Growth dynamics of geographically different arbuscular mycorrhizal fungal isolates belonging to the 'Rhizophagus clade' under monoxenic conditions

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Abstract: The growth dynamics of extraradical mycelium and spore formation of 14 ''*Rhizophagus*'' isolates from different sites in Argentina were evaluated under monoxenic conditions. A modified Gompertz model was used to characterize the development of mycelium and spores for each isolate under the same conditions. The lag time, maximal growth rate and total quantity of both extraradical hyphae and spores were determined. Wide variability among isolates was detected, and all growth parameters were significantly altered by fungal isolate. Discriminant analysis differentiated isolates primarily based on the extent of extraradical hyphae produced, yet such differences did not conclusively correspond to phylogenetic relationships among closely related isolates based on partial SSU sequences. Given that the ''*Rhizophagus*'' isolates were grown under controlled conditions for many generations, the expression of phenotypic variability could be attributed to genetic differences that are not completely resolved by phylogenetic analysis employing the small ribosomal gene.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi (phylum Glomeromycota) are obligate biotrophs that establish mutualistic symbioses with roots of most plant species and play key roles in ecosystem functioning (Smith and Read 2008). The mycelial network of AM fungi increases the surface area for nutrient uptake, produces many spores and is a source of inoculum for colonization of new host plants. The exchange of genetic material also takes place in the extraradical mycelium (ERM) by hyphal anastomosis (Avio et al. 2006). The ERM, spores and intraradical mycelium are the main fungal structures for propagation and survival of *Glomerales* species (Klironomos and Hart 2002, Schalamuk and Cabello 2010).

Based on molecular surveys and morphological identification, the most widespread and abundant AM fungal species belong to the genus *Rhizophagus* (former *Glomus* Group Ab, ''*Rhizophagus intraradices* clade'') (Schüßler and Walker 2010). Within the ''*Rhizophagus* clade'' (Schwarzott et al. 2001) *R. intraradices* and *R. irregularis* are the AM species most frequently studied as fungal models (Martin et al. 2008, Fernández et al. 2009, Ehringer et al. 2012). However, little is known concerning developmental traits that reflect life-history strategies and phenotypic plasticity in this group.

The study of phenotypic variance among closely related AM fungal isolates has been problematic, mainly due to their limited distinguishing characteristics and the experimental protocols used. Hart and Reader (2005) observed that total ERM length did not differ among six *R. intraradices* isolates from different locations. In contrast, Avio et al. (2006) found variation in total hyphal length between two geographically different isolates of *R. intraradices*. These studies used a pot-culture system with inherent risks of measuring artifacts due to growing conditions, contaminants and host physiological differences. In addition, studies of phenotypic variance performed with only a few AM fungal strains are not sufficiently representative of AM fungal population variability. These limitations were partly overcome by using root-organ cultures (ROCs) of Ri T-DNA-transformed

carrot roots colonized by AM fungi (Declerck et al. 2005). This method allows fungal growth over several generations under identical conditions, thus removing environmental influences and highlighting the role of genetics. Koch et al. (2004) showed that individuals from a *R. irregularis* population varied in total ERM length, spore number and growth rates with the use of ROCs. These authors concluded that considerable phenotypic and genetic variation exists at the population level. However, the range of phenotypic variation among AM fungal isolates within the ''*Rhizophagus* clade'' from different geographic populations remains unclear. To date no studies have analyzed the in vitro growth dynamics of ERM development and spore formation among a wide assemblage of AM isolates belonging to the ''*Rhizophagus* clade''. The isolation process involved in obtaining many fungal strains, their maintenance under monoxenic cultures for many generations and the time-consuming process of monitoring their growth under controlled conditions have impeded studies of this nature.

One possibility to investigate in more detail the growth dynamics of AM fungal structures is through the application of a mathematical function to fungal growth curves. This approach can be used to validate biological observations and quantify growth parameters. The mathematical function of Gompertz reparameterized by Zwiertering et al. (1990) first was used by Declerck et al. (2001) for modeling sporulation dynamics of three *Glomeraceae* species under monoxenic cultures. Declerck et al. (2004) used the same equation for studying the growth dynamics of extraradical structures of a *Gigasporaceae* species. In both studies AM fungal development followed a classical lag-exponential-plateau phase, and lag time, maximum growth rate and the total quantity of spores and hyphae could be predicted.

An important parameter of fungal growth is the lag phase, in that it involves the early colonization of substrate and host roots and subsequently the successful establishment of the AM symbiosis (Bonfante and Genre 2010). However, whether variation in the lag phase exists among closely related AM fungal isolates is unknown. In addition, the rapidity and extent at which the external mycelia develop in soil could be related to the ability of the AM fungus to survive under different environmental conditions. In turn, the rate of spore formation and the total number of spores produced are likely key factors that govern short- and long-term survival of AM fungi. We suggest that phenotypic variability in such ecologically important life-history traits may contribute to the propagation and colonization of AM fungal isolates within ''*Rhizophagus* clade'' in heterogeneous environments.

The aim of this study was to investigate the growth dynamics of ERM and spore production among AM fungal isolates belonging to the ''*Rhizophagus* clade'' from different geographic populations under monoxenic conditions. In addition, the link between these phenotypic characters and the molecular phylogeny based on the ribosomal small subunit gene was investigated with discriminate analysis. Characterizing fungal development under environmentally controlled conditions may help resolve taxonomic difficulties within this important and cosmopolitan *Rhizophagus* group.

MATERIALS AND METHODS

Biological material.—Isolation of AM fungi. We isolated AM fungi from different geographic habitats of Argentina, using mycorrhizal roots from trap plants as initial inoculum for the establishment of monoxenic cultures. Bulk soil samples (approx. 200 g) were randomly collected (to a depth of 20 cm) from different environments (TABLE I). Trap cultures consisted of pots (1000 mL) with field soil as AM fungal inoculum (100 g per trap plant) mixed with an autoclaved substrate (100 C for 1 h, three consecutive days) composed of perlite: vermiculite: soil $(1:1:1, v/v/v)$ (pH 7.1; total C 12.08 and N 1.1 $(g \ kg^{-1})$; P 34.2 mg kg^{-1}; K 0.9, Ca 7.5, Mg 1.7 and Na 0.2 $[cmol \ kg^{-1}]$). Several pregerminated seeds of *Solanum lycopersicum, Solanum melongena* and *Pisum sativum* were planted in each pot. Plants were grown in a greenhouse 6 mo with natural light and ambient temperature, watered when necessary and fertilized every 15 d with 20 mL nutritive solution (Hewitt 1952). After 6 mo, root samples from each trap plant were cleared in KOH (10% w/v 15 min, 90 C) and stained with trypan blue in lactic acid (0.02% 10 min, 90 C) to observe AM root colonization.

Establishment of monoxenic culture. Mycorrhizal root samples from trap plants were surface-sterilized, cut into pieces and each root piece was incubated in drops of Gel-Gro® medium, as described in Silvani et al. (2008). Each root piece with hyphal regrowth of AM fungi in the absence of other contaminant microorganisms was placed in the vicinity of a Ri T-DNA-transformed carrot root growing on minimum medium (MM) (Bécard and Fortin 1988) and incubated in an inverted position at 25 C in the dark. Monoxenic cultures were monitored weekly under a binocular microscope for the development of ERM and spore production. New monoxenic cultures were initiated by transferring a single spore to a transformed carrot root culture to ensure that only one isolate was present. These single-spore cultures were propagated for at least six generations under identical conditions, as suggested by Koch et al. (2004), and used for all subsequent work reported here. Each AM isolate in monoxenic culture was characterized morphologically from spores and by molecular technique.

Morphological characterization of AM fungal isolates. To obtain many healthy spores from monospecific pot cultures, a piece of monoxenic culture (containing mycorrhizal root fragments, extraradical mycelia and spores) was inoculated

TABLE I. Identification of AM fungal isolates, GenBank accession number, site descriptions, ecological characterization, properties of sampled soils and host plants

Isolate name[a]	GenBank No.	Geographic position[b]	Latitude and longitude	Biome	Climate[c]	Soil type[d]	Host plant
GA2	JX049517	Campo Quijano, Salta	24°53′6″S; 65°38′10″W	grassland	temperate (703.4 mm, 17.0 C)	alfisols, clay loam	unknown *Poaceae*
GA3	JX049518	Campo Militar, Salta	24°45′10″S; 65°24′45″W	grassland	temperate (741.1 mm, 17.2 C)	alfisols, clay loam	*Poa* sp., *Festuca* sp.
GA5	GU140042	San Lorenzo, Salta	24°43′12″S; 65°30′18″W	woodland	temperate (727.7 mm, 16.8 C)	alfisols, clay loam	*Fabaceae, Lauraceae*
GA10	JX051853	Chicoana, Salta	25°07′03″S; 65°33′28″W	woodland	temperate (722.6 mm, 17.5 C)	inceptisols, silt loam gravel	*Jacaranda mimosifolia, Erithrina* sp.
GA11	JX049519	Chicoana, Salta	25°07′03″S; 65°33′28″W	woodland	temperate (722.6 mm, 17.5 C)	inceptisols, silt loam gravel	*Jacaranda mimosifolia, Erithrina* sp.
GX3	JX049526	Huerta Grande, Córdoba	31°04′59″S; 64°30′02″W	grassland	temperate (827.8 mm, 18.1 C)	entisols, loamy sand	*Bromus* sp.
GX7	JX049527	Huerta Grande, Córdoba	31°04′52″S; 64°28′14″W	grassland	temperate (839.2 mm, 18.0 C)	mollisols, silt loam	*Bidens pilosa*
GX10	JX049528	Villa Giardino, Córdoba	31°03′51″S;64°30′49″W	grassland	temperate (827.8 mm, 18.0 C)	entisols, sandy loam	*Chloris* sp.
GB1	JX049520	Matheu, Buenos Aires	34°32′22″S; 58°50′15″W	grassland	temperate (1025.5 mm, 16.8 C)	mollisols, silt loam	unknown *Poaceae*
GB2	JX049521	San Martín, Buenos Aires	34°32′34″S; 58°33′17″W	grassland	temperate (1023.7 mm, 17.0 C)	mollisols, silt loam	*Sellaginela* sp.
GC1	JX049522	Ciudad Universitaria, Buenos Aires	34°32′27″S; 58°26′27″W	grassland	temperate (1022.4 mm, 17.0 C)	urban soil	*Ricinus communis*
GC2	JX049523	Ciudad Universitaria, Buenos Aires	34°32′27″S; 58°26′27″W	grassland	temperate (1022.4 mm, 17.0 C)	urban soil	*Picris echioides*
GC3	JX049524	Ciudad Universitaria, Buenos Aires	34°32′22″S; 58°26′41″W	grassland	temperate (1022.4 mm, 17.0 C)	urban soil	*Ricinus communis*
GC4	JX049525	Ciudad Universitaria, Buenos Aires	34°32′22″S; 58°26′42″W	grassland	temperate (1022.4 mm, 17.0 C)	urban soil	*Medicago lupulina*

[a] Accession number in Banco de Glomeromycota In Vitro (http://www.bgiv.com.ar/).
[b] Locality and province.
[c] In parenthesis: mean annual precipitation and temperature.
[d] According to Soil Survey Staff. 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington DC.

to plantlets of *Sorghum halepense* and *Trifolium repens* grown in pots with a sterile substrate as previously described. After 6 mo AM spores were extracted by wet sieving and decanting.

Approximately 200 spores from pot cultures were mounted in polyvinyl-alcohol glycerol (PVLG) and a mixture of PVLG and Melzer reagent (1:1, v/v) to observe their morphological characters and subcellular structure with a Nikon Optiphot-2 microscope. Identification was made under supervision of Dr Gisela Cuenca, (Instituto Venezolano de Investigación Científica, Caracas, Venezuela) in accordance with species descriptions and the online guide provided by INVAM (http://invam.caf.wvu.edu) and Dr Blaszkowski's website (http://agro.ar.szczecin.pl/ wjblaszkowski/index.html). Taxonomic assignments were done according to the Index Fungorum. Permanent slides were deposited as voucher material at the Banco de Glomeromycota in Vitro (BGIV, Buenos Aires, Argentina).

Molecular characterization of AM fungal isolates. For fungal DNA extraction, a bicompartmentalized culture system was used to obtain pure AM fungal propagules (St Arnaud et al. 1996). Spores and external mycelia were recovered from the root-free compartment through dissolution of the growth medium with sodium citrate buffer and washed with sterile distilled water (Cranenbrouck et al. 2005).

A cluster of spores and mycelium of each isolate was crushed in 40 μL sterile Tris-EDTA buffer 10 mM (pH 8.0) with a pipette tip and 10 μL Chelex® 100 Resin (BioRad, California) 20% (w/v) was added to the solution. The mixture was incubated (95 C, 10 min), immersed in ice (2 min) and centrifuged (11 000 rpm, 5 min); supernatants were transferred to sterile tubes and stored at −18 C until use as template. The partial SSU nrDNA was amplified using AM1 and NS31 primers (Simon et al. 1992, Helgason et al. 1998).

Reactions were performed in a final volume of 25 μL containing 5 μL DNA extracts, 0.02 U/μL high-fidelity DNA iproof BioRad® polymerase (BioRad, California), PCR buffer 1× iproof HF, 200 mM dNTPs (Invitrogen, Carlsbad, California) and 0.5 μM each primer. Amplification was performed in a thermo-cycler Gene Bioer Pro® (Bioer, Hangzhou, China). The PCR conditions consisted of an initial denaturing cycle (98 C, 5 min), 35 amplification cycles (98 C, 5 s; 58 C, 15 s; 72 C, 1 min) and a final elongation cycle (72 C, 10 min). The amplified products of all AM isolates were cloned into the P-Gem T easy vector® 2.1 (Promega, Madison, Wisconsin) following the manufacturer's instructions and transformed into competent *Escherichia coli* DH5α cells. Per isolate, three colonies of putative positive transformants were picked and directly subjected to another PCR amplification using the primers and reaction program as described above. The transformed bacterial colonies showing correct insert size were grown overnight at 37 C with shaking (200 rpm) in 3 mL Luria-Bertani medium supplemented with 100 mg/mL ampicillin. The plasmids were isolated with the MO BIO UltraClean® Mini Plasmid Prep Kit (MO BIO, Carlsbad, California), according to the manufacturer's protocol. Sanger sequencing was performed by the Servicio de Secuenciación y Genotipificado of Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires) on an ABI 3130XL 16-capillary sequencer using big dye 3.1 sequencing chemistry. Sequences were submitted to the EMBL database under accession numbers GU140042, JX049517- JX049528, JX051853 (TABLE I).

The sequences were compiled with BioEdit Sequence Alignment Editor 7.0 software and compared with sequences from the GenBank database. DNA similarity was analyzed with the NCBI BLAST server (http://www.ncbi.nlm.nih.gov) and the MaarjAM database for the phylum *Glomeromycota* (http://maarjam.botany.ut.ee). The sequences obtained together with identified species of the genera *Rhizophagus* (former *Glomus* group Ab, ''*Rhizophagus intraradices* clade'' including sequences of references isolates of *R. intraradices* [FL208] and *R. irregularis* [DAOM 197198/MUCL43194]), and the most similar sequences to our clones obtained from BLAST queries were aligned with the program Clustal W. A neighbor-joining consensus tree was constructed with MEGA 4.0 software assessing Kimura 2-p distances model and 1000 replicates of non-parametric bootstrapping. *Funneliformis mosseae* was used as outgroup. The individual model parameters were estimated with Modeltest 3.7. Phylogenetic trees were viewed and edited by Tree Explorer and a CorelDraw 11.

Experimental design and measurement of variables.—A total of 14 *Rhizophagus* isolates were used for this assay. The *Rhizophagus* isolates GC1, GC2, GC3 and GC4 (http://www.bgiv.com.ar/strains/) previously isolated in Silvani et al. (2008) also were included in the analyses. Each experimental unit consisted of a 9 cm Petri plate with two 5 cm long transformed roots from a 2 wk old root culture growing in MM and inoculated with a 1 cm³ plug of culture medium from a 6 mo old monoxenic culture. For assuring the same amount of fungal propagules in each replicate, plug locations were selected in the monoxenic culture with similar number of extraradical spores (approx. 150–200 spores), mycorrhizal root fragments and ERM length. Replicate subcultured material prepared in this way (many spores and hyphae, rather than just one spore) is stable and ensures the reproducibility in experiments (Ehringer et al. 2012). Monoxenic cultures were incubated horizontally in the dark at 25 C in an inverted position for 20 wk. Five replicates were included for each isolate. The ERM development and the number of newly formed spores were quantified for each replicate at regular intervals from week 3 after initiation of dual cultures and then every 2 wk until week 20. The length of ERM per Petri plate was measured with the gridline intersect method of Marsh (1971), and the number of intersects of 1 cm² squares between hyphae and lines was included in the formula of Newman (1966). An estimation of spore production per Petri plate was obtained for each replicate by adding the number of spores counted individually in five 1 cm³ squares located randomly in each Petri plate. All measurements were made with a binocular microscope (Nikon OPTIPHOT-T2) at 3.2× magnification.

Application of the Gompertz growth model.—Growth curves were fitted by the mathematical model of Gompertz reparameterized by Zwiertering et al. (1990) to study the kinetics of in vitro development of the AM fungal isolates for each replicate plate. The formula of the

Gompertz model was applied to each curve as follows: $E(Yt) = A\exp(-\exp[(\mu_m e/A)(\lambda - t) + 1])$, where $E(Yt)$ is the expected growth for the variable Y at time t, and the parameters can be interpreted as: (A) number of spores/ERM when the fungus reached the stationary phase, (μ_m) maximum rate of spores/ERM production and (λ) lag time of the curve. The Gompertz model was fitted to the data by a nonlinear regression algorithm using the Nelder-Mead optimization to minimize the residual sum of squares, which is the sum of the squared differences between the measured and the predicted values. The nonlinear curve fitting was carried out by the SOLVER option in Microsoft Excel software, which allowed the application of an iterative algorithm to approximate the optimal solution. The values of the three parameters (A, μ_m and λ) were recorded for ERM and spore production.

The frequency (%F) and intensity (I%) of mycorrhizal colonization was measured at the end of the experiment (Declerck et al. 2004). To accomplish this, transformed carrot roots were removed and cleared and stained as described above. Fifty randomly selected root pieces were mounted on microscope slides in groups of 10 and examined with a Nikon binocular microscope at 100× magnification.

Statistical analysis.—For each growth curve, the coefficient of determination (R^2) was obtained to evaluate the goodness of fit between the model applied and the data observed. The means and standard errors of each parameter (A, μ_m and λ) for both variables (ERM length and spore production) were calculated over the five replicates for each isolate. For intraspecific comparison, the parameters obtained for each isolate were analyzed by multivariate ANOVA (MANOVA), using isolate as factor. Assumptions of homoscedasticity and normality were evaluated with Levene test and Shapiro-Wilkes test. The data on AM root colonization (I%) were arcsine square root-transformed before analysis. The least significant difference (LSD) test was performed to compare the means of each parameter among isolates with a significance of $P \leq 0.05$. Discriminant function analysis was performed to visualize distribution patterns according to the phenotypic variation among isolates (Johnson and Wichern 2001).

The A, μ_m, λ, %F and I% data were included in this analysis. Finally, to determine whether ERM and spores developed simultaneously ($\lambda_{ERM} = \lambda_{spores}$), the lag parameters ($\lambda$) of ERM and spores were compared with Student's t-test analysis at a significance of $P \leq 0.05$ for each isolate. Statistical procedures were carried out with the software package SPSS 17.0 and Microsoft Excel for Windows XP.

RESULTS

Monoxenic cultures of AM fungal isolates.—Fourteen AM fungal isolates from different regions in Argentina were obtained in monoxenic culture from a single spore (TABLE I). Spore characters of all isolates were consistent with the morphological descriptions of species within the genus *Rhizophagus* (phylogenetic group *Glomus* Group Ab). Glomoid spores formed

in loose clusters in roots and soil, with a three-layered wall composed of two sloughing, hyaline outer layers and an innermost laminated, yellow layer (SUPPLEMENTARY FIGS. 1, 2). Likewise, phylogenetic analysis of partial SSU nrDNA of the 14 isolates, including sequences of reference isolates of *R. intraradices*, FL208 and *R. irregularis*, MUCL 43194/DAOM 197198, indicates that all isolates cluster within the *Rhizophagus* clade and are separated from *F. mosseae* and a *Glomus* sp. from Argentina with high bootstrap support (100%) (FIG. 1).

A cluster was composed of GX3, GX7, GA2, GA5, GC3, GA11, GB1, GC2, GX10 and GA10 isolates, including *R. irregularis* AFTOL-ID 845 and. *R. irregularis* MUCL 43194/DAOM 197198 (84%). However, based on spore morphology, only the GA10 isolate clearly belongs to *R. irregularis*, given the intense reactivity of the laminate innermost spore wall layer in Melzer's reagent (SUPPLEMENTARY FIGS. 1G–H) and the production of abundant irregular spores with apical cap-like swellings of the outermost wall layer. All spores of the rest of the isolates were globose to subglobose, and their wall has neither depressions nor swelling at the spore apex (SUPPLEMENTARY FIGS. 1, 2). The GC4 isolate was grouped together with *R. intraradices* −FL208 (100% bootstrap support). In concordance with the species description (Blaszkowski et al. 2008), the spores of this isolate were globose to subglobose, with the mucilaginous outermost layer reactive in Melzer's reagent and the third laminate layer composed of separating sublayers that were not reactive in Melzer's. (SUPPLEMENTARY FIGS. 2K–L). GC1 and GB2 isolates were positioned within the *Rhizophagus* clade with an uncertain position between both *Rhizophagus* species, while the GA3 isolate was clustered together with an uncultured *Glomus* sp. that originated from Cuba, supported by a bootstrap value of 94%. The spore morphology of these three isolates was not consistent with *R. irregularis* but was similar to *R. intraradices*, given the similar size and shape of spores and the positive reaction in Melzer's of the outer wall layer (FIGS. 2C–F; SUPPLEMENTARY FIGS. 1C, D).

Description of growth curves and application of the Gompertz model.—Growth curves of ERM development and sporulation for each isolate grown under identical monoxenic conditions are illustrated (FIG. 2). In general, all isolates followed a typical sigmoid curve for the development of both ERM and spores, and three main phases could be distinguished: lag, exponential and stationary.

Microscopic observations revealed that spore germination and hyphal regrowth from ERM and internal mycelia from mycorrhizal root fragments
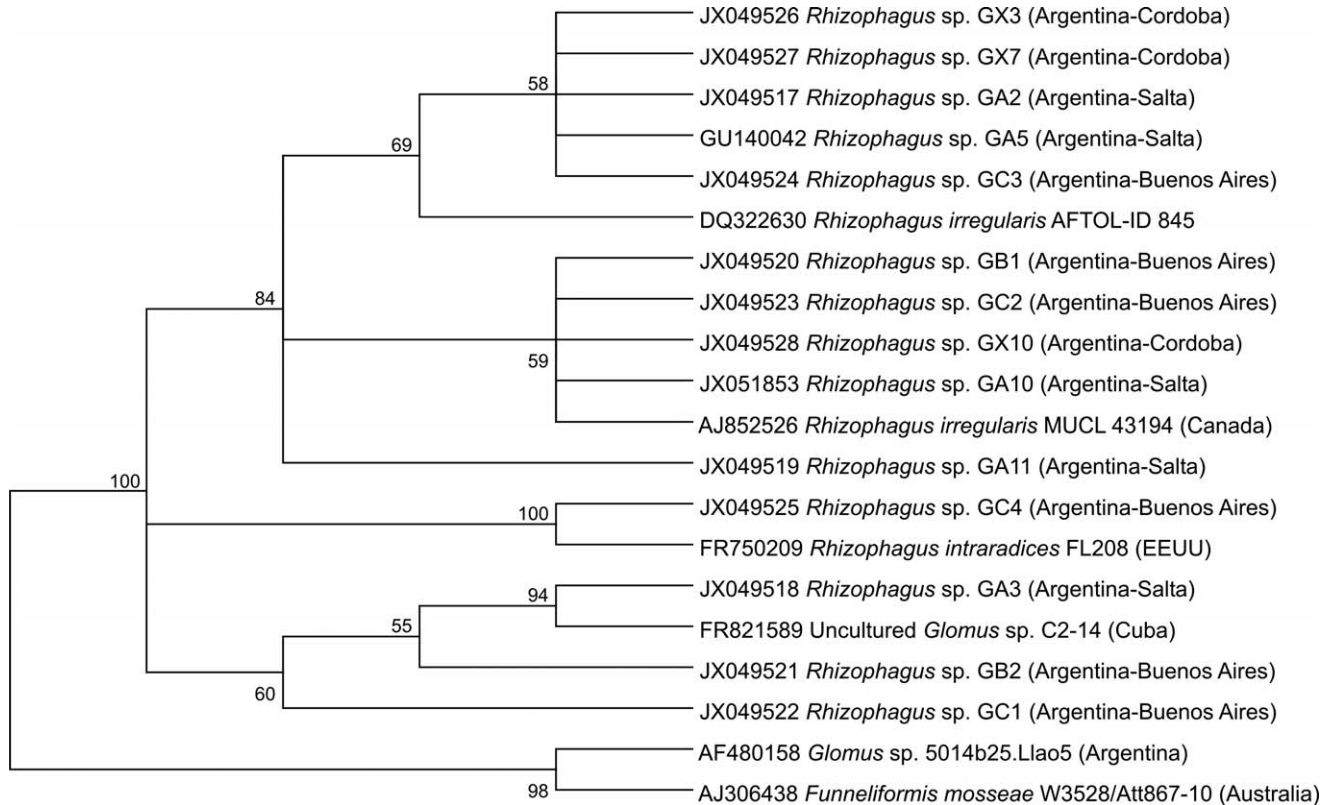
FIG. 1. Neighbor-joining tree showing the phylogenetic positions of AM fungal isolates within *Rhizophagus* clade (former *Glomus* group Ab) inferred from nrDNA SSU sequences with *Funneliformis mosseae* as outgroup. GenBank accession numbers and geographic origin (in parentheses) are provided. Values above branches have NJ bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

took place during the lag phase. Therefore, hyphal extension gave rise to the ERM network, which rapidly increased during the exponential phase until reaching the maximal slope of the growth curve. The first spores occurred through hyphal differentiation during the lag phase, and their number then increased exponentially. Finally, a stationary phase was reached with no increases in ERM length or spore number. The Gompertz model had a good fit for all isolates with R^2 values of 0.973–1.00. The values of the parameters (λ, μ_m, A) for each isolate after applying the Gompertz model in both growth curves are provided (TABLE II).

Growth dynamics of AM fungal isolates.—Variation in the development of ERM and spores among the 14 closely related *Rhizophagus* isolates from different regions in Argentina was observed in monoxenic culture (FIG. 2). Significant variation was detected in all the model parameters fitted to ERM length and sporulation patterns among isolates (TABLE II).

The lag phase of ERM development (λ_{ERM}) differed significantly among isolates (TABLE II). The λ_{ERM} periods varied approximately 1–5 wk, with most isolates 2–3 wk. The GA11 and GC2 isolates had the shortest λ_{ERM} (approx. 1 wk), whereas GA10, GA3, GX3, GB2 and GC3 had the longest λ_{ERM} (approx. 5 wk). GA2 started to develop the ERM exponentially approximately on the second week, GX7, GC1 and GC4 between the second and third week, whereas the rest of the isolates began this phase between the third and fourth week (GA5, GX10, GB1).

The lag phase for sporulation (λ_{SPORES}) varied significantly also among isolates (TABLE II). The λ_{SPORES} ranged between three and 11 wk, with most isolates ranging between the fourth and seventh week. GC1, GB1 and GX7 started to produce spores exponentially earlier from approximately the third week of monoxenic culture. In contrast, GA3 showed a longer λ_{SPORES}. Spores of GA11, GX10, GC2 and GC3 began to be produced exponentially on the fourth week, GA2 and GC4 during the fifth, GA5, GB2, GA10 between the sixth and seventh week and GX3 in the eighth week. The sporulation continued until reaching maximum production in the stationary phase.

The duration of the exponential phase for both growth curves varied among isolates, 4–12 wk. After that period, the slope of the curves decreased until

the stationary phase was reached. GC4 produced both AM fungal structures exponentially for approximately 4 wk, GA11, GB1, GB2 and GC1 approximately 6 wk and GX3, GX7 and GA10 8 wk. GC2 showed the largest exponential phase (approx. 10 wk). The exponential formation of ERM network of GC3 lasted 8 wk, while the exponential production of spores occurred up to 12 wk. The exponential ERM growth phase of GA2 lasted 8 wk, that of GA3 and GX10 6 wk and that of GA5 10 wk. GX10, GA2 and GA5 produced spores for 8 wk in their exponential phase.

The sporulation of some isolates (GA2, GA3, GA5, GA10, GA11, GX7, GX10, GB2, GC1, GC2, GC4) was delayed with respect to the growth of ERM (λ_{SPORES} values were significantly longer than those of λ_{ERM}). GC1 and GA10 started to produce spores exponentially approximately 1 wk later than ERM, GX7, GX10 and GB2 approximately 2 wk, GA5, GA11 and GC2 approximately 3 wk and GA2, GA3, GC4 and GX3 approximately 4 wk. In contrast, GX3 and GC3 synchronously developed both extraradical structures (the λ_{ERM} values were not significantly different from the λ_{SPORES} values).

The maximum growth of ERM ($\mu_{m\ ERM}$) varied significantly among isolates and ranged from a mean of 143.3–784.6 cm/wk (TABLE II). The ERM network of GA10 and GA3 spread slowly throughout the entire Petri plate, but both AM fungal isolates did not differ significantly from GA11, GX7, GX10 and GC1. The latter four isolates together with GA5, GC4, GB2 and GX3 reached $\mu_{m\ ERM}$ values of 216–544 cm/wk. The highest value of $\mu_{m\ ERM}$ was recorded for GC3, GA2, GB1 and GC2.

The maximum production rate of spores ($\mu_{m\ SPORES}$) also differed among isolates, 67–436 spores/wk (TABLE II). GB2 and GX7 isolates had the lowest rates of spore production but did not statistically differ from the GC2, GA10, GA3, GX10 and GC4 isolate. In contrast, GX3 produced more spores than any other isolate. The remaining isolates had intermediate values of $\mu_{m\ SPORES}$, 147–184 spores/wk.

The AM fungal isolates produced different amounts of ERM and spores when they reached the stationary phase (FIG. 2). The maximum production of ERM (A_{ERM}) differed among isolates and ranged from an average of 821 cm to 5777 cm (TABLE II). The isolates that produced the highest values of A_{ERM} were GC2, GC3, GA5 and GA2. The extraradical mycelial network of these isolate was composed mainly of numerous runner hyphae (RH) and hyphal branches (HB). Some differences in the pattern of hyphal ramifications were observed among isolates. GC3 produced the highest amount of branched absorbing structures (BAS) (Bago et al. 1998b), while GC2 developed a large number of short HB, but few of the highly ramifying BAS. GA5 and GA2 formed

more BAS than short HB at 20 wk. GA10 produced the lowest amount of ERM with few RH and HB, although the A_{ERM} value was not statistically different from GA3, GC4, GC1, GX10 and GA11 isolates. The remaining isolates (GB1, GX3, GB2, GX7) reached intermediate A_{ERM} mean values, but GX3 and GX7 developed more numerous RH than GB1 and GB2.

There were significant differences in the maximum production of spores (A_{SPORES}) among isolates at the stationary phase (TABLE II). The A_{SPORES} parameter varied, 273–1848 spores produced per dish. GA3, GC4 and GB2 produced the least number of total spores and GC3 and GX3 produced the greatest number of spores. The remaining isolates had intermediate values of A_{SPORES}.

The frequency (%F) and intensity (%I) of root colonization differed among isolates in monoxenic culture (TABLE II). Roots colonized by GA10 isolate had the lowest %F, while GX10 had the highest. Other isolates had intermediate frequencies of colonization. The %I of intraradical colonization at harvest was lower in GA3 than all other isolates.

Results from the discriminant analysis showed that some isolates could be distinguished from others, although many isolates overlapped (FIG. 3). In the canonical axis 1, the A_{ERM} contributed the most in separating the isolates, while all growth parameters of sporulation curves (λ_{SPORES}, $\mu_{m\ SPORES}$ and A_{SPORES}) had similar weight contributing to the canonical axis 2 (data not shown). GC2, GC3, GB2 and GA3 isolates were clearly separated from the other isolates, whereas there was less clear separation among the remaining isolates. GA2, GA5 and GX7 clustered together, while other clustering was found for GA10, GA11, GB1, GC1, GX10, GX3 and GC4 (FIG. 6). However within the last group, GC4 isolate was separated along the second axis.

The differences between some isolates and the clustering among other isolates within the ordination space did not correspond well to either the geographic locations where the fungi were isolated or to the phylogenetic relationship of the isolates. For example, GC2 was closely related to GA10 based on phylogenetic analysis (FIG. 1), but these two isolates were on opposite extremes of axis 1 based on ERM growth characters (FIG. 3).

DISCUSSION

In the present study we describe new insights into the growth dynamics of geographically different AM fungal isolates belonging to the "*Rhizophagus* clade". A similar approach to model sporulation dynamics using the modified Gompertz function was applied in three *Glomeraceae* species grown under monoxenic
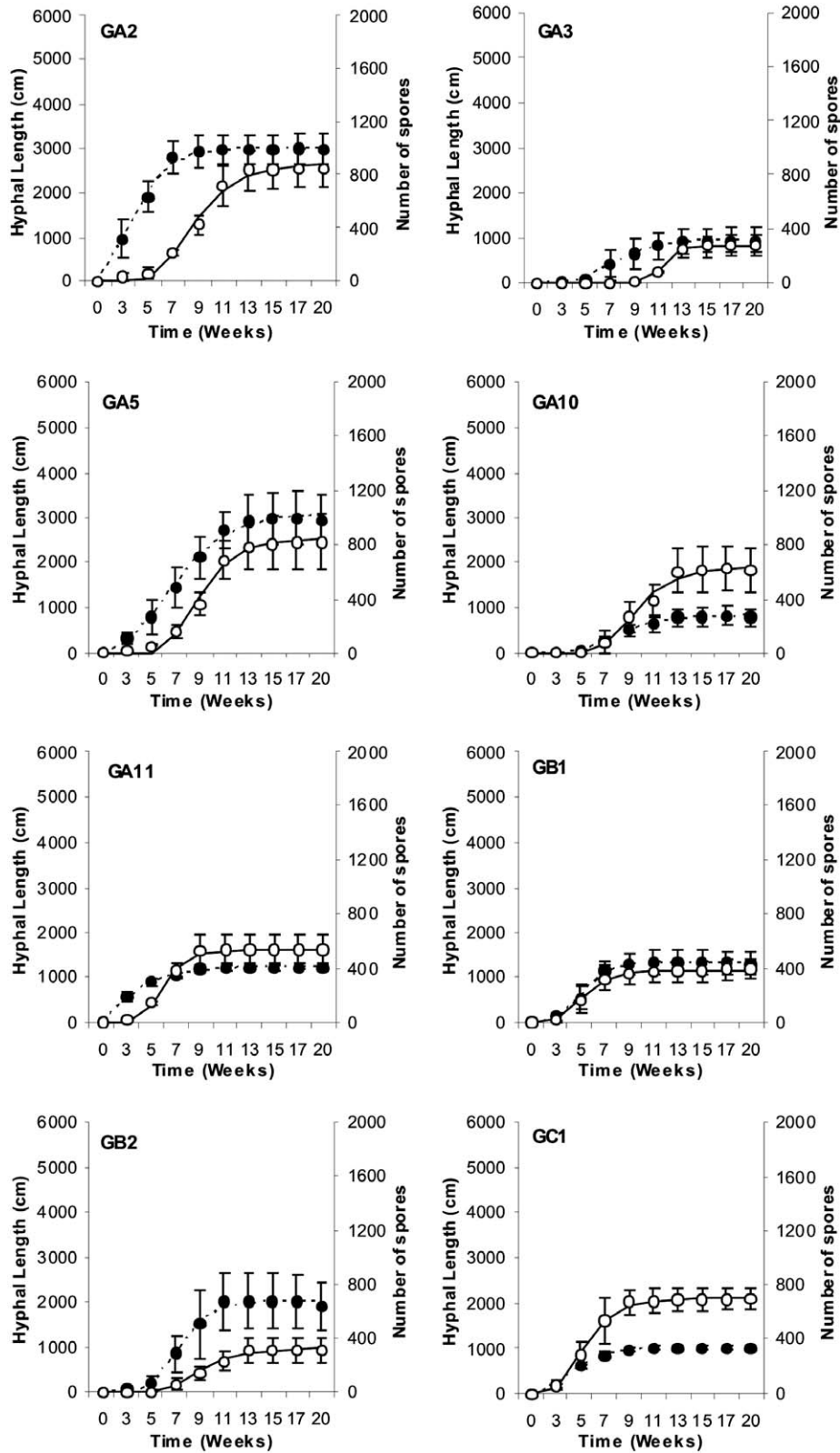
FIG. 2. Production of extraradical mycelium and spores for each isolate associated with transformed carrot roots grown in minimal medium. Each curve represented the average values of extraradical hyphal length (cm) (filled circles) and the number of spores (empty circles) in time (n = 5). Both curves were fitted with the Gompertz model (solid line for mycelium curve or dotted line for sporulation curve).
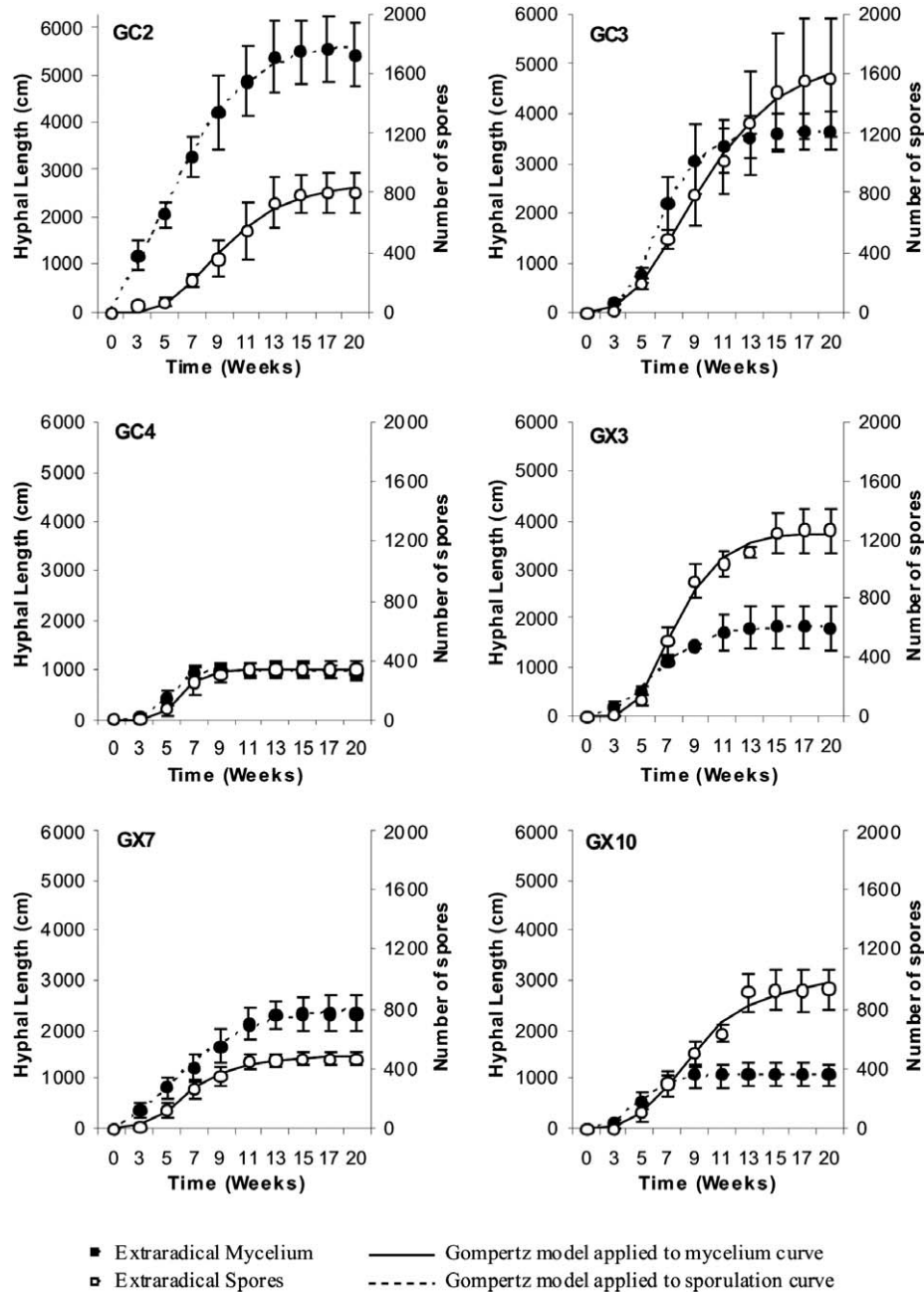
- ■ Extraradical Mycelium —— Gompertz model applied to mycelium curve
- □ Extraradical Spores ----- Gompertz model applied to sporulation curve

FIG. 2. Continued.

conditions (Declerck et al. 2001) and a *Gigasporaceae* species (Declerck et al. 2004). Our work expanded this analysis to include 14 AM fungal isolates within a single clade, demonstrating its utility for modeling mycelial and spore growth of *Rhizophagus* isolates. In agreement with studies based on monoxenic cultures of *R. irregularis* (MUCL 41833 and MUCL 43194/ DAOM 197198 isolates) (Bago et al. 1998a, Declerck et al. 2001), all *Rhizophagus* isolates followed a sigmoid growth curve with the three distinguishable phases (lag, exponential, stationary).

Little is known about the extent of the lag phase that occurs during the early stages of the AM fungal life cycle. Our results show that the *Rhizophagus* isolates varied in lag phase periods in mycelia growth and sporulation. These data suggest that variations in the lag phase are related to different propagule germination rates, presymbiotic mycelial extension and formation of primary infection units in roots of each *Rhizophagus* isolate. It has been documented that Diversisporales species initiate in vitro sporulation after reaching a critical ERM biomass (Diop et al. 1992,

TABLE II. Parameters values (means ± SEM) of lag phase (λ), maximum growth rate (μ_m) and maximum total growth (A) for extraradical mycelium (ERM) and spore production in 14 'Rhizophagus' isolates derived from the Gompertz model, and frequency (F %) and intensity (I %) of transformed carrot root colonization by each isolate after 20 wk

Isolate	λ_{ERM} (wk)	λ_{spores} (wk)	$\mu_{m\ ERM}$ (cm wk^{-1})	$\mu_{m\ spores}$ (spores wk^{-1})	A_{ERM} (cm)	A_{spores} (N° spores)	F (%)	I (%)
GA2	1.7 ± 0.2 b	5.5 ± 0.2 b,c	741.8 ± 2.8 e	147.2 ± 1.4 b	2994.8 ± 3.8 e	880.1 ± 2.4 f,g	39.8 ± 0.6 b,c	52.4 ± 5.9 a
GA3	4.9 ± 0.1 e	10.3 ± 0.1 d	192.4 ± 2.2 a,b	112.6 ± 1.3 a,b	958.9 ± 3.1 a,b	273.3 ± 1.7 a	38.8 ± 0.5 b,c	28.0 ± 1.2 b
GA5	3.3 ± 0.2 c,d	6.4 ± 0.2 b,c,d	409.1 ± 1.1 c,d	165.6 ± 1.4 b	3075.4 ± 5.0 e	836.0 ± 2.9 f,g	45.5 ± 3.6 d,e	45.5 ± 0.6 a
GA10	5.2 ± 0.1 e	6.6 ± 0.2 b,c,d	143.3 ± 1.3 a	103.5 ± 0.9 a,b	820.9 ± 2.9 a	636.9 ± 2.7 d,e,f	27.4 ± 0.5 a	52.5 ± 1.3 a
GA11	0.7 ± 0.1 a	4.2 ± 0.1 a,b	243.0 ± 1.5 a,b,c	183.9 ± 1.9 b	1221.7 ± 1.7 a,b	541.6 ± 2.2 c,d,e	50.0 ± 2.3 e	66.4 ± 2.8 a
GX3	4.6 ± 0.2 e	7.9 ± 0.4 c,d	544.5 ± 2.7 d	436.0 ± 2.8 c	1815.9 ± 4.2 c	1267.6 ± 2.5 h,i	45.2 ± 1.4 d,e	60.3 ± 2.0 a
GX7	2.1 ± 0.2 b,c	3.7 ± 0.2 a	275.5 ± 1.4 a,b,c	85.1 ± 0.7 a,b	2415.9 ± 3.7 d	477.2 ± 1.4 b,c,d	42.2 ± 1.0 c,d	59.8 ± 4.6 a
GX10	3.0 ± 0.1 c,d	4.6 ± 0.2 a,b	285.2 ± 1.2 a,b,c	118.3 ± 0.7 a,b	1096.5 ± 3.0 a,b	985.2 ± 2.6 g,h	61.8 ± 1.2 f	57.1 ± 1.6 a
GB1	3.6 ± 0.2 d	3.5 ± 0.2 a	618.5 ± 4.1 d,e	166.0 ± 2.0 b	1334.0 ± 3.3 b	386.6 ± 1.8 b,c	45.3.6 ± 0.6 d,e	66.7 ± 1.0 a
GB2	5.0 ± 0.2 e	6.5 ± 0.2 b,c,d	448.9 ± 2.9 c,d	66.6 ± 1.1 a	2026.4 ± 4.9 c,d	315.6 ± 1.9 a,b	40.8 ± 0.5 b,c,d	53.1 ± 6.0 a
GC1	2.1 ± 0.1 b,c	3.3 ± 0.1 a	215.9 ± 1.3 a,b	171.1 ± 1.2 b	1013.4 ± 1.6 a,b	699.4 ± 1.8 e,f,g	36.6 ± 0.4 b	52.4 ± 4.6 a
GC2	1.4 ± 0.2 a,b	4.9 ± 0.2 a,b	639.7 ± 2.8 d,e	106.7 ± 1.5 a,b	5776.6 ± 5.2 g	918.0 ± 3.0 g,h	39.8 ± 0.7 b,c	58.4 ± 2.6 a
GC3	3.9 ± 0.1 d,e	4.1 ± 0.2 a,b	784.6 ± 3.5 e	162.5 ± 1.3 b	3662.1 ± 3.7 f	1848.2 ± 5.0 i	48.2 ± 1.0 e	54.3 ± 4.5 a
GC4	2.5 ± 0.2 b,c	5.2 ± 0.2 b,c	438.2 ± 2.8 c,d	130.0 ± 1.5 a,b	969.6 ± 2.3 a,b	340.8 ± 1.5 a,b	36.4.8 ± 2.1 b	49.3 ± 2.5 a

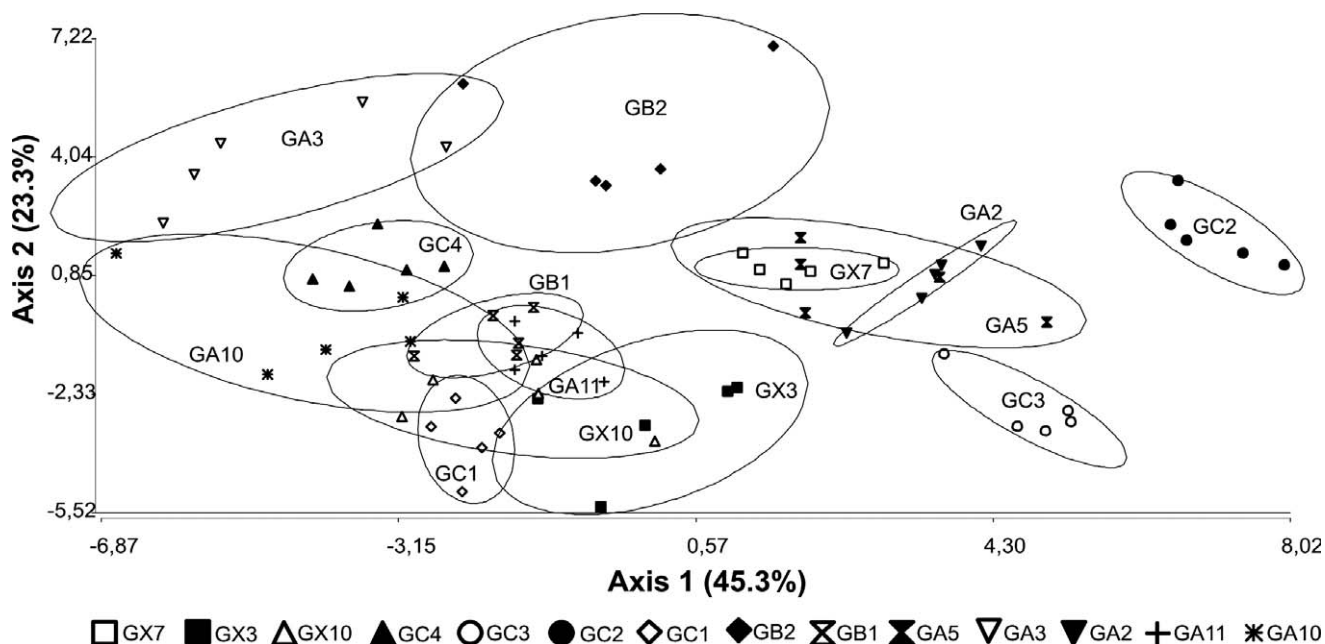Values followed by the same letter within the same column are not significantly different ($P < 0.05$).

FIG. 3. Discriminant analysis biplot showing patterns of distribution of AM fungal isolates based on growth parameters (λ, μ_m, A) from the Gompertz model of extraradical mycelium and spore production in monoxenic culture.

Declerck et al. 2004, Fernández Bidondo et al. 2012). However, this was not observed for all isolates tested here. For example, GX3 and GC3 did not require long periods or a maximal biomass of ERM to initiate sporulation but produced spores and developed ERM simultaneously. This developmental trait suggests an adaptive value for these AM fungal isolates to grow and rapidly reproduce in a particular set of conditions.

Studies have shown variations in ERM length and spore number between and within AM fungal species under different experimental protocols (Hart and Reader 2002, 2005; Koch et al. 2004; Munkvold et al. 2004; Avio et al. 2006). We documented that AM fungal isolates belonging to ''*Rhizophagus* clade'' also differed in their maximal growth rate and amount of extraradical structures reached at the stationary phase under monoxenic conditions. These phenotypic variations among phylogenetically closely related isolates could reflect several life-history traits and might have a significant impact on host root and soil colonization. The ability of certain *Rhizophagus* isolates to rapidly produce an extensive hyphal network into the growth substrate might provide a competitive advantage over other isolates with limited mycelial growth by increasing the absorptive area for nutrient acquisition, colonizing new roots and trans-locating nutrients to host plants. The capacity of certain AM fungal isolates to produce abundant extraradical propagules likely leads to an increase in inoculum potential and colonization of new host roots. In Declerck et al. (1996) spore production of

Glomus versiforme in monoxenic culture was strongly correlated with the internal colonization of trans-formed carrot roots. In the present study we found that differences in the rapidity and total production of ERM and spores were not related to the extent of carrot root colonization by the different *Rhizophagus* isolates (correlation coefficients below 0.2, data not shown).

Despite high overall diversity in the growth patterns among the isolates examined here, it was possible to distinguish certain phenotypic groups. These groups could be differentiated as either fast or slow coloniz-ers or those with an intermediate pattern of growth. A high growth rate and ability to produce a large amount of ERM (e.g. GC2), a large quantity of spores (e.g. GX3) or both (e.g. GC3) could be discerned from those isolates characterized by a longer lag phase, a slow growth and a limited production of ERM and spores (e.g. GA3, GB2 or GA10). Other isolates such as GA11, GC1 and GX10 showed intermediate patterns between these extremes.

Comparing the phenotypes of isolates based on growth patterns to the phylogenetic analysis pro-duced mixed results. In one case, the phylogenetic clade that clustered with the *R. irregularis* AFTOL-ID 845 isolate that included GA2, GA5, GX3, GX7 and GC3 isolates matched the phenotypic classification based on discriminant analysis of growth parameters. However, other phylogenetically closely related iso-lates (GC2, GA10) displayed the most divergent pattern of ERM development observed here. Our

findings are consistent with Munkvold et al. (2004) showing wide variation in mycorrhizal effectiveness to deliver phosphorus to a common host plant among AM fungal isolates within a species. The phenotypic diversity of closely related AM fungi cannot be adequately represented by examining phylogenetic diversity based on ribosomal genes. Further work should be aimed at elucidating taxonomic relationships among such phylogenetically related isolates by adding another DNA regions and phenotypic traits.

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