16–152 µm, mean 84 × 74 µm for 561 intraradical spores. For ATT4 spores in ROC, 556 extraradical spores were measured (30–182 × 30–178 µm, mean 98 × 97 µm). There were insufficient intraradical spores in ROC to make meaningful measurements. Another 71 spores from PCs were measured (55–141 × 50–179 µm, mean 93 × 91 µm) that could not be assigned with certainty as either intraradical or extraradical spores. Taking all these measurements into account, the overall spore dimensions from 1 915 spores were 18–234 × 16–179 µm, mean 94 × 90 µm.

The new isolate (ATT 1102) in pot culture produced extraradical spores of 26–383 × 26–398 µm mean 94 × 93 µm (n = 250) and intraradical spores of 48–383 × 34–398 µm, mean 112 × 150 µm (n = 150), and in ROC (extraradical only) of 48–123 × 46–122 µm, mean 88 × 88 µm (n = 145), giving an overall size range of 26–383 × 26–398 µm, mean 99 × 97 µm (n = 545).

Spore wall structure

Wall structure comparisons used in descriptions of glomeromycotan spores are primarily based on light microscopy of PVLG-mounted specimens, with occasional observations in water or glycerol. In these mounting media, spores from both PC and ROC were similar, except that in the former, some older specimens developed what appeared to be a new outer wall component, formed by the growth of what appear to be bacterial colonies. These manifested themselves as an apparent ornamentation of rounded surface bumps or scrobicular patterning (Figs 3N, 4N).

Whether in glycerol or water mounts, not subject to the effects of the acidic PVLG-based mounting media, or in PVLG and PVLG-M, the wall structure is of an outer, evanescent component, up to 1 µm thick, overlaying a colourless to pale yellow finely laminated component (1–4 µm thick) that sometimes

misleadingly appears unitary (*i.e*., without laminations). A third, darker yellow to brown laminated component then becomes evident, at first appearing as a unit component, but later developing laminae that may be tightly adherent, or loosened to varying degrees. In many specimens, the laminae become very loosely associated, often to the point of seeming to consist of many different unit components (Fig. 11F). This third component is indeterminate in thickness because it lays down new laminae with time. These laminae may be very loosely associated or become separated. Occasionally, when mounted in PVLG, the outer wall component can react, apparently depending on the age and condition of the particular specimen. In very young spores, component 1 sometimes can expand in PVLG to become up to 8 µm thick, and to give the appearance of a doublet (Figs 5I, 10C), but later this may not be observable in the mounting medium. In middle aged spores, it may or may not expand slightly, but it usually reacts with Melzer's reagent (in PVLG-M) to become pink or red. In what seem to be fully mature spores, this component remains evident, but does not always react to Melzer's reagent. In very old spores, this component disappears, hence its designation as evanescent. The reaction to Melzer's reagent is thus variable and seemingly inconsistent. In some specimens, there is no reaction at all. In others, the outer evanescent component reacts rapidly to become pink, whereas in others it turns purple, and in yet others, it does not react at all. The innermost laminated component also can react to become rust red in some specimens, but it did not react at all in most specimens examined. The only consistency seems to be that the first laminated component [which appears as a unit component in some ROC cultures (W6517 & W6158)] does not react at all to Melzer's reagent.

Germination

Spores of *R. intraradices* germinate (Fig. 9R) by hyphae emerging through the broken end of the subtending hypha. New hyphae may also emerge from hyphal fragments in the substrate.

Mycorrhiza

Rhizophagus intraradices forms arbuscular mycorrhizas (Fig. 17), often producing spores in the root cortex. It may form vesicles (thin-walled balloon-shaped structures), but there is a difficulty in defining the latter. Here, we define spores in the roots as having a multiple wall structure, normally with relatively thick walls. Vesicles (temporary storage organs, such as those in the genera *Ambispora, Acaulospora* or *Funneliformis*) lack such thickening. It is not always possible to know for *R. intraradices*, if such thin-walled structures in a root are vesicles (and thus will not proceed in development any further) or immature spores (in which case their wall will thicken and differentiate in the same manner as extraradical spores). Vesicles may occur near penetration points and arbuscules (thus active mycorrhizas), whereas the thick-walled spores in roots are not associated with active structures such as these. Mycorrhizas were not compared systematically, but they are not known to provide species-discriminating characters. Comparisons were not made either with other AMF, or with the same organism under different hosts, conditions and developmental stages: consequently, the images in Fig. 17 should not be considered as typical of the species.

Publications in which structures described as vesicles were recognised and used as inoculum do not indicate why they were referred to as vesicles (*e.g*., Plenchette & Strullu 2003) and most provide neither description nor illustration. Given the current knowledge of the genus *Rhizophagus*, it seems most likely the authors were actually referring to spores (identified at the time as *G. intraradices*, although possibly not identified correctly). One publication (Diop *et al.* 1994) does, in their fig. 13, illustrate the so-called vesicles, showing thickened walls typical of spores formed by *Rhizophagus* spp. within roots. Similarly, extraradical and intraradical propagules have been distinguished, respectively, as spores and vesicles (Declerck *et al.* 1998), but one illustrated as a 'vesicle of *Glomus intraradices*' (their fig. 3) is a thick-walled intraradical spore.

TAXONOMY

The genus *Rhizophagus* was described with illustrations of arbuscules and spores in roots and it has long been accepted that its assignment as a chytrid-like pathogen of poplar was erroneous. Based on the accumulated wisdom of biologists throughout the 20th century, it has been widely accepted as such (*e.g.*, Petri 1919, Peyronel 1923, Butler 1939, Kelly 1950, Greenall 1963). However, it was later considered a synonym of *Glomus* by Gerdemann & Trappe (1974). Subsequently, when *Glomus* was circumscribed in a narrow sense from molecular analysis (Schüßler & Walker 2010), *Rhizophagus* was resurrected as belonging in a separate clade. It was then assigned to the clade accommodating *R. intraradices inter alia*, a move that has been very widely accepted. There was no requirement for a physical type specimen when the genus, based on *Rhizophagus populinus* was published (Dangeard 1896), and there were no known culture lines representing that species. The lack of a type was rectified by Walker *et al.* (2017) when illustrations by Dangeard (1900) were designated as neotype.

Although no new information had been published since the widespread acceptance of the application of the genus *Rhizophagus* (Schüβler & Walker 2010) for organisms in the clade GlGrAb (as defined in Stockinger *et al.* 2009), Sieverding *et al*. (2014) proposed the name *Rhizoglomus*, reiterating that *R. populinus* was a pathogenic organism. The ICNafp states (Preamble 12) that "The only proper reasons for changing a name are either a more profound knowledge of the facts resulting from adequate taxonomic study or the necessity of giving up a nomenclature that is contrary to the rules." No such profound knowledge, taxonomic study or nomenclatural error was demonstrated with this name change. The genus *Rhizoglomus* is therefore herein formally designated as a later heterotypic synonym of *Rhizophagus.*

Rhizoglomus venetianum

The organism named *Rhizoglomus venetianum* by Turrini *et al.* (2018) shares morphological characters with *R. intraradices* and *R. irregularis*. In the protologue, the species is described as having a wall consisting of four layers. The illustrations provided, however, are not sufficiently detailed to understand how this conclusion was reached, but the ultrastructure of the walls of *R. intraradices* (Maia & Kimbrough 1994; the culture studied in this publication, LITR 208, most likely represents *R. intraradices*) shows that the laminated component can separate into what appear to be separate layers, and it would be easy to misinterpret these, at the light microscopic level, as different components. It

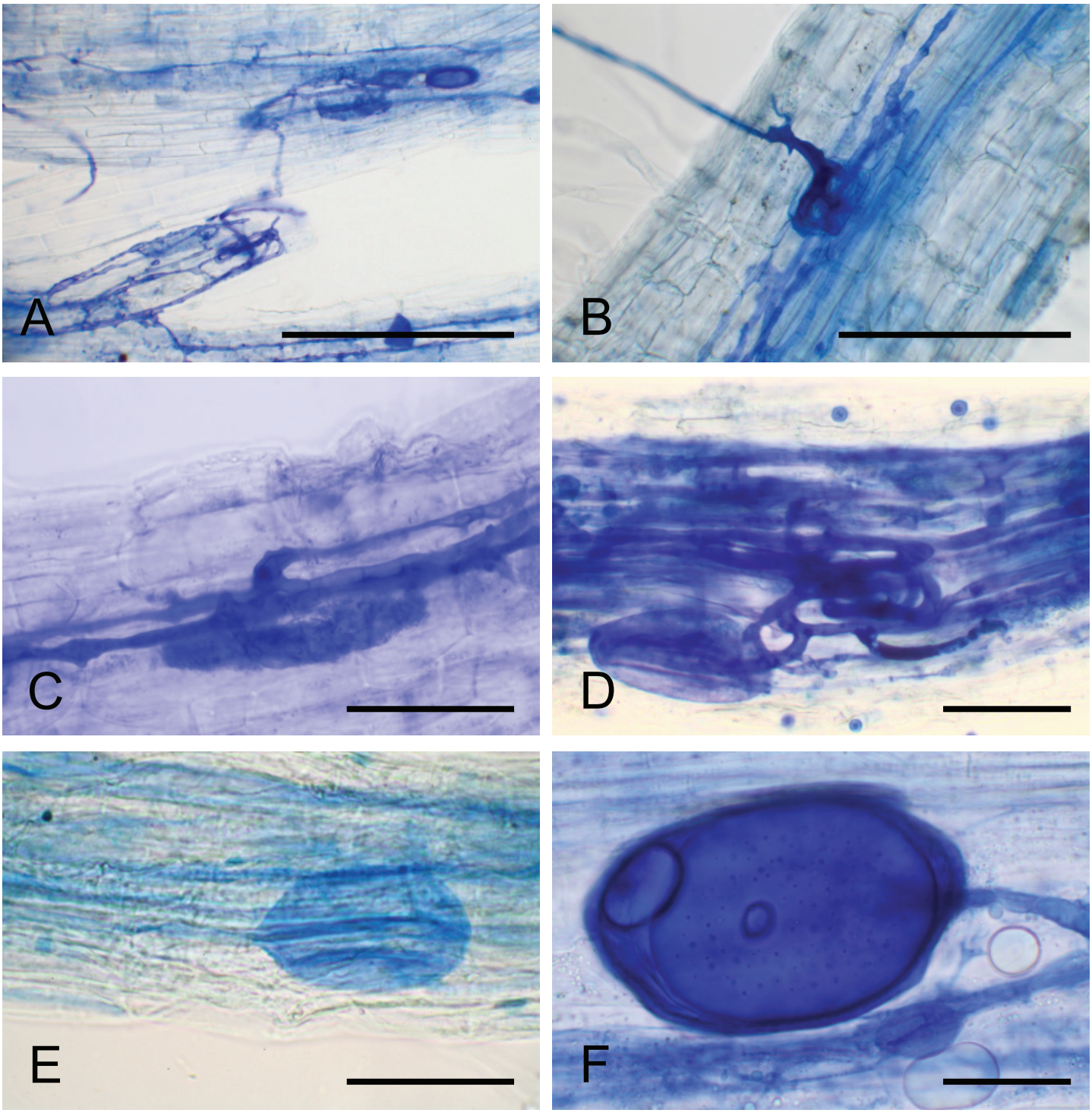


Fig. 17. *Rhizophagus intraradices* mycorrhiza with *Plantago lanceolata* as host, cleared with KOH and stained with 0.02 % methyl blue in 0.1 M HCl. **A.** Crushed fine root showing arbuscular mycorrhiza and intraradical spores. **B.** Appressorium at entry point into root cortex. **C.** Finely branched arbuscule. **D.** Entry point showing hyphal coils in outer cortical cells and a thin-walled structure, either a vesicle or an immature spore. **E.** Thin-walled structure in the cortex which could be interpreted as a vesicle, but may be an intraradical spore in the earliest development stage. **F.** Thick-walled mature intraradical spore. Scale bars: A = 250 µm, B = 125 µm, C–F = 50 µm.

is likely that the fourth wall component, used in the protologue to distinguish *Rhizoglomus venetianum* from both *R. intraradices* and *R. irregularis,* is an innermost loose lamina of the second laminated wall component (see Figs 3K, 10F).

The phylogenetic analysis of *Rhizoglomus venetianum* in the protologue showed a well-supported separate clade when compared with a widely-used Canadian isolate of *R. irregularis* (DAOM 197198, also labelled MUCL 46241). However, when analysed amongst a more comprehensive sequence sampling representing the intraspecific variability of *R. irregularis*, including also sequence variants characterised in a genome sequencing project (Maeda *et al.* 2018), it becomes obvious that the '*Rhizoglomus venetianum*' sequences merely represent a ribotype within the clade representing *R. irregularis* (Fig. 2C). There are, thus, neither substantial morphological, nor molecular phylogenetic characters that separate it from *R. irregularis*, and it therefore is placed as conspecific with that species.

Rhizophagus P.A. Dang., *Botaniste* **5**: 43 (1896) [1896–1897] *sensu* Schüßler & Walker, *The Glomeromycota*: 19 (2010).
Synonyms: *Rhizoglomus* Sieverd. *et al., Mycotaxon* **129**: 377 (2015) [2014]
Stigeosporium C. West, *Ann. Bot., Lond.* **30**: 357 (1916).

Rhizophagus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & Schüßler, *The Glomeromycota*: 19. 2010. Figs 2–10.
Basionym: *Glomus intraradices* N.C. Schenck & G.S. Sm., *Mycologia* **74**: 78. 1982. (holotype OSC40255).
Synonym: *Rhizoglomus intraradices* (N.C. Schenck & G.S. Sm.) Sieverd. *et al.*, *Mycotaxon* **129**: 378. 2015. (2014).

Chlamydospores (thick-walled one-celled asexual resting spores) produced in the substrate or in root cortical cells: overall length by width 18–383 × 16–398 µm. *Extraradical spores* formed singly, in loose to dense clusters (fascicles), clustered around roots; sometimes occupying voids such as empty seeds or arthropod integuments in soil. Fascicles up to approx. 2 × 2 mm in planar view. *Spores* colourless to white to pale yellow to yellow to brownish yellow to pale yellow-brown to yellow-brown to dark yellowish brown to dark brown. Spore shape globose, subglobose, broadly ellipsoid, ellipsoid, oval, sometimes subangular, pyriform, spatulate, subcardioid, reniform or subreniform, peanut shaped, obovoid, ovoid or often misshapen (irregular); sometimes partly flattened by juxtaposition with other spores in dense clusters; 26–383 × 26–398 µm. *Intraradical spores* very variable in size and shape, formed singly, or in clusters either entirely within the root cortex, or bursting through the cortex to form spore clusters around the roots. Globose, subglobose, broadly ellipsoidal, ellipsoidal, ovoid, obovoid, reniform, peanut shaped, bottle shaped, subrectangular, or irregular; colourless to pale yellow to yellow brown to dark yellowish brown; 18–234 × 16–202 µm.

Subtending hypha very variable in size and shape, often curved or sharply recurved, frequently constricted at the spore base or expanded distally, sometimes straight, parallel sided or funnel shaped. Open-pored, or occluded by a proximal or distal septum. *Spore wall* structure of three components (1–3) in one wall group. Component 1 sometimes appearing unitary, sometimes expanding in acidic mounting media, and sometimes evanescent, and in older spores often covered by bacteria that can produce the impression of an ornamented outermost component. Wall component 2 colourless to very pale yellow, 1–5 µm thick, under light microscopy, sometimes appearing unitary, but mostly finely laminated. Component 3 laminated, sometimes with very easily separable laminae, yellow to brown, increasing in thickness depending on the age of the spore to become up to 5 µm thick. Both intraradical and extraradical spores reacting variably to Melzer's reagent in PVLG-M. In young spores usually rapidly pink (outer component), with the middle component not reacting, and the inner laminated component darkening, sometimes becoming dark rust red. In some spores the reaction absent, or developing slowly over several days. Reaction fading over time with storage in polymerised PVLG-M.

Molecular phylogenetic analyses of the SSU-ITS-LSU sequences (Fig. 2) as the extended DNA barcode for glomeromycotan fungi (Stockinger *et al.* 2010) separate the clade with sequences from *R. intraradices* from any other clade representing other *Rhizophagus* species (Figs 2A, S2) and the culture lines studied here fall into the monophyletic clade that represents *R. intraradices* (Fig. 2C. The closest relative, based on present knowledge, is *Rhizophagus prolifer*. *Rhizophagus irregularis* is clearly separated, despite the absence of any clear morphological characters distinguishing the two species.

Specimens examined: **USA**, Florida, Orlando, Clermont-Mineola (approx. 28°30'31''N 81°46'15''W), *Paspalum notatum*, from a pot culture established with roots of *Citrus* sp. coll. *S. Nemec* (*N.C. Schenck & G.S. Smith*), **holotype** OSC 40255 (5 May 1981) and numerous ex-type cultures PC and ROC) with various hosts in North America and Europe (*C. Walker* ATT 4) (Fig. S1); Florida, Orlando, Clermont-Mineola (approx. 28°30'31''N 81°46'15''W), from a pot culture with *Plantago lanceolata* established with spores from a soil trap culture with *P. lanceolata* (*C. Walker*, UK, New Milton, Hampshire) and numerous ex-type cultures (PC and ROC) with various hosts in Great Britain and mainland Europe (*C. Walker* ATT 1102-0 (established 14 Oct. 2001) and subcultures) (Fig. S1); [**epitype** here designated, W 5719 (E), IF 553332], 30 Mar. 2010, from a ROC, with the ex-type culture in its ancestry, cultivated with *Cichorium intybus* as host (MUCL 52327, M 5F1A4, ATT 4-83), GenBank sequence registration numbers HE817873, HE817874 HE817875 (all from the same gathering).

Synonymisation of *Rhizophagus* spp.

Having synonymised *Rhizoglomus* with *Rhizophagus*, five species named within the former genus must be formally transferred as new combinations:

Rhizophagus dalpeae (Błaszk. *et al.*) C. Walker & Schüßler, ***comb. nov.*** IF 551357.
Basionym: *Rhizoglomus dalpeae* Błaszk. *et al.*, *Mycologia* **111**: 972. 2019.

Rhizophagus dunensis (Błaszk. & Kozłowska) C. Walker & Schüßler, ***comb. nov.*** IF 551358.
Basionym: *Rhizoglomus dunense* Błaszk. & Kozłowska, *Botany* **95**: 636. 2017.

Rhizophagus maiae (Jobim *et al.*) C. Walker & Schüßler ***comb. nov.*** IF 551359.
Basionym: *Rhizoglomus maiae* Jobim *et al*., *Mycologia* **111**: 973. 2019.

Rhizophagus silesianus (Magurno *et al.*) C. Walker & Schüßler, ***comb. nov.*** IF 551360.
Basionym: *Rhizoglomus silesianum* Magurno *et al*., *Mycologia* **111**: 976. 2019.

Rhizophagus variabilis (Corazon-Guivin *et al.*) C. Walker & Schüßler, ***comb. nov.*** IF 551361.
Basionym: *Rhizoglomus variabile* Corazon-Guivin *et al.*, *Sydowia* **71**: 185. 2019.

DISCUSSION

Rhizophagus intraradices and related species

The type material of *G. intraradices* (*R. intraradices*) appears as if it was not made from freshly collected material, but perhaps from material that had been kept after extraction for some time before preservation. It was heavily degraded, and the wall structure was difficult to determine. It was also much darkened

in colour due to the action of lactophenol. Nevertheless, it was possible to see most of the characteristics used by the original authorities to describe the species, although it was much more varied than the species description and illustrations in the protologue indicated.

In the original protologue (Schenck & Smith 1982), the species was described as forming spores singly or in clusters in roots, and '… rarely formed outside the root …'. Spore shape was said to be '… predominantly globose, but frequently subglobose …', and their dimensions were given as (40.5–)98.5(–190.5) µm diam when globose, and 93–119 × 112–131 µm when subglobose. Our independent new measurements of spores from the type material resulted in spore dimensions of 29–224 × 18–154 µm and our new measurements herein extend the range further to 18–383 × 16–398 µm. The images supporting the description are few, and do not show the degree of variation that the species can manifest. Two uncrushed spores are illustrated, one subglobose, and the other obovoid. There is an image showing the base of one spore with a 'tubaeform flare' formed by the 'walls of the spore extending into the hyphal attachment…' at the junction of the subtending hypha and the spore base. The subtending hypha, however, is described as '9–33 µm wide with a wall thickness of 1.5–2.5 µm at the base … occasionally constricted 2–3 µm' basally. The fourth illustration is of spores densely occupying the cortex of a root. There is no overall description of spore colour, but the spore wall is described as yellow to grey brown, with a greenish brown appearance in transmitted light. The use of transmitted light to assess colour can be misleading, as it depends on the colour temperature of the light source, and, for this reason, is best avoided in species descriptions. The wall structure of spores is described as of '... 1 or 2, occasionally up to 4 laminated walls ...', with '... on young spores an additional, hyaline, ephemeral outer wall (1–2 µm) ...'. It is difficult to interpret the wall structure from this description. The illustrations are of too low a magnification to see any detail, and only show a pale outer component overlaying a series of up to 7 coarse laminae. Observations of *R. irregularis* over many years (C. Walker, unpublished) show a range of spore colour from colourless to white to various shades of yellow to brown.

In a publication by Stürmer & Morton (1997), spore developmental patterns were used in re-description of a fungus identified as '*G. intraradices*'. The ex-type INVAM culture FL 208 was cited in the materials and methods. However, it was not specifically described or illustrated, but another culture, designated KS 906, was illustrated, for which ITS sequences (AF185669-73) were available that provides verification of its species identity. The spore wall was described as being at first of a 'mucilaginous' layer and a 'semiflexible layer', neither of which had any distinguishable structure. Later, a pale-yellow laminated component is described as developing which gradually increases in thickness as new laminae are developed with age. In the murographic illustration (their fig. 1), this is indicated as a second laminated component. The latter is concordant with our interpretation of an evanescent and two distinct laminated structural components.

From our study, we consider that the spore wall has three components at the light microscope level: an ephemeral (evanescent) outer component, a persistent pale-yellow component that initially does not have obvious layers, but later can be seen to be finely laminated, and a multi-layered 'laminated' main structural component that increases in thickness with age by addition of more laminae, some of which may separate by splitting. The outermost of these is colourless, and usually reacts to become pink in PVLG-M. It behaves sometimes as a unitary component, sometimes as an evanescent component, and sometimes as an expanding component, rendering these descriptive terms of considerably less use in species description than was first proposed by Walker (1983). In very young specimens, the outermost component may expand in PVLG to produce the impression of an extra component (Fig. 11 A).

The outer, colourless ephemeral component may be colonised by bacterial clusters which can be so dense, in older spores, that they may appear to form an additional outer wall component. Similar bacterial colonies, attributed predominantly to the genus *Azotobacter* were described for *R. fasciculatus* (as *Glomus fasciculatus*) by Gerdemann & Trappe (1974). Maia & Kimbrough (1994) illustrated them for *R. intraradices* culture LITR208, suggesting that they are responsible for degradation of the wall component, though experimental evidence for this is lacking. We have not observed these amongst *in vitro* cultures and experimental investigation would be required to determine if this is so.

Spore colour in reflected light is extremely variable. The greenish brown tint in transmitted light, referred to (Schenck & Smith 1982) in the protologue, was rarely noted because only observations with reflected light were used for colour determination, but one sample of spores (Fig. 5B) did show a greenish tint.

The original species description does not encompass all the morphological variation present in the type collection. The study of newly collected ex-type material and a second conspecific isolate revealed much more variation than displayed by the type material. Ideally, new species of glomeromycotan fungi should be described from cultures that have been grown sufficiently long to produce spores encompassing, as far as possible, the morphological variation within the species concerned. For many species that are recalcitrant or impossible to culture, this aim cannot be achieved, but it appears that many species in *Rhizophagus* are relatively easy to establish in both PC and ROC, and thus are amenable to being described in this way.

Błaszkowski *et al.* (2008), in the species description of *R. irregularis* (as *Glomus irregulare*), compared two pot cultured fungi from the same geographic area, maritime sand dunes at Bornholm, one given the new name, and the other determined as *G. intraradices*. These authors concluded that the morphological differences between the specimens they examined were sufficient to distinguish the two species from each other. However, these two fungi were both the same species (Stockinger *et al.* 2009), and thus the comparison was between two different cultures of *R. irregularis*. Consequently, the variation in the seven properties that were provided to separate these two species must represent intraspecific differences between the two cultures. Indeed, from our study, *R. intraradices* also shares all seven of these properties, that is: 1) presence of both terminal and intercalary intraradical spores; 2) spore colour varies from almost colourless, through yellow, to brown (the 'greenish tint' used as a character is unreliable); 3) some spores possessing an 'apical cap' caused by thickening of the colourless outer wall component; 4) spores with an outer wall component that disintegrates, and may be rough or smooth, depending on age; 5) a laminated spore-wall component that may be inseparable, or may separate under pressure when mounted on a microscope slide; 6) a variable reaction to Melzer's reagent, to which both outer and inner components can react,

seemingly dependent on age and condition; 7) spores produced both in roots, and in the substrate. Since, for both species, spore colour, spore wall structure, subtending hyphal form, spore shape, spore size, or reaction to Melzer's reagent are similarly variable, we suggest that molecular evidence is required to separate these, and probably some other species in the genus.

The *Rhizophagus intraradices* sequences of all culture lines studied here, including the newly isolated strain from the type locality, form a monophyletic clade at the species level, clearly separated from other species in the genus, supporting the analyses of Stockinger *et al.* (2009, 2010) and Krüger *et al.* (2012) that the species is not phylogenetically sister to *R. irregularis*.

This survey of *R. intraradices*, in culture over a very long period, shows that several phenotypic characteristics of spores, particularly spore shape and size, can be affected by external factors such as culture type and host plant. The nature of the present survey does not allow explanation of how this can be so, but there are implications for species descriptions. A new species may be described from a single sample, and the rules of nomenclature dictate that the type material must be from 'a single gathering', and thus protologue descriptions are unlikely to cover all the variation that might occur within the species. Over time, this can be rectified if the type culture, and others that can be shown, *e.g.*, by molecular analysis, to be conspecific, are maintained as living cultures. In addition, caution should be exercised when describing new species based solely on morphological comparison with the species description alone, or even after examination of type material. There is considerable scope for experimental studies to examine the effects of such factors on spore morphology.

CONCLUSIONS

Molecular analyses show that sequences of *R. intraradices* culture-lines, derived from the ex-type culture and from a re-isolation from the type locality, occupy a monophyletic clade and represent the same species. The original species description required considerable amendment, and a new description and designation of an epitype is made to provide a sound basis for further studies of this and similar organisms.

We propose that, although not a requirement of the ICNafp, it is most important to have molecular evidence, including characterisation of intraspecific variability, to define many of the *Rhizophagus* species. In several genera in the *Glomeromycota* species are found that form different spore morphs, and for species such as *R. intraradices* spore morphology is extremely plastic and variable.

This paper highlights the need for intermittent redescriptions of species in the *Glomeromycota* as more knowledge of the variation within a species is gathered. The type specimen of a species is often unlikely to encompass the entire degree of variation, hence the specific statement in the ICNafp that the nomenclatural type is not necessarily the most typical or representative element of a taxon. Original species descriptions are likely to encompass only a subset, perhaps a very small subset, of the variation within a species. We offer this re-description, based on precisely defined isolates, as a reliable foundation for further investigations into the taxonomy, systematics, and, eventually, functional diversity of species in *Rhizophagus*.

The variation in anatomical characters within this single species, indeed within single isolates of the species, implies that the following characteristics, particularly if based on a small point sample, cannot be used as reliable characteristics for separating the species of *Rhizophagus* with yellow to yellow-brown to brown spores [some species, such as *R. clarus* are always pale, and others, such as *R. neocaledonius* are described as being dark-coloured with bleaching necessary to visualise spore walls (Crossay *et al.* 2018)]:

- spore dimensions,
- spore shapes,
- production of spores in roots,
- production of spores in fascicles or small 'sporocarps',
- spore colour,
- subtending hypha morphology, including mode of occlusion,
- germination characteristics,
- reaction to Melzer's reagent.

Molecular phylogenetic analyses of the SSU-ITS-LSU marker can robustly differentiate the known *Rhizophagus* species. However, the example of '*Rhizoglomus venetianum*' shows that, beside the morphological plasticity, the intraspecific DNA sequence variability of a species must be considered in the characterisation. There are several recently described species names that are characterised by only one major sequence variant, often with some sub-variants within the range of the PCR error rate. This is very unfortunate (and not congruent with the concept of DNA barcoding) because species can only be reliably identified based on DNA sequences if their intraspecific variability, at least of the major sequence variants, is characterised. Further analysis will be required to examine if it will be necessary to synonymise some of these with existing species.

For future studies, ex-type material of *R. intraradices* will be available as descendant cultures of the original root trap pot culture from INVAM as FL 208, and from GINCO-BEL (ATT 4 above), the Belgian *Glomeromycota in vitro* collection (https://www.mycorrhiza.be/ginco-bel/index.php), hosted within the BCCM/MUCL collection (https://bccm.belspo.be/about-us/bccm-mucl), under two different numbers, MUCL 49413 for cultures directly descended from a multi-spore ROC, and MUCL 52327 for those from the ex-epitype single spore isolate. For operational reasons, the GINCO-BEL cultures are temporarily unavailable (October 2021), but an ex-epitype culture derived from MUCL 52327 is registered with the Microorganisms Section of the National Institute of Agrobiological Sciences, Japan as MAFF 520088 (www.gene.affrc.go.jp/databases-micro_search_en.php). The *R. intraradices* culture (ATT 1102, above), established independently from the type locality of the species, is available from GINCO-BEL as MUCL 49410.

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Supplementary Material: http://fuse-journal.org/

Fig. S1. *Rhizophagus intraradices*: culturing history of the type and successful ex-type culture attempts (ATT 4) and a new isolate (ATT 1102) from the type location established approx. 30 years later. Both pot cultures (PC) and root organ cultures (ROC) are shown with dates of establishment and voucher numbers for samples that yielded specimens for preservation in herbaria. Sun bags are item B7062, Sigma Aldrich (https://www.sigmaaldrich.com). Location of cultures: Forestry Commission Northern Research Station or other localities in UK; Université catholique de Louvain (UCL); Ludwig Maximilian University of Munich (LMU). Gel refers to a small portion of substrate from a parent ROC, with a single spore, several spores, or root fragments (usually with attached mycelium).

Fig. S2. Phylogenetic maximum likelihood phylogenetic tree of *Rhizophagus* species and isolates characterised for the SSU-ITS-LSU rDNA region, with *Sclerocystis* as outgroup. Bootstrap (BS) values below 60 % and BS values of terminal sister relations are not shown. "*Rhizoglomus venetianum*" (= *R. irregularis*) sequences are marked in red, sequence variants characterised in a *Rhizophagus irregularis* genome project are marked in blue. Sequences of *Rhizophagus intraradices* cultures derived from the ex-type culture FL 208, including the epitype (voucher W 5719 from MUCL 52327 = ATT 4-83), are shown in green and sequences of the new isolate collected from the type locality (MUCL 49410 = ATT 1102-12) in brown.

Table S1. *Rhizophagus intraradices*: lengths and widths (µm) of extra- and intraradical spores from two strains, ATT 4 and ATT 1102 spores with inferential statistics (number of spores observed (n), minimum value (Min), first quartile of the data (Q1), median, third quartile of data (Q3), maximum value (Max), mean, standard deviation (SD) and % coefficient of variation (CV %)).

Table S2. *Rhizophagus intraradices*: spore colours from two strains (ATT 4 – type and ex-type) and ATT 1102 (new culture from type locality) from pot cultures (PC) and root organ cultures (ROC). Where possible, colours were matched with charts from Royal Botanic Garden Edinburgh, Munsell, or Methuen Handbook of Colour.

Fig. S1-1. *Rhizophagus intraradices*. ATT 4. Culture history.

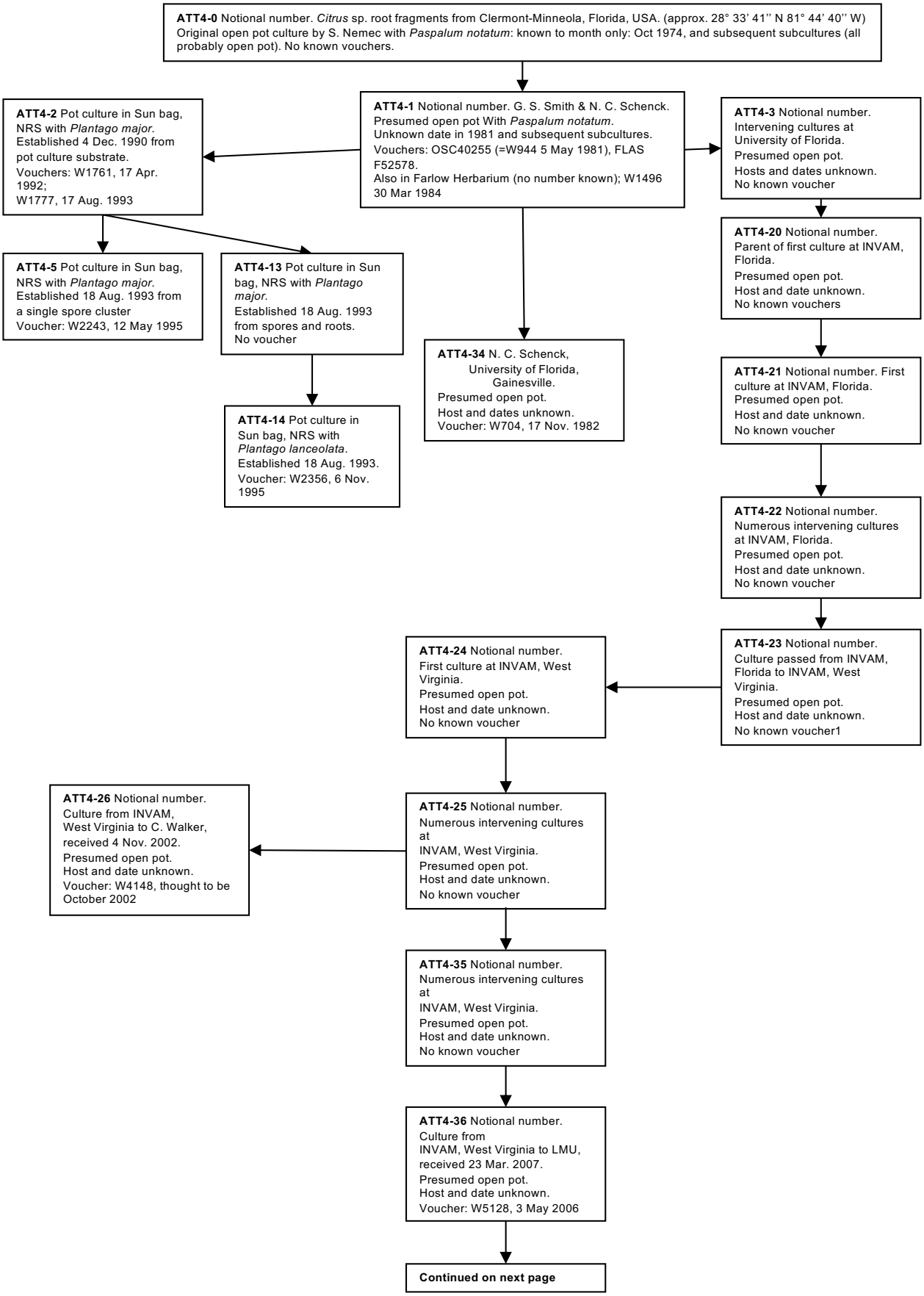

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Fig. S1-2. *R. intraradices*. ATT 4. Culture history (Continued).

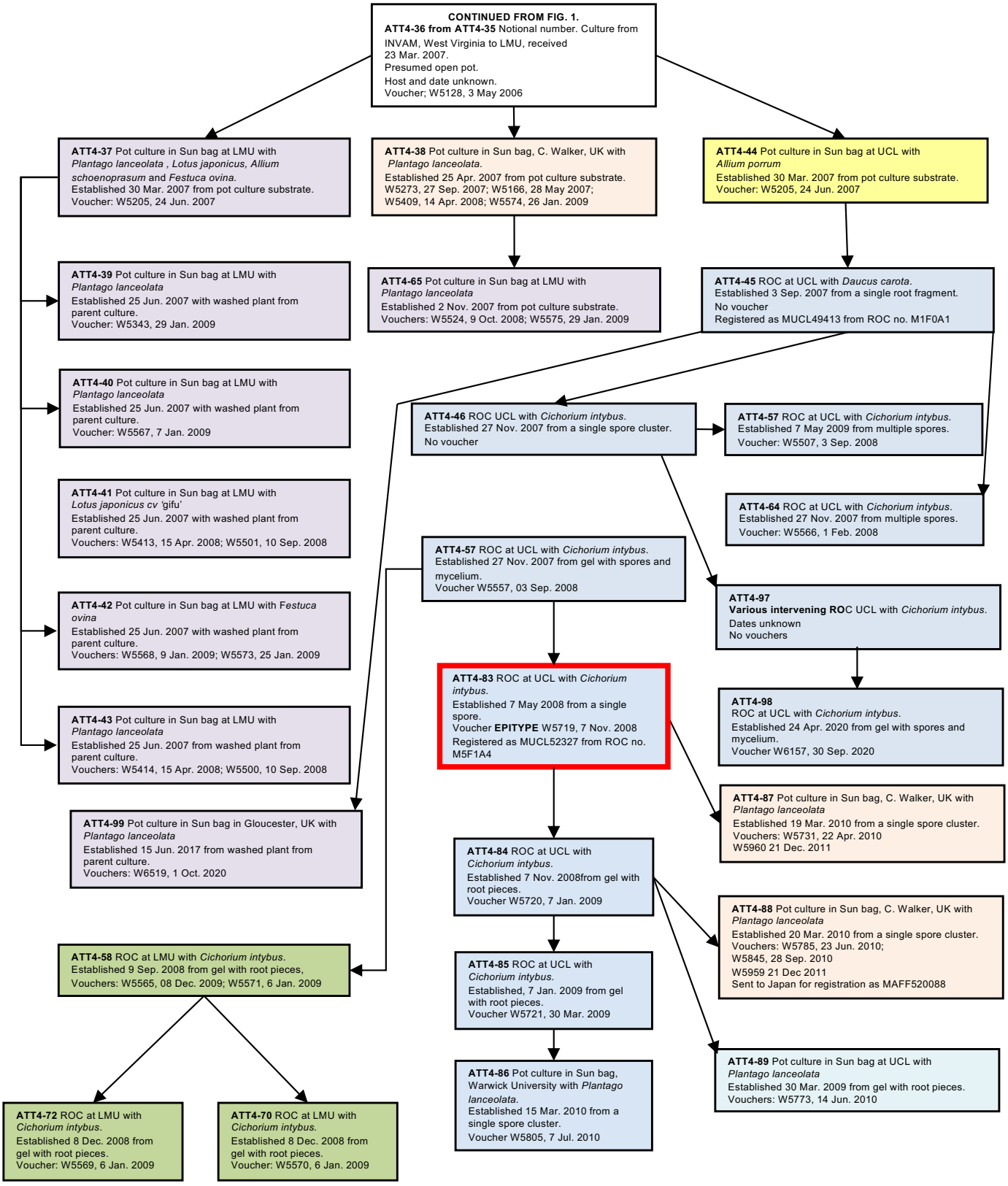

Fig. S1. (Continued).

Fig. S1-3. *R. intraradices*. ATT 1102. Culture history.

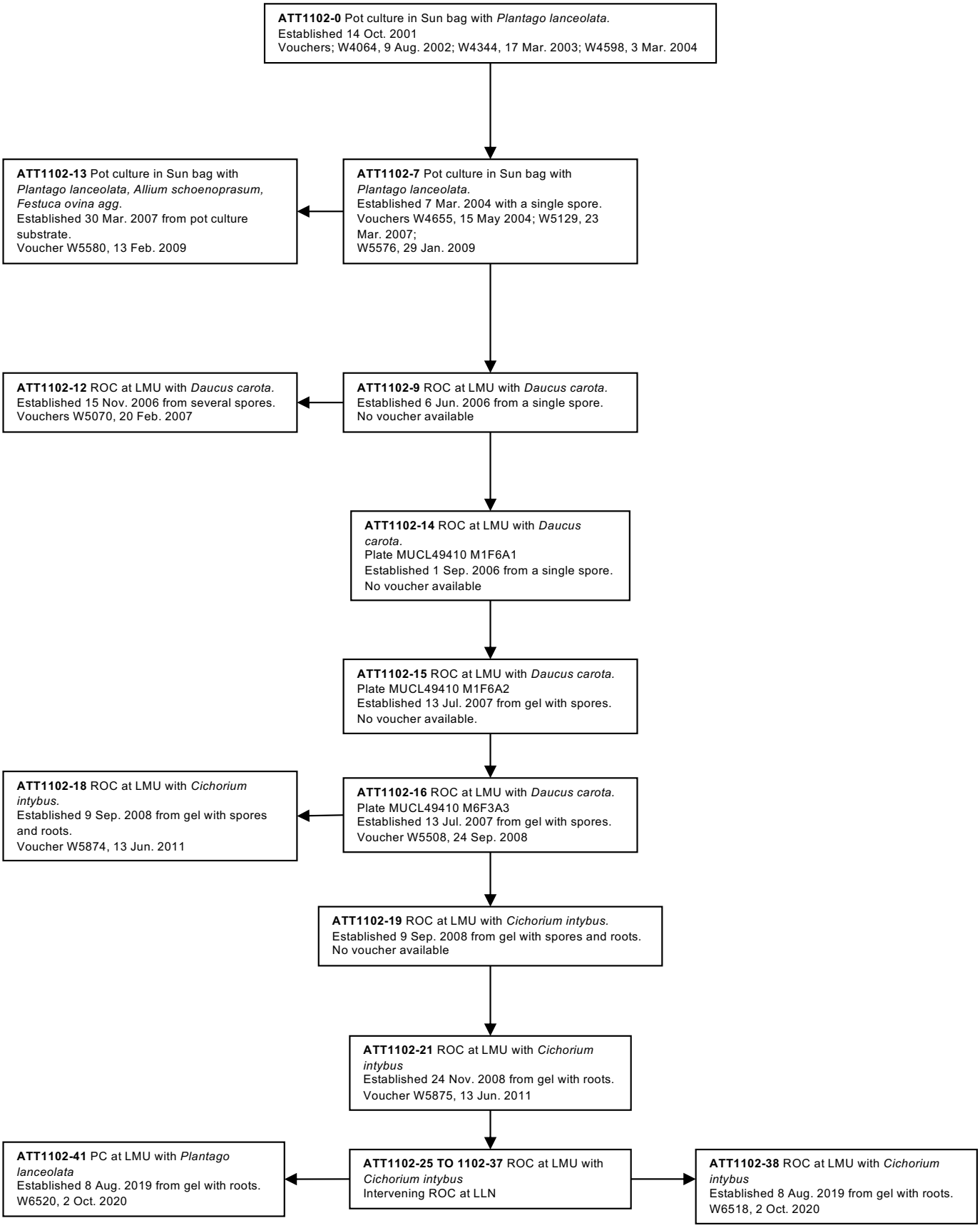

Fig. S1. (Continued).

Fig. S2

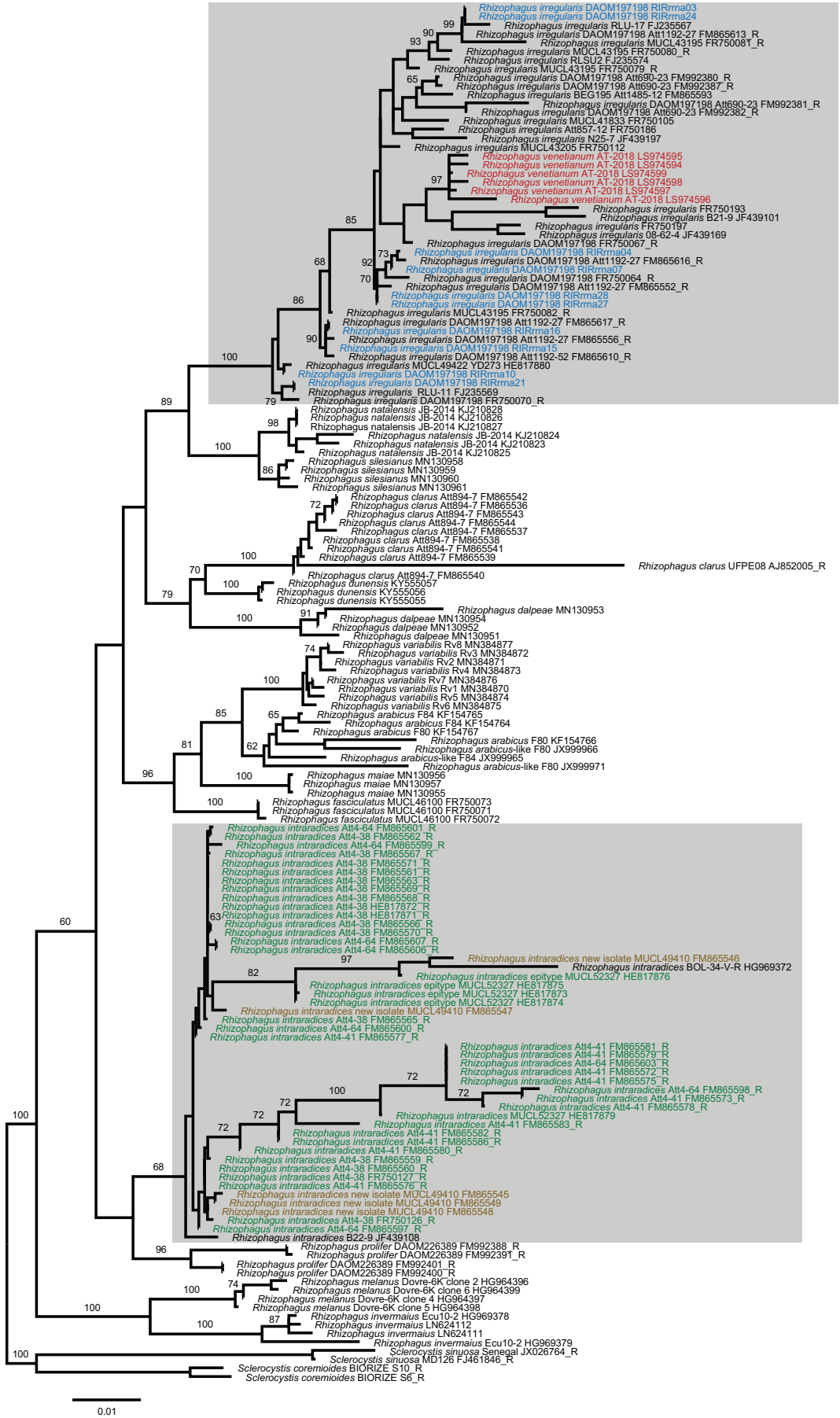

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Table S1. *Rhizophagus intraradices*: lengths and widths (µm) of extra- and intraradical spores from two strains, ATT 4 (type and ex-type) and ATT 1102 (new culture from type locality) with inferential statistics (number of spores observed (n), minimum value (Min), first quartile of the data (Q1), median, third quartile of data (Q3), maximum value (Max), mean, standard deviation (SD) and % coefficient of variation (CV %)).

Table S1

						Spore length (µm)							Spore width (µm)						
Voucher	**ATT-culture**	**Type**	**Spore position**	**Age (days)**	**n**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**Mean ± SD**	**CV%**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**Mean ± SD**	**CV%**
W5070	1102-12(9)	ROC	extraradical	97	100	48	80	91	100	123	90 ± 15	17	48	78	91	101	122	89 ± 15	17
W5580	1102-13(7)	PC	extraradical	686	100	48	80	103	135	383	114 ± 56	49	43	80	108	134	398	115 ± 57	50
W5508	1102-16(15)	ROC	extraradical	126	45	48	77	83	96	107	85 ± 13	16	46	77	82	99	110	85 ± 14	16
W6518	1102-38(37)	ROC	extraradical	421	100	61	92	106	120	147	106 ± 19	18	58	91	104	117	146	104 ± 19	18
W6520	1102-41(40)	PC	extraradical	716	100	42	78	90	103	130	88 ± 21	24	43	75	87	106	144	89 ± 22	25
W4655	1102-7(0)	PC	extraradical	944	100	26	54	74	96	117	74 ± 24	32	26	51	72	94	117	71 ± 23	32
W5576	1102-7(0)	PC	extraradical	1789	50	43	79	92	109	154	94 ± 25	27	43	78	90	111	149	93 ± 25	27
W944	4-1(0)	PC	extraradical	n/a	52	42	86	103	113	224	104 ± 30	29	42	86	98	111	154	99 ± 22	23
W4148	4-26(25)	PC	extraradical	n/a	65	54	104	120	140	182	121 ± 24	20	52	100	116	134	160	117 ± 22	19
W5128	4-36(35)	PC	extraradical	182	63	67	99	112	124	171	111 ± 21	19	45	95	106	123	162	107 ± 23	22
W5574	4-38(36)	PC	extraradical	642	100	37	75	87	102	147	89 ± 22	25	37	72	87	101	152	88 ± 21	24
W5567	4-40(37)	PC	extraradical	562	100	51	101	109	128	157	112 ± 23	20	51	99	109	129	165	111 ± 23	21
W5501	4-41(37)	PC	extraradical	443	50	56	92	101	114	205	104 ± 24	23	61	92	103	112	155	102 ± 20	20
W5568	4-42(37)	PC	extraradical	564	27	56	78	88	116	152	98 ± 27	27	56	77	95	114	144	95 ± 24	26
W5507	4-57(46)	ROC	extraradical	119	90	51	94	107	120	171	107 ± 23	21	53	89	109	122	171	107 ± 24	22
W5565	4-58(57)	ROC	extraradical	90	100	42	85	93	103	168	97 ± 22	23	43	83	93	103	173	97 ± 24	24
W5571	4-58(57)	ROC	extraradical	119	50	64	83	92	104	178	95 ± 21	22	61	82	93	104	178	95 ± 22	23
W5524	4-65(38)	PC	extraradical	342	50	48	83	100	112	147	98 ± 27	27	43	85	98	112	149	97 ± 27	27
W5570	4-70(58)	ROC	extraradical	29	50	43	76	98	112	182	95 ± 30	31	42	76	97	109	176	94 ± 31	33
W5719	4-83(57)	ROC	extraradical	508	100	30	77	91	107	130	89 ± 22	25	30	75	90	104	131	88 ± 23	26
W5720	4-84(83)	ROC	extraradical	447	100	37	86	96	107	162	94 ± 22	23	32	83	95	104	163	92 ± 23	25
W5721	4-85(84)	ROC	extraradical	303	64	67	99	117	128	152	113 ± 20	18	62	99	116	127	149	112 ± 22	20
W5960	4-87(83)	PC	extraradical	642	100	29	58	78	91	149	77 ± 26	34	29	59	78	91	149	77 ± 26	33
W5785	4-88(84)	PC	extraradical	95	100	43	72	85	99	146	85 ± 20	23	37	72	85	100	147	85 ± 21	25
W5959	4-88(84)	PC	extraradical	641	20	48	71	95	115	138	95 ± 29	30	48	71	95	116	139	95 ± 29	31
W6517	4-98(97)	ROC	extraradical	161	100	40	80	96	109	168	93 ± 23	25	37	76	93	106	168	90 ± 24	26
W5580	1102-13(7)	PC	intraradical	686	100	28	69	83	108	183	89 ± 32	36	25	63	74	93	150	78 ± 26	34
W6520	1102-41(40)	PC	intraradical	716	100	40	80	99	123	190	101 ± 31	31	29	64	82	96	149	82 ± 25	30
W4655	1102-7(0)	PC	intraradical	944	50	56	95	134	165	218	131 ± 45	34	34	74	98	152	202	109 ± 43	40
W5576	1102-7(0)	PC	intraradical	1789	50	18	66	81	93	160	82 ± 27	33	40	66	79	90	157	79 ± 24	30
W944	4-1(0)	PC	intraradical	n/a	100	29	56	71	93	186	79 ± 31	39	18	44	61	86	147	66 ± 29	43

Table S1. (Continued).

						Spore length (µm)							Spore width (µm)						
Voucher	**ATT-culture**	**Type**	**Spore position**	**Age (days)**	**n**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**Mean ± SD**	**CV%**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**Mean ± SD**	**CV%**
W704	4-34(1)	PC	intraradical	n/a	40	27	66	91	116	160	89 ± 32	36	27	50	70	84	131	70 ± 26	37
W5128	4-36(35)	PC	intraradical	182	52	48	77	106	123	147	100 ± 30	30	35	58	83	105	152	84 ± 28	33
W5574	4-38(36)	PC	intraradical	642	100	18	61	74	100	234	82 ± 33	40	16	54	67	86	122	69 ± 23	33
W5567	4-40(37)	PC	intraradical	562	40	43	71	93	117	144	94 ± 28	29	24	66	90	107	131	86 ± 27	32
W5568	4-42(37)	PC	intraradical	564	29	32	53	64	67	80	67 ± 23	35	18	32	42	64	104	48 ± 25	53
W5960	4-87(83)	PC	intraradical	642	100	42	74	85	98	138	85 ± 19	23	27	66	82	94	128	80 ± 23	29
W5959	4-88(84)	PC	intraradical	641	100	21	59	83	98	128	80 ± 23	29	19	58	80	98	147	77 ± 25	32

Table S2

Table S2. *Rhizophagus intraradices*: spore colours from two strains (ATT 4 – type and ex-type) and ATT 1102 (new culture from type locality) from pot cultures (PC) and root organ cultures (ROC). Where possible, colours were matched with charts from Royal Botanic Garden Edinburgh, Munsell, or Methuen Handbook of Colour.

Attempt-no. (parent)	Establishment date	Sample date	Age (days)	Type of culture	Voucher (W no.)	Colour	Chart used	Extra- or intraradical
4-1(0)	Unknown	5 May 1981	unknown	PC	TYPE	Yellow to grey-brown, appearing greenish brown with transmitted light (FROM PROTOLOGUE)	None	Extraradical
4-1(0)	Unknown	5 May 1981	unknown	PC	944	Sienna to fulvous probably darkened by lactophenol storage (11-12)	RBG	Extraradical
4-1(0)	Unknown	5 May 1981	unknown	PC	944	Yellowish cream to sienna, probably darkened by lactophenol storage (5-11)	RBG	Intraradical
4-34(1)	Unknown	17 Nov. 1982	unknown	PC	704	Colourless to yellow (colourless-10YR 7/8)	Munsell	Extraradical
4-34(1)	Unknown	17 Nov. 1982	unknown	PC	704	Colourless to yellow (colourless-10YR 7/8)	Munsell	Intraradical
4-1(0)	Unknown	30 Mar. 1984	unknown	PC	1496	Not recorded	not relevant	Intraradical
4-2(0)	4 Dec. 1990	17 Apr. 1992	500	PC	1761	Yellow	None	Not noted
4-2(0)	4 Dec. 1990	17 Aug. 1993	987	PC	1777	Colourless to pale ochraceous to ochraceous (colourless-6-8)	RBG	Extraradical
4-5(2)	18 Aug. 1993	12 May 1995	632	PC	2243	Colourless to white to pale ochraceous to ochraceous (colourless-1-6-8)	RBG	Not noted
4-17(16)	24 Aug. 1993	15 May 1995	629	PC	2250	Colourless to pale yellowish cream to pale ochraceous to sienna (colourless-3-6-11)	RBG	Not noted
4-14(13)	25 Jan. 1995	6 Nov. 1995	285	PC	2356	Yellowish cream to pale ochraceous to ochre (5-6-8)	RBG	Not noted
4-14(13)	24 Aug. 1993	6 Nov. 1995	804	PC	2360	Colourless to ochre to sienna (colourless-9-11)	RBG	Not noted
4-14(13)	24 Aug. 1993	14 Dec. 1995	842	PC	2397	Colourless to straw (colourless-50)	RBG	Not noted
4-26(25)	Unknown	4 Nov. 2002	unknown	PC	4148	Yellow to reddish yellow (5Y7/8 - 7.5 YR 8/6 - 7.5 YR 7/8)	Munsell	Extraradical
4-36(35)	2 Nov. 2005	3 May 2006	182	PC	5128	Colourless to ivory to buff to hazel (colourless-2-52-27)	RBG	Extraradical
4-37(36)	30 Mar. 2007	24 Jun. 2007	86	PC	5205	Colourless to pale yellowish cream to pale ochraceous to fulvous (colourless-3-6-12)	RBG	Extraradical
4-38(36)	25 Apr. 2007	27 Sep. 2007	155	PC	5273	Colourless to pale pinkish cream to pale fulvous (colourless-4-pale 12)	RBG	Mixed
4-39(37)	25 Jun. 2007	29 Jan. 2008	218	PC	5343	Colourless to pale yellow	None	Extraradical
4-38(36)	25 Apr. 2007	27 Sep. 2007	155	PC	5409	Colourless to pale yellow to yellow to brownish yellow to yellowish brown (Colourless-2.5Y 8/4-8/6-7/6-10YR 6/8-5/8)	Munsell	Mixed
4-41(37)	25 Jun. 2007	15 Apr. 2008	295	PC	5413	Reddish yellow (7.5YR 7/6-7/8-6/8)	Munsell	Mixed
4-43(37)	25 Jun. 2007	15 Apr. 2008	295	PC	5414	Yellow (10YR 7/6-7/8)	Munsell	Extraradical
4-43(37)	25 Jun. 2007	10 Sep. 2008	443	PC	5500	Yellow (2.5Y 7/6)	Munsell	Not noted

Table S2. (Continued).

Attempt-no. (parent)	Establishment date	Sample date	Age (days)	Type of culture	Voucher (W no.)	Colour	Chart used	Extra- or intraradical
4-41(37)	25 Jun. 2007	10 Sep. 2008	443	PC	5501	Colourless to yellow to yellowish brown to dark yellowish brown (10YR 7/6-5/8-3/4)	Munsell	Extraradical
4-57(46)	7 May 2008	3 Sep. 2008	119	ROC	5507	Yellow to brownish yellow to yellowish brown (10YR 7/6-6/6-5/6)	Munsell	Extraradical
4-65(38)	2 Nov. 2007	9 Oct. 2008	342	PC	5524	Colourless to yellow (colourless-2.5 Y 8/4-10YR 8/8)	Munsell	Extraradical
4-58(57)	9 Sep. 2008	8 Dec. 2008	90	ROC	5565	Colourless to yellow to brownish yellow (10YR 8/6-10YR 7/8-6/6)	Munsell	Extraradical
4-64(45)	27 Nov. 2007	1 Feb. 2008	66	ROC	5566	Reddish yellow (7.5YR 7/8-6/8)	Munsell	Extraradical
4-40(37)	25 Jun. 2007	7 Jan. 2009	562	PC	5567	Very pale brown to yellow (10YR 8/4-8/8-7/8-5/8)	Munsell	Extraradical
4-40(37)	25 Jun. 2007	7 Jan. 2009	562	PC	5567	Very pale brown to yellow (10YR 8/4-8/8-7/8-5/8)	Munsell	Intraradical
4-42(37)	25 Jun. 2007	9 Jan. 2009	564	PC	5568	Colourless to very pale brown to yellow to brownish yellow (10YR 8/3-7/6-6/8	Munsell	Extraradical
4-42(37)	25 Jun. 2007	9 Jan. 2009	564	PC	5568	Colourless to very pale brown to yellow to brownish yellow (10YR 8/3-7/6-6/9	Munsell	Intraradical
4-72(58)	8 Dec. 2008	6 Jan. 2009	29	ROC	5569	Colourless to pale pink (colourless-7.5YR 8/3)	Munsell	Extraradical
4-70(58)	8 Dec. 2008	6 Jan. 2009	29	ROC	5570	Colourless to reddish yellow [but very pale] (colourless-very pale 7.5YR 8/6)	Munsell	Extraradical
4-58(57)	9 Sep. 2008	6 Jan. 2009	119	ROC	5571	Reddish yellow (7.5YR 8/6-7/8)	Munsell	Extraradical
4-38(36)	25 Apr. 2007	14 Apr. 2008	355	PC	5574	Reddish yellow to strong brown (7.5YR 6/8-5/8-4/6)	Munsell	Extraradical
4-38(36)	25 Apr. 2007	26 Jan. 2009	642	PC	5574	Reddish yellow to strong brown (7.5YR 6/8-5/8-4/6)	Munsell	Intraradical
4-65(38)	2 Nov. 2007	29 Jan. 2009	454	PC	5575	Colourless to very pale brown to yellow to brownish yellow (Colourless-10YR 8/4-8/6-8/8-7/6-7/8-6/8)	Munsell	Mixed
4-83(57)	7 Nov. 2008	30 Mar. 2010	508	ROC	5719	Yellow to brownish yellow to yellowish brown (10YR 7/8-6/8-5/8)	Munsell	Extraradical
4-84(83)	7 Jan. 2009	30 Mar. 2010	447	ROC	5720	Yellow to brownish yellow to yellowish brown to dark yellowish brown (10YR 8/8-6/8-5/8-3/6)	Munsell	Extraradical
4-85(84)	30 Mar. 2009	27 Jan. 2010	303	ROC	5721	Yellow to brownish yellow to yellowish brown (10YR 7/8-6/8-5/8)	Munsell	Extraradical
4-87(83)	19 Mar. 2010	22 Apr. 2010	34	ROC from PC	5731	Colourless	not relevant	Extraradical
4-38(36)	25 Apr. 2007	14 Jun. 2010	1146	PC	5739	Pale yellow to dark yellowish brown	None	Mixed
4-89(84)	30 Mar. 2009	14 Jun. 2010	441	ROC	5773	Brown	None	Mixed
4-88(84)	20 Mar. 2010	23 Jun. 2010	95	ROC	5785	Mostly colourless to pink to very pale brown (colourless (mainly)-7.5YR 8/4-10YR 8/3)	None	Extraradical

Table S2 (Continued).

Table S2. (Continued).

Attempt-no. (parent)	Establishment date	Sample date	Age (days)	Type of culture	Voucher (W no.)	Colour	Chart used	Extra- or intraradical
4-86(85)	15 Mar. 2010	7 Jul. 2010	114	ROC	5805	Mostly colourless with few spores very pale yellow	None	Extraradical
4-86(85)	15 Mar. 2010	7 Jul. 2010	114	ROC	5805	Colourless	not relevant	Intraradical
4-88(84)	20 Mar. 2010	28 Sep. 2010	192	ROC	5845	Mostly colourless, a few yellow (mostly colourless, a few 10YR 8/6-8/8)	Munsell	Mixed
4-68(58)	8 Dec. 2008	14 Jun. 2011	918	ROC	5876	Greyish orange to yellowish brown (5B5-5E8)	Methuen	Extraradical
4-69(58)	8 Dec. 2008	13 Jun. 2011	917	ROC	5877	Brownish orange to yellowish brown (5C4-5E5)	Methuen	Extraradical
4-88(84)	20 Mar. 2010	21 Dec. 2011	641	ROC	5959	Colourless to yellow to yellowish brown to dark yellowish brown (colourless-2.5Y 8/6-10YR 5/8-4/6)	Munsell	Intraradical
4-88(84)	20 Mar. 2010	21 Dec. 2011	641	ROC	5959	Colourless to yellow to yellowish brown to dark yellowish brown (colourless-2.5Y 8/6-10YR 5/8-4/6)	Munsell	Extraradical
4-87(83)	19 Mar. 2010	21 Dec. 2011	642	ROC	5960	Colourless to yellow (10YR 8/6-7/8)	Munsell	Extraradical
4-87(83)	19 Mar. 2010	21 Dec. 2011	642	ROC	5960	Colourless to yellow (10YR 8/6-7/8)	Munsell	Intraradical
1102-0	16 Oct. 2001	3 Mar. 2004	869	PC from field	4598	Pale ochraceous to fulvous (6-12)	RBG	Mixed
1102-7(0)	7 Mar. 2004	15 May 2004	69	PC	4655	Colourless to pale ochraceous to ochraceous to ochre to sienna (colourless-6-8-9-11)	RBG	Extraradical
1102-7(1)	7 Mar. 2004	15 May 2004	69	PC	4655	Colourless to pale yellow to pale brown	None	Intraradical
1102-12(9)	15 Nov. 2006	20 Feb. 2007	97	ROC	5070	Pale ochraceous (6)	RBG	Extraradical
1102-7(0)	8 Mar. 2004	23 Mar. 2007	1110	PC	5129	Colourless to pale yellow	None	Extraradical
1102-16(15)	21 May 2008	24 Sep. 2008	126	ROC	5508	Yellow to brownish yellow (10YR 8/6-8/8-7/8-6/8)	Munsell	Extraradical
1102-7(0)	8 Mar. 2004	29 Jan. 2009	1788	PC	5576	Colourless to yellow to brownish yellow to yellowish brown to dark yellowish brown (10YR 7/8-6/8-5/8-4/6)	Munsell	Extraradical
1102-7(0)	8 Mar. 2004	29 Jan. 2009	1788	PC	5576	Colourless to yellow to brownish yellow to yellowish brown to dark yellowish brown (10YR 8/6-8/8-7/8-6/8-5/8-4/6)	Munsell	Intraradical
1102-13(7)	30 Mar. 2007	13 Feb. 2009	686	PC	5580	Yellow to reddish yellow (10YR 8/6-7/8 to 7.5YR 7/8-6/8)	Munsell	Mixed