Applied Soil Ecology 115 (2017) 1–9

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Tracing native and inoculated *Rhizophagus irregularis* in three potato cultivars (Charlotte, Nicola and Bintje) grown under field conditions

Catherine Buysens[a], Pierre-Louis Alaux[a], Vincent César[b], Stéphanie Huret[c], Stéphane Declerck[a,*], Sylvie Cranenbrouck[c]

[a] Université Catholique de Louvain, Earth and Life Institute, Applied Microbiology, Mycology, Croix du Sud 2, Box L7.05.06, 1348 Louvain-la-Neuve, Belgium
[b] Walloon Agricultural Research Centre, Life Sciences Department, Breeding and Biodiversity Unit, Rue du Serpont 100, B-6800 Libramont, Belgium
[c] Université catholique de Louvain, Earth and Life Institute, Applied Microbiology, Mycology, Mycothèque de l'Université catholique de Louvain (MUCL[1]), Croix du Sud 2, Box L7.05.06, 1348 Louvain-la-Neuve, Belgium

ARTICLE INFO

Article history:
Received 22 December 2016
Received in revised form 16 February 2017
Accepted 2 March 2017
Available online 27 March 2017

Keywords:
Arbuscular mycorrhizal fungi
Rhizophagus irregularis
Real-time quantitative PCR
Mitochondrial LSU
Traceability

ABSTRACT

Crop inoculation with arbuscular mycorrhizal fungi (AMF) is a promising option to increase plant yield. However, in most cases, the inoculated strains could not be traced in the field and their contribution to root colonization separated from native AMF. Therefore, there is no clear indication that growth promotion is strictly related to the inoculated isolates. Here, *Rhizophagus irregularis* MUCL 41833 was inoculated on three potato cultivars (Bintje, Nicola, Charlotte) under field conditions in Belgium. Inoculum was encapsulated into alginate beads and mycorrhizal infective potential (MIP) estimated with a dose-response relationship under greenhouse conditions before field experiment. Mitochondrial Large SubUnit (mtLSU) of inoculated *R. irregularis* MUCL 41833 was characterized to design haplotype- (inoculated *R. irregularis* haplotype) and species-specific (native or inoculated *R. irregularis)* markers. The magnitude of detection of the markers, determined by Real-Time quantitative PCR, was linked to the stage of colonization of potato cv. Bintje grown under greenhouse conditions. Under field conditions, the inoculant *R. irregularis* MUCL 41833 was detected at a very low level (between 10^{-5} and 10^{-7} ng/ ng total DNA) in a marginal number of plants, in contrast to native *R. irregularis* strains that were detected at higher levels (between 10^{-4} and 10^{-6} ng/ ng total DNA) in all plants of the three cultivars. This suggested that the inoculated strain was almost absent in the plants due either to the environmental conditions, competition with indigenous AMF or inadequate placement of inoculum. This was corroborated by the absence of growth promotion and differences in root colonization between inoculated versus non-inoculated potato plants. This study validated the mtLSU markers to detect/trace and quantify AMF inoculants as native strains in plants grown under field conditions and further supported that potato cultivars in the same field conditions differed in root colonization.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil micro-organisms that form symbiotic associations with the majority of land plants (Smith and Read, 2008), including most agricultural crops (*e.g.* Douds et al., 2007). Their major benefits to plants include increased acquisition and accumulation of nutrients (*e.g.* P, N) (Smith and Read, 2008) and improved tolerance/resistance to biotic and abiotic stresses (Gianinazzi et al., 2010).

Despite their clear economic and ecological significance, the number of studies that indubitably report their potential benefits to plants in the field remains scarce (24%) as compared to the numerous results obtained in greenhouse (65%) or growth chamber (4%) conditions (Berruti et al., 2016). It is obvious that under field conditions, inoculated AMF have to compete with native AMF communities (Verbruggen et al., 2013) and may be influenced by numerous agricultural as well as environmental factors (see Gosling et al., 2006) impacting their capacity to improve plant yield and health. Meta-analysis of Berruti et al. (2016) showed that fungal colonization gain was more frequent in the greenhouse as compared to open-field conditions because field soil contains AMF propagules that could colonize the non-inoculated controls, contrarily to the

* Corresponding author.
E-mail address: stephan.declerck@uclouvain.be (S. Declerck).
[1] Part of the Belgian Coordinated Collection of Micro-organisms (BCCM).

http://dx.doi.org/10.1016/j.apsoil.2017.03.007
0929-1393/

controls in pot experiments usually filled with sterilized substrates free of AMF.

Rhizophagus irregularis is a worldwide distributed AMF, commonly used for commercial applications owing to its easy mass-production (Ijdo et al., 2010) and life history strategy adapted to agricultural soils (Chagnon et al., 2013). A recent analysis of 231 field experiments in North America and Europe where potatoes were inoculated with *R. irregularis* DAOM 197198 (syn. MUCL 43194), using a liquid suspension of AMF spores sprayed onto potato seeds (71 spores/seed piece), showed a significant increase in the production of marketable potato tubers (Hijri, 2016). An increase in potato yield was also observed in a potato culture succeeding to a cover crop of *Medicago sativa* inoculated with *R. irregularis* MUCL 41833 and *Trichoderma harzianum* MUCL 29707 encapsulated into alginate beads (20 AMF propagules and 5 conidia/bead with ±30 beads/potato seed) (Buysens et al., 2016). However, even if it was supposed that growth promotion was related to the inoculated isolates, it could not be ruled out that other AMF belonging to the same species or different species could be involved. Indeed, *R. irregularis* is a ubiquitous AMF species and crops naturally become colonized by native AMF species making it difficult to distinguish the effects of applied inoculum as opposed to indigenous AMF species, in particular if the local communities harbor similar species to the inoculated ones.

Microscopically, it is difficult to distinguish different AMF species or different AMF isolates of the same species based on fungal structures within roots. Molecular tools have been developed to differentiate AMF at family and species level (*e.g.* Gollotte et al., 2004; Helgason et al., 1999; Krüger et al., 2009; Redecker, 2000). Four nuclear (nr) ribosomal (r) DNA regions, individually or in combination, are used as molecular markers: the partial small subunit rRNA gene (SSU), the partial large subunit rRNA gene (LSU), 5.8S rRNA gene (5.8S) and the Internal Transcribed Spacers (ITS). Kohout et al. (2014) compared four routinely-used AMF-specific primers covering (i) the partial small subunit (SSU), (ii) the partial large subunit (LSU), (iii) the partial SSU, ITS and 5.8S and (iv) the partial SSU-ITS-5.8S-partial LSU region (Krüger et al., 2009). These last primers used by Krüger et al. (2009) seemed to yield higher AMF diversity than the SSU primers. Recently Schläppi et al. (2016) took advantage of these AMF-specific PCR primers and sequenced the resulting amplicons with single molecule real-time (SMRT) sequencing in order to detect all major AMF families and discriminate closely related AMF species. With this method they could trace an introduced AMF inoculum and study the impact on the native community. Intraspecific markers were also developed to detect different isolates of *R. irregularis* by amplifying the mitochondrial Large SubUnit (mtLSU) (Croll et al., 2008; Koch et al., 2004; Raab et al., 2005). This allowed the discrimination of different haplotypes of this specific fungus in the field (Börstler et al., 2008, 2010). The problems of apparent polymorphism of nr rDNA and protein-coding genes within single spores could be circumvented by using this independent genetic system within the fungal organism. AMF mitochondrial DNA (mtDNA) is homogeneous within single isolates (Raab et al., 2005), making it a good target for marker development. The exon phylogeny of a region of the mtLSU showed superior resolution among subclades of *R. irregularis* compared to nuclear-encoded rDNA ITS (Börstler et al., 2008). Particularly, the mtLSU introns were shown to be highly sensitive molecular markers to genotype different isolates of *R. irregularis* (sensu lato) and it was used to differentiate mtLSU haplotypes directly from colonized roots (Börstler et al., 2008) which is a promising approach to better understand the diversity and dynamics of field communities and populations. Börstler et al. (2008) revealed with a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach and sequencing that the diversity of mtLSU haplotypes of *R. irregularis* was very high in field populations as well as in isolates collected worldwide. Real-time quantitative (q) PCR in the large subunit of mtDNA was recently used by Krak et al. (2012) to study the dynamics of two coexisting isolates of *R. irregularis* under greenhouse conditions. To our knowledge this technique was not yet applied on field samples in order to distinguish inoculated from native *R. irregularis* isolates and for their absolute quantification.

Potato is the most important tuber crop worldwide (FAO, 2013). This crop is characterized by heavy mechanization and huge applications of fertilizers and pesticides. The effect of a direct inoculation of AMF on potato was investigated by Douds et al. (2007), Bayrami et al. (2012) and Hijri (2016) under different fertilization treatments. These authors noticed that inoculation with *R. intraradices, R. irregularis* or *Funneliformis mosseae* significantly increased potato yield and root colonization under low fertilization treatments. However, none of these studies could firmly demonstrate that the increased yield was strictly related to the inoculated isolate. Indeed, these authors could not separate the contribution of native from inoculated AMF to root colonization.

The aims of the present study were (1) to characterize the mtLSU of the AMF isolate *R. irregularis* MUCL 41833 used as inoculant in our field experiment; (2) to validate the markers on inoculated potato plants under greenhouse conditions (3) to evaluate field inoculation success at potato planting by quantifying introduced *R. irregularis* MUCL 41833 isolate as opposed to the native *R. irregularis*; and (4) to compare AMF root colonization abundance by microscopic and molecular techniques between different potato cultivars.

2. Materials and methods

2.1. Biological material

Solanum tuberosum L. cv. Bintje, cv. Nicola and cv. Charlotte (Euroseeds, Belgium) were used in this study. These cultivars are amongst the most widely cultivated in Belgium and Europe (www.fiwap.be). After storage at low temperature (3–4 °C), the potato tubers were placed three weeks at 10–15 °C before planting.

Medicago truncatula Gaertn. cv. Jemalong A 17 (Sardi, Australia) seeds were used in the MIP test.

The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker *in vitro* collection (BCCM/MUCL/GINCO – http://www.mycorrhiza.be/ginco-bel) was cultured *in vitro* as detailed in Cranenbrouck et al. (2005) and subsequently mass-produced during 6 months on Zea mays L. cv. ES. Ballade (Euralis, France) grown under temperate greenhouse conditions in pots containing pulverized lava (DCM, Belgium) supplemented with Osmocote®.

Six other *R. irregularis* strains (MUCL 46241; MUCL 46239; MUCL 43194; MUCL 43204; MUCL 46240; MUCL 43195) and four closely related species to *R. irregularis* (*Rhizophagus diaphanus* MUCL 43196; *Rhizophagus intraradices* MUCL 49410; *Rhizophagus clarus* MUCL 46238; *Rhizophagus fasciculatus* MUCL 46100) were supplied by the Glomeromycota *in vitro* collection for the tests of specificity.

2.2. Entrapment of AMF and mycorrhizal infective potential

R. irregularis was entrapped in alginate beads following the method described in De Jaeger et al. (2011) slightly modified with a filler (see Supplementary material Fig. 1) to maintain the shape of the beads after drying. Each gelled bead had a volume of 34 μl and contained an approximate 20 AMF propagules (*i.e.* isolated spores or root fragments containing spores/vesicles). Beads were air-dried to 35% of their initial weight.

Table 1
Parameters of the Real-Time PCR used for the quantification of mtLSU of *R. irregularis* species-specific or MUCL 41833 haplotype-specific isolates.

Target region, isolate	Primer names (forward/reverse)	Primer sequences (5′ ≥ 3′)	Primer conc. (μM)	Annealing temp. (°C)	Amplicon size (bp)	Amplification efficiency† (SD)
mtLSU, *R. irregularis*	BF7/BR5	TGGTCTAAACATGGTTGAAAAAT/ ACGCTATCGCTAAAAGGTGG	0.2/0.2	62	67	96.2 (5.3)
mtLSU, *R. irregularis* MUCL 41833 haplotype	BF8/BR6	AAGTCCTCTAGGTCGTAGCA/ ACAGGTATTTATCAAATCCTTCCC	0.2/0.2	62	121	93.2 (4.5)

† Amplification efficiency was calculated from ten independent 5-fold dilutions of DNA template.

The mycorrhizal infective potential (MIP) of the beads was evaluated using the method described in Declerck et al. (1996) slightly modified. The method was based on a dose-response relationship and involved the cultivation of a population of mycotrophic plantlets on a range of inoculum density under controlled conditions. Briefly, beads (1, 3, 10, 30 and 100– logarithmic scale), were thoroughly mixed in 200 cm^3 pots containing autoclaved Terragreen® (a calcinated attapulgite clay soil conditioner; Oil-Dri, Cambridgeshire, UK; see Hodge, 2001). Seeds of *M. truncatula* (10 per pot) were sown into the pots and grown for 20 days in a growth chamber set at 22/18 °C (day/night) with 70% relative humidity, a 16 h photoperiod and a photosynthetic photon flux (PPF) of 300 $\mu mol\ m^{-2}\ s^{-1}$. The plantlets were then harvested and roots cleared (10% KOH at 50 °C for 90 min) and stained (5% blue ink (Parker®, USA) diluted in vinegar (7% acidity) at 50 °C for 60 min) (Walker, 2005). The presence/absence of AMF was evaluated in each plantlet under stereo-microscope (Olympus SZ40, Olympus Optical GmbH, Germany) at 1–40x magnification. Three replicates were set up. Data were expressed as the percentage of mycorrhizal plants (% MP) per pot. The % MP was plotted against the logarithm of bead number. Regression curves were used to determine the number of beads needed to colonize 90% of the plants (*i.e.* ID_{90}). This number was 13 (see Supplementary material Fig. 2).

2.3. Molecular characterization of R. irregularis MUCL 41833

Fungal DNA was extracted from a mix of ±1000 spores and mycelium of *R. irregularis* MUCL 41833 produced *in vitro* using innuPREP Plant DNA kit (Analytikjena, Germany) and following manufacturer recommendations with slight modifications (the homogenization step was adapted to fungal material as explained below). Spores/mycelium of 2-month-old cultures were extracted from the Modified Strullu- Romand (MSR) medium (Declerck et al., 1998) following solubilization of the gelling agent by citrate-buffer (Doner and Bécard, 1991). They were further isolated with a micropipette under a dissecting microscope, placed in a 1.5 ml plastic tube (Eppendorf, Germany), repeatedly centrifuged to eliminate water and air-dried. The tip of the tube was put in liquid nitrogen and spores/mycelium were crushed with a plastic pestle (Eppendorf, Germany) before lysis with the buffer. Extracted DNA was then subjected to PCR amplification of the mitochondrial large ribosomal subunit (mtLSU) RNA gene. The region was amplified by nested PCR using the primers RNL28a (Börstler et al., 2008) and RNL5 (Raab et al., 2005) in the first step and then RNL29 (Börstler et al., 2008) and GImt4510R (Krak et al., 2012) in the second step (see Supplementary material Table 1). The PCRs were conducted using a total volume of 20 μl and contained 0.2 mM of each dNTP (Applied Biosystems, USA), 0.5 U of AmpliTaq® DNA polymerase (Applied Biosystems, USA), 1 × Taq buffer with KCL (Applied Biosystems, USA), 1 ng of genomic DNA and reaction-specific concentrations of primers and $MgCl_2$ (see Table 1). An initial denaturation step at 94 °C (4 min) was followed by 35 cycles of denaturation (94 °C, 30 s), annealing (59 °C in the first step and 53 °C in the second step, 30 s), and extension (72 °C, 1.5 min), with a final extension step at 72 °C (10 min). The resulting PCR products were sequenced using the original PCR and additional internal primers (see Supplementary material Table 1). One sequence type was obtained (accession number KY347874).

2.4. Primer design

Two primer pairs targeted to the mtLSU region (Table 1) were designed. One primer pair was designed to amplify mtLSU of all *R. irregularis* strains and another to amplify mtLSU of the specific target strain. Specific primers were designed by aligning the sequence obtained together with mtLSU sequences of *R. irregularis sensu lato* as well as other species that are representative of all published haplotypes (www.ncbi.nlm.nih.gov/) using Mega6 software (http://www.megasoftware.net/). After a first specificity assessment by checking *in silico* the absence of significant similarity of our primers with other known DNA sequences using BLAST in the NCBI GenBank public database, tests in qPCR were performed using genomic (g) DNA of various AMF taxa from the Glomeromycota *in vitro* collection or root samples (colonized or not by AMF) from *in vitro* or *in vivo* collection as templates (see Table 2). All primers were tested for dimer formation using AmplifX 1.7.0 (by Nicolas Jullien; CNRS, Aix-Marseille Université – http://crn2m.univ-mrs.fr/pub/amplifx-dist).

2.5. Optimization of qPCR assay

To prepare standards for the qPCR experiments, DNA extract from *R. irregularis* MUCL 41833 spores and mycelium grown *in vitro* was used. The concentration of DNA was measured with Quant-iT™ PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA) and diluted to 100 pg gDNA.

The qPCR using LightCycler® Fast Start DNA Master SYBR Green I kit (Roche, Switzerland) was performed in 10 μl reaction mixtures on the LightCycler® 96 Real Time PCR instrument (Roche, Switzerland). The following cycling conditions were used: 10 min at 95 °C, followed by 45 cycles of denaturation (95 °C, 10 s), annealing (62 °C, 10 s), and extension (72 °C, 10 s). The cycling was finalized by a standard melting curve analysis.

The efficiencies of the qPCR assays were estimated from standard calibration curves based on serial 5-fold dilutions of genomic DNA standards (10^{-1}–10^{-5} ng μl^{-1}). The absolute quantification of the target sequences was performed based on the standard calibration curves using the LightCycler® 96 software, version SW1.1 (Roche, Switzerland). The resulting concentrations were expressed as ng of *R. irregularis* DNA or ng of *R. irregularis* MUCL 41833 haplotype DNA / ng total DNA.

2.6. Greenhouse validation of molecular markers to trace and quantify R. irregularis MUCL 41833 haplotype or R. irregularis species in potato roots

A greenhouse experiment was set up to validate molecular markers and to quantify the mtLSU genes at different stages of potato root colonization by *R. irregularis* MUCL 41833. Potato tubers of the cultivar Bintje were inoculated with *R. irregularis* MUCL

Table 2
Arbuscular mycorrhizal fungal strains and root samples (colonized or not by AMF) included in the test of specificity of primers.

AMF isolates/root samples					mitochondrial genome sequence	qPCR	
Species		Isolates		Origin			
Recent classification	Old classification	GINCO strain code	Other codes		accession number	BF7/BR5	BF8/BR6
Rhizophagus irregularis	*Glomus intraradices*	MUCL 41833 (spores/mycelium *in vitro*)	/	Spain, Canary Islands	KY347874	+	+
R. irregularis	*G. intraradices*	MUCL 41833 (spores of *in vivo* culture)	/	Spain, Canary Islands	KY347874	+	+
R. irregularis	*G. intraradices*	MUCL 41833 (roots of maize colonised by strain *in vivo*)	/	Spain, Canary Islands	KY347874	+	+
R. irregularis	*G. intraradices*	MUCL 41833 (roots of potato colonised by strain *in vivo*)	/	Spain, Canary Islands	KY347874	+	+
R. irregularis	*Glomus irregulare*	MUCL 46241	DAOM 234180/4348YD	Canada, Quebec	JQ514225 (Formey et al., 2012) GQ204983 (Sokolski et al., 2010)	+	–
R. irregularis	*G. irregulare*	MUCL 46239	DAOM 234181/4375YD	Canada, Quebec	JQ514223.2 (Formey et al., 2012) GQ204981.1 (Sokolski et al., 2010)	+	–
R. irregularis	*G. irregulare*	MUCL 43194	DAOM 197198/DAOM 181602	Canada, Quebec	KF848212.1 (Borriello et al., 2014) KI297408.1 KI274799.1 KI296156.1 ...	+	–
R. irregularis	*G. irregulare*	MUCL 46240	DAOM 234179/4341YD	Canada, Quebec	KC164354.1 (Beaudet et al., 2013)	+	–
R. irregularis	*G. irregulare*	MUCL 43195	DAOM 212349/3938 (YD)	Canada, Ontario	/	+	–
R. irregularis	*G. irregulare*	MUCL 43204	DAOM 229457/CC-4	Canada, Ontario	JQ514224 (Formey et al., 2012) GQ204984.1 (Sokolski et al., 2010) AM950206.1 AM950205.1 AM950204.1 (Börstler et al., 2008)	+	+
Rhizophagus diaphanus	*Glomus* sp.	MUCL 43196	DAOM 229456/SD1 (SD)	Unknown	JX065416.1 (Beaudet et al., 2013) GQ204985.1 HE583398.1	+	+
Rhizophagus clarus	*Glomus clarum*	MUCL 46238	INCAM 12	Cuba, Pinar del Rio	/	–	–
Rhizophagus intraradices	*Glomus intraradices*	MUCL 49410	/	USA, Florida	/	–	–
Rhizophagus fasciculatus	*Glomus fasciculatum*	MUCL 46100	/	Unknown	/	+	–
potato roots not colonized by AMF from *in vitro* and *in vivo* culture	/	/	/		/	–	–

41833 *via* an *in vivo* mycelium donor plant (MDP) system for fast colonization of potato plantlets (adapted from Voets et al., 2009) under greenhouse conditions. Briefly, maize cv. ES Ballade plantlets were inoculated with a suspension of spores from an *in vitro* culture of *R. irregularis* MUCL 41833. The plants were cultured for two months in pots of 8 dm^3 filled with sand and vermiculite (1 v/1 v) to set up a profuse and active extraradical mycelium network. Seedlings of *M. truncatula* were then placed in the active network, for 5 weeks to produce extensively colonized small-sized plantlets that can be easily used as mycelium donors. A pre-germinated tuber and 3 donors *M. truncatula* plants were placed in biodegradable pots (6 cm, Jiffy®, Netherland) filled with autoclaved sand/vermiculite (1V/1V) (see Supplementary material Fig. 3). Roots of potato were harvested 2–5 weeks after planting in order to have plants colonized at different colonization stages. They were washed with deionized water, dried at room temperature under laminar flow and stored at −20 °C before DNA extraction and root staining.

2.7. Field experimental setup

The field trial was conducted in Libramont (Belgium) at the Walloon Agricultural Research Centre, Life Sciences Department, Breeding and Biodiversity Unit (49°55′N, 5°22′E). The trial was conducted from May 2014 to September 2014. The climate of the region is temperate with an annual mean precipitation of 1191 mm $year^{-1}$ and an annual mean temperature of 8.3 °C (Pameseb asbl. 2014). The soil was a Dystic Cambisol (IUSS

Working Group WRB, 2014). The soil had a pH of 6.3 and 5.3 in H_2O and KCl, respectively. Soil organic matter and C content were 48 g kg^{-1} and 28 g kg^{-1}, respectively.

The soil was covered with a mown grassland during three consecutive years before trial. Individual subplots were arranged in a randomized complete block design replicated four times (Supplementary material Fig. 4). The trial was conducted under conventional agricultural system but no fertilizers were added. The timeline of the trial and the conventional agricultural practices and treatments applied are presented in Supplementary material Table 2.

The trial was conducted with three different potato cultivars (*i.e.* Bintje, Nicola and Charlotte). Two inoculation treatments were applied (*i.e.* beads containing *R. irregularis* MUCL 41833 (referred as Ri41833)) and beads without AMF (*i.e.* the control). Each potato tuber was inoculated with 120 beads. Inoculation was done on the 19th of May 2014 in the planting hole close to the tuber. Each individual subplot had a surface of ±21 m^2 (3 × 7 m) with 60 plants and was separated from a neighboring subplot by a distance of 2 m. At the end of the experiment the subplots were chemically and mechanically defoliated before tuber harvest on the 24th of September 2014.

The roots of sixteen randomly-selected plants (*i.e.* four potato plants per plot over the 4 blocks) of each potato cultivar (*i.e.* Bintje, Nicola and Charlotte) and each inoculation treatment (Ri41833; Control) were harvested at the flowering stage (the 15th of July 2014), just before defoliation (the 27th of August 2014) and at harvest (the 24th of September 2014). Whole plants were harvested but only the roots were analyzed. These plants were not considered for the final harvest. They were washed with deionized water, dried at room temperature under laminar flow and stored at −20 °C until analysis.

2.8. Estimation of potato tuber yield, size and number

At harvest, tubers (four rows in each plot, *i.e.* 40 plants per subplot) were collected. Tubers were counted, weighted and their size distribution (<60 mm or >60 mm) determined.

2.9. Estimation of root colonization of potato by AMF

Roots were stained following the method of Walker (2005). Root colonization was subsequently assessed following the method of McGonigle et al. (1990). Two hundred intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20–40× magnifications. Total root colonization (%RC), abundance of arbuscules (%A), intraradical spores/vesicles (%V) and/or hyphae only (%H) were determined. Total root colonization (%RC) means the frequency of observation of hyphae, vesicles/spores or arbuscules (alone or together) on 200 observations on a root sample.

2.10. Traceability and/or quantification of haplotype R. irregularis MUCL 41833 and R. irregularis species in potato with the mitochondrial marker

Frozen root samples of the potatoes were crushed in liquid nitrogen, and DNA of 500 mg crushed root samples was extracted using the innuPREP Plant DNA kit (Analytikjena, Germany) according to the manufacturer recommendations with slight modifications (700 µl of SLS buffer and 25 µl of proteinase K were directly added to the crushed roots for the lysis). The concentration of DNA was measured with Quant-iT™ PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA), and 10 ng of total gDNA was used as the template in qPCR.

qPCR was performed as described above. Standard curves constructed from 5-fold dilutions of genomic standard (*i.e.* DNA extract from *R. irregularis* MUCL 41833 spores and mycelium grown *in vitro*) were included in each run. The amplification of each dilution of genomic standard and experimental samples were performed in triplicate.

2.11. Data analysis and statistics

Data analysis was performed with the SAS statistical software version 9.3 (SAS Inc., Cary, NC). Data of the preliminary greenhouse experiment were analyzed by one-way ANOVA. Data of the field experiment was analyzed using a Linear Mixed Model (PROC MIXED) where "cultivar" and "inoculation treatment" were regarded as fixed factors, and block as a random factor. If necessary, data were either logarithmically or arcsine transformed prior to statistical analyses to meet the requirements of normal distribution and homogeneity of variance (as determined by Levene's test). Differences between means were tested by Tukey's test ($P \leq 0.05$).

3. Results

3.1. R. irregularis specific Real-Time qPCR markers

Specificity of the primer pairs is detailed in Table 2. The first primer pair was able to amplify all *R. irregularis* strains tested (but also one *Rhizophagus fasciculatus* and one *Rhizophagus diaphanus* strain) and the second primer pair was able to amplify the specific target strain *R. irregularis* MUCL 41833 and *R. irregularis* MUCL 43204 but not the other *R. irregularis* strains. This last marker detected also *Rhizophagus diaphanus* MUCL 43196. More specific primers could not be found in the mtLSU region sequenced.

3.2. Validation of qPCR assay

Standard curve, qPCR amplifications and melting peak for each primer pair are illustrated in the Supplementary material Figs 5 and 6. qPCR amplification with each primer pair was confirmed by Sanger sequencing. CT values of potato roots in the greenhouse and in the field ranged from 21 to 36 and fit with the standard curves. Amplification efficiency was calculated from ten independent 5-fold dilutions of DNA template and corresponded to 96.2% for mtLSU *R. irregularis* species-specific primer pair and 93.2% for mtLSU *R. irregularis* MUCL 41833 haplotype primer pair (see Table 1).

3.3. Greenhouse validation of molecular markers to trace and quantify the haplotype R. irregularis MUCL 41833 or species-specific R. irregularis in potato roots

Three different stages of colonization (early, intermediate and late stage – according to Gallou et al., 2010) could be distinguished in the thirteen potato plants sampled from the greenhouse experiment (see Supplementary material Table 3). Three samples (harvested at week 2) were in the early stage of root colonization, characterized by the presence of few hyphae (*i.e.* 18 ± 9%) and absence of arbuscules and vesicles/spores. Four samples (harvested at week 2, 5 and 6) were in the intermediate stage, with hyphae (*i.e.* 18 ± 4%), a few arbuscules (*i.e.* 2.2 ± 0.3%) and no vesicles/spores. Similarly, four samples (harvested at week 3 and 4) were in the intermediate stage with the presence of hyphae (*i.e.* 22 ± 7%), some arbuscules (*i.e.* 10 ± 3%) and the first vesicles/spores (*i.e.* 1 ± 0%). The two intermediate stages were thus discriminated by the presence of vesicles/spores. Finally, two samples (harvested at week 5) were in the late stage of colonization with higher

amounts of hyphae (*i.e.* 25 ± 13%), arbuscules (*i.e.* 15 ± 3%) and vesicles/spores (*i.e.* 8 ± 0%).

Whatever the colonization stage, no significant differences were noticed in %RC or %H. To the contrary, significant differences were noticed in %A and %V among the three stages, with the highest values observed in the second intermediate and late stage for %A and late stage for %V. With the mitochondrial markers, significant differences were noticed in mtLSU detection with the lowest concentration ($3.9\ 10^{-6} \pm 2.1\ 10^{-6}$ ng mtLSU_MUCL41833/ng DNA *vs*. $3.5\ 10^{-6} \pm 2.0\ 10^{-6}$ ng mtLSU_RI/ng DNA) in the early stage of root colonization, and with the highest concentration ($7.4\ 10^{-3} \pm 5.1\ 10^{-3}$ ng mtLSU_MUCL41833/ng DNA *vs* $7.8\ 10^{-3} \pm 4.1\ 10^{-3}$ ng mtLSU_RI/ng DNA) in the late stage. An intermediate concentration of mitochondrial markers ($5.7\ 10^{-5} \pm 1.7\ 10^{-5}$ ng mtLSU_MUCL41833/ng DNA *vs*. $4.6\ 10^{-5} \pm 1.8\ 10^{-5}$ ng mtLSU_RI/ng DNA and $3.0\ 10^{-4} \pm 1.2\ 10^{-4}$ ng mtLSU_MUCL41833/ng DNA *vs*. $2.8\ 10^{-4} \pm 1.4\ 10^{-4}$ ng mtLSU_RI/ng DNA) was found in the intermediate stages. Pearson's correlation coefficients (r) between mtLSU of *R. irregularis* MUCL 41833 and AMF root colonization parameters (%RC, %A, %V and %H) were calculated for the thirteen samples (Supplementary material Fig. 7). A significant positive correlation was observed between mtLSU_MUCL41833 and %RC ($r = 0.5967$; $P = 0.0240$), %A ($r = 0.6228$; $P = 0.0168$) and %V ($r = 0.7797$; $P = 0.0004$) but not between mtLSU_MUCL41833 and %H.

3.4. Field experiment

3.4.1. Potato tuber yield, size and number

Potato tuber yield, size and number results and statistics are presented in the Supplementary material Table 4. The average tubers yield per plant ranged from 1.91 ± 0.12 kg to 2.31 ± 0.04 kg among the different treatments and a significant effect of the factor "cultivar" was observed ($P < 0.01$). To the contrary, no significant effect on tubers yield was observed for the factor "inoculation treatment" and there was no interaction between both factors. As the factor "inoculation treatment" was not significant, average tubers yield per plant was calculated for the "Ri41833" and "Control" treatments combined. Potato tubers yield was significantly higher in Bintje (*i.e.* by 11%) and in Nicola (*i.e.* by 10%) as compared to Charlotte.

The % tubers >60 mm ranged from 14 ± 2% to 30 ± 2% among the different treatments. A significant effect ($P < 0.0001$) of the factor "cultivar" was observed. To the contrary, no significant effect on the % tubers >60 mm was observed for the factor "inoculation treatment" and there was no interaction between both factors. As the factor "inoculation treatment" was not significant, % tubers >60 mm was calculated for the "Ri41833" and "Control" treatments combined. The % tubers >60 mm was significantly higher for cultivar Bintje as compared to cultivar Nicola ($P < 0.001$) or Charlotte ($P < 0.0001$) and % tubers >60 mm was significantly

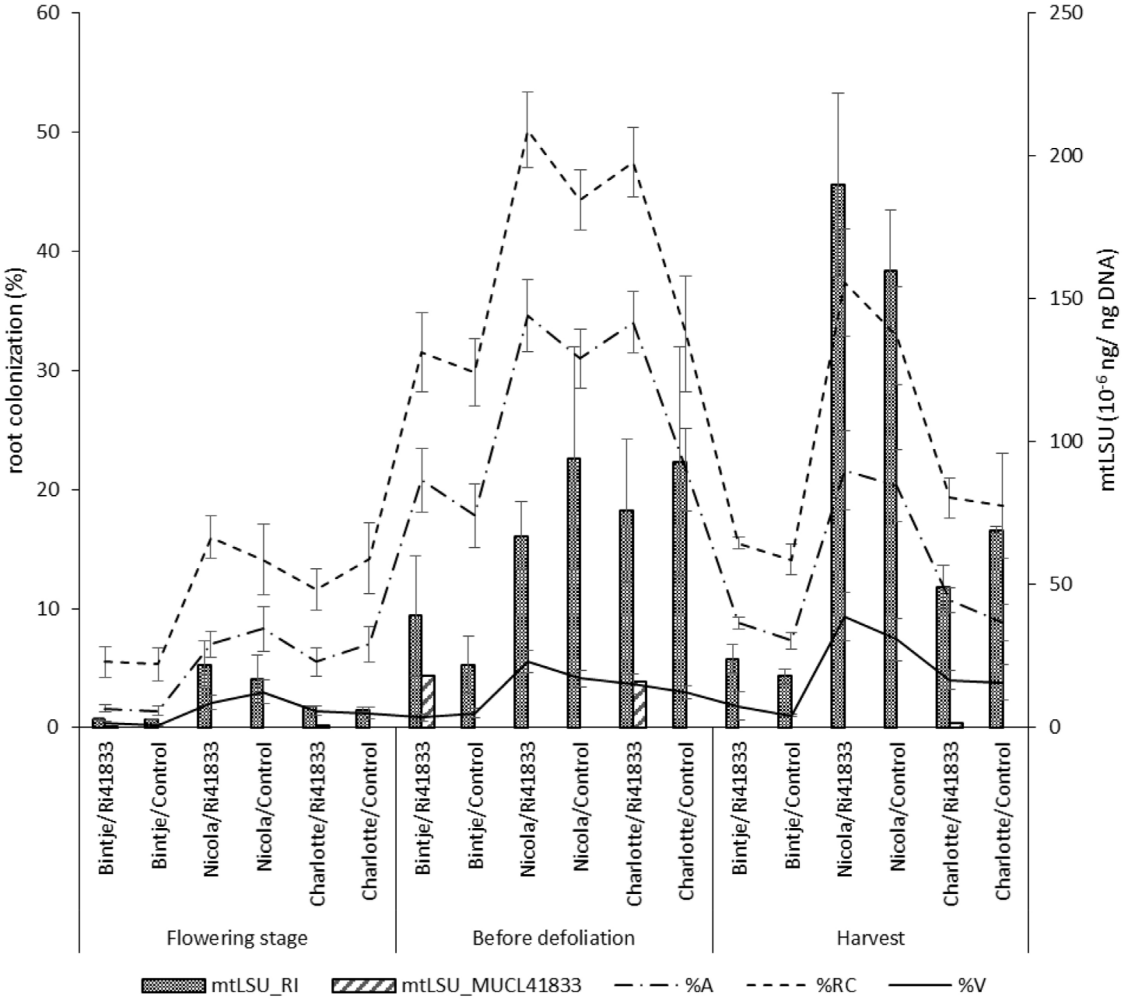

Fig. 1. Root colonization (Total root colonization (%RC), Arbuscules (%A) and Vesicles/Spores (%V)) by AMF of potato cultivars Bintje, Nicola and Charlotte and mtLSU DNA concentration of *R. irregularis* (mtLSU-RI) or *R. irregularis* haplotype MUCL 41833 (mtLSU_MUCL41833) at flowering stage, before defoliation and at harvest.

higher for cultivar Nicola as compared to cultivar Charlotte ($P < 0.05$).

The number of tubers per plant ranged from 14 ± 1 to 16 ± 1 among the different treatments. However, no significant effect on the number of tubers per plant was noticed for the factors "cultivar" or "inoculation treatment".

3.4.2. Root colonization by AMF

Potato root colonization at flowering stage, before defoliation and at harvest is presented in Fig. 1. and statistical analysis in Supplementary material Table 5. At flowering stage, the %RC ranged from $5.3 \pm 1.4\%$ to $16.0 \pm 1.8\%$ and the %A from $1.4 \pm 0.4\%$ to $8.3 \pm 1.9\%$. The %V remained low (from 0.2 ± 0.1 to $3.0 \pm 1.0\%$). A significant effect ($P < 0.0001$) of the factor "cultivar" was noticed on the %RC, the %A and the %V. In contrast, the factor "inoculation treatment" and the interaction of both factors had no significant effect on %RC, %A or %V. The %RC, %A and %V were calculated for the "Ri41833" and "Control" treatment combined. Significant ($P < 0.0001$) higher %RC and %A were noticed for cultivars Nicola and Charlotte in comparison to cultivar Bintje, while no differences were observed between Nicola and Charlotte. In addition, significant higher %V was noticed for cultivars Nicola ($P < 0.0001$) and Charlotte ($P < 0.05$) in comparison to Bintje, while no differences were observed between Nicola and Charlotte.

Before defoliation the %RC ranged from $29.9 \pm 2.8\%$ to $50.2 \pm 3.2\%$, %A from 17.8 ± 2.7 to 34.6 ± 3.0 and %V from $0.8 \pm 0.3\%$ to $5.6 \pm 0.9\%$. Similarly to the flowering stage, a significant effect ($P < 0.0001$) of the factor "cultivar" was noticed on the %RC, the %A and the %V. The factor "inoculation treatment" had no significant effect on %RC, %A or %V. An interaction ($P < 0.05$) was noticed between the two factors for %RC but no interaction was noticed for %A or %V. Similarly as above, %RC, %A and %V were calculated for the "Ri41833" and "Control" treatment combined. Significant ($P < 0.0001$) lower %RC, % A and %V were noticed for cultivars Bintje in comparison to cultivar Nicola. Significant ($P < 0.05$) lower % RC, %A and %V were noticed for cultivars Charlotte in comparison to cultivar Nicola and significant ($P < 0.05$) lower %RC, %A and %V were noticed for cultivar Bintje in comparison to cultivar Charlotte.

At harvest the %RC ranged from $14.1 \pm 1.3\%$ to $37.4 \pm 4.5\%$, %A from $7.3 \pm 0.7\%$ to $21.6 \pm 3.3\%$ and %V from 1.0 ± 0.1 to $9.3 \pm 2.0\%$. A significant effect ($P < 0.0001$) of the factor "cultivar" was noticed on the %RC, the %A and the %V. The factor "inoculation treatment" had no significant effect on %RC, %A or %V. No interaction was noticed between the two factors for %RC, %A or %V. %RC, % A and %V were thus calculated for the "Ri41833" and "Control" treatment combined. Significant ($P < 0.0001$) lower %RC and %A were noticed for cultivars Bintje and Charlotte in comparison to cultivar Nicola, while no differences were observed between Bintje and Charlotte. Moreover, significant different %V were noticed between the three cultivars, with the highest %V for cultivar Nicola ($8.3 \pm 1.3\%$), then for cultivar Charlotte ($3.9 \pm 0.8\%$) and the lowest %V for cultivar Bintje ($1.4 \pm 0.2\%$).

3.4.3. Traceability and/or quantification of native and inoculated R. irregularis *in potato within field*

MtLSU detection levels of all *R. irregularis* strains or just the target isolate at flowering stage, before defoliation and at harvest are presented in Fig. 1. MtLSU of haplotype MUCL 41833 (mtLSU_41833) was detected at a very low level at the flowering stage in only one plant of cultivar Bintje ($2.2\ 10^{-7} \pm 1.7\ 10^{-7}$ ng/ng DNA) and Charlotte ($7.0\ 10^{-7} \pm 1.2\ 10^{-7}$ ng/ng DNA) inoculated with this strain. Before defoliation, detection was again noticed in only one plant of the inoculated cultivar Bintje ($1.8\ 10^{-5} \pm 8.0\ 10^{-6}$ ng/ng DNA) and inoculated cultivar Charlotte ($1.6\ 10^{-5} \pm 1.5\ 10^{-5}$ ng/ng DNA). At harvest, detection was only noticed in one inoculated plant of cultivar Charlotte ($1.5\ 10^{-6} \pm 2.9\ 10^{-7}$ ng/ng DNA). MtLSU of haplotype MUCL 41833 was never detected in cultivar Nicola, whatever the stage of development. Inoculated strain was never detected in the control plants.

The species-specific mtLSU region of *R. irregularis* (mtLSU_RI) was detected in all the potato plants (Fig. 1). Statistical analyses of these results are presented in the Supplementary material Table 6. At flowering stage, before defoliation and at harvest, significant effects ($P < 0.001$) of the factor "cultivar" were observed. To the contrary, no significant effect for the mtLSU_RI was observed for the factor "inoculation treatment" and there was no interaction between both factors. As the factor "inoculation treatment" was not significant, detection of mtLSU_RI was calculated for the "Ri41833" and "Control" treatments combined. The detection of mtLSU_RI was significantly higher for cultivar Nicola ($P < 0.001$) and Charlotte ($P < 0.01$) than for cultivar Bintje at the flowering stage and before defoliation. At harvest, detection of mtLSU_RI of cultivar Nicola was significantly higher than for cultivar Charlotte ($P < 0.001$) and cultivar Bintje ($P < 0.001$). In addition, detection of MtLSU_RI of cultivar Charlotte was significantly higher ($P < 0.0001$) than for cultivar Bintje.

Pearson's correlation coefficients (r) between mtLSU of *R. irregularis* and AMF root colonization parameters (%RC, %A and %V) were calculated for all the treatments. A significant positive correlation was observed between mtLSU and %RC ($r = 0.6544$; $P = 0.0019$), %A ($r = 0.59750$; $P = 0.0062$) and %V ($r = 0.9184$; $P < 0.0001$).

4. Discussion

This study is the first, to our knowledge, that traces and quantifies native and inoculated *R. irregularis* by Real-Time quantitative PCR of mtLSU on three different potato cultivars grown under field conditions. MtLSU of the inoculant *R. irregularis* MUCL 41833 was characterized and two different markers designed. The first marker was a haplotype-specific marker (to trace the field-inoculated isolate) and the second marker a *R. irregularis* species-specific marker (to trace either the field-inoculated isolate or all other native isolates belonging to the species *R. irregularis*). Prior to field experiment, the markers were validated on potato plants grown under greenhouse conditions and the quantification of mtLSU paralleled with the root colonization stage as defined by Gallou et al. (2010). Even if in our field experiment markers were specific enough to trace inoculated or native strains, complete specificity of these primers could not be found in the mtLSU region sequenced. Other regions of mitochondrial genome should be further explored for more specific primers.

Significant correlation was noticed between mtLSU_-MUCL41833 and total, arbuscular or vesicular colonization within roots of potato c.v. Bintje inoculated with *R. irregularis* MUCL 41833 in the greenhouse. This suggested that the haplotype marker was a good indicator of inoculation success of this specific isolate as well as a good indicator of the stage of root colonization. Krak et al. (2012) compared this mtDNA-based quantification to nrDNA-based quantification, which is used in numerous studies (Alkan et al., 2004; Filion et al., 2003; Gamper et al., 2008). They found that the ratio of mtLSU to nrLSU copy numbers was constant across the root colonization of various ages. But the higher-resolution power of the mtLSU compared to that of nrLSU (Börstler et al., 2010) increased the chances of distinguishing an introduced isolate from a native background. The mtLSU marker was already used for the characterization of *R. irregularis* strains in pure cultures (Börstler et al., 2008; Raab et al., 2005) as well as for diversity studies (Börstler et al., 2010) or tracing of introduced isolates (Sýkorová et al., 2012) in the field.

The absolute quantification of the mtLSU gene of an introduced isolate was to our knowledge never performed on field samples. In our field trial, the haplotype *R. irregularis* MUCL 41833 was not detected in the non-inoculated controls suggesting that this haplotype was not present in the field before the trial. Moreover, the haplotype-specific molecular marker revealed the low detection of the inoculated *R. irregularis* MUCL 41833 isolate in the cultivars Charlotte and Bintje and its non-detection in the cultivar Nicola. By contrast, the species-specific marker allowed to detect *R. irregularis* in all potato roots. This indicated that *R. irregularis* was indigenous to the experimental field since it was never inoculated in this specific location. The low detection of the inoculant *R. irregularis* MUCL 41833 could be explained by various factors such as the viability/infectivity of introduced isolate or its incompatibility with the soil environment or plant host (Oehl et al., 2010; Öpik and Moora, 2012). Berruti et al. (2016) observed that the occurrence of root colonization gain was more frequent in response to inoculation with native species than with commercial or collection inoculants (such as in our study). The competition with other microorganisms including the native AMF, lack of time for adequate and significant root colonization (Dickie et al., 2012) or the inadequate localization of the inoculant versus the tuber may similarly explain the low to absence of root colonization by *R. irregularis* MUCL 41833. From our experiment, it appears that the viability/infectivity of inoculum was high and probably not responsible for the low to absent colonization of the inoculated strain. The number of beads necessary to infect 90% of the plants was 13 (estimated by the MIP trial) and 120 beads were applied per potato plant. Competition between isolates of the same species is another factor that was proposed by Krak et al. (2012). These authors observed a decrease in mtLSU levels for one of two coexisting isolates from the same species. This hypothesis could not be verified in our experiment. The distance of inoculum to the roots of the germinating tubers was possibly responsible for the low colonization. Beads were placed next to the potato tuber but the distance with the roots was probably not optimal for good establishment. Propagule germination is generally restricted to less than one mm (D'Souza et al., 2013) and it is not excluded that the first large roots, produced by reserves from the tuber, are less adequate to be colonized by AMF than secondary roots that are at a higher distance from the tuber. However, in other studies (Hijri, 2016) potato seeds were sprayed with a liquid suspension of spores and apparently, this way of inoculation led to increased AMF root colonization and subsequent increased tuber yield. As described above a lot of factors can influence inoculation success. For example, inoculation of *Medicago sativa* cover crop with *R. irregularis* combined with *Trichoderma harzianum* and co-encapsulated into alginate beads increases AMF colonization and yield of subsequently-grown potato under low nutrient conditions (Buysens et al., 2016).

The three potato cultivars were colonized with *R. irregularis* at flowering stage, before defoliation and at harvest as determined by species-specific mtLSU detection. Interestingly, AMF root colonization and *R. irregularis* mtLSU detection at flowering stage, before defoliation or at harvest was in most cases the highest for Nicola, followed by Charlotte and Bintje. These results are in accordance with Bhattarai and Mishra (1984) who observed a difference in root colonization between potato cultivars. They suggested that the highly disease resistant cultivars showed an earlier establishment and more rapid development of AMF than susceptible ones. Previous studies also showed that crop breeding programs selecting for high yield varieties under fertilized conditions may have inadvertently select genotypes that are less responsive to mycorrhiza (Johnson and Pfleger, 1992). But a meta-analysis on studies from 1981 to 2010 (Lehman et al., 2012) observed that new cultivars were less intensively colonized but were more mycorrhiza-responsive (and possibly dependent) compared to ancestral genotypes. Although this could not be proved in our experiment it may support the interest to consider the association with AMF within breeding strategies (Rengel, 2002).

5. Conclusion

In conclusion, our study demonstrated, for the first time, that mtLSU-based Real-Time qPCR assays could be used in the field for quantification of native and inoculated AMF strains in addition or potentially in substitution to root colonization estimation by microscopic observations. MtLSU amount was correlated with colonization level (especially % of arbuscules) and is thus a good indicator of colonization success. This study showed that the effectiveness of AMF inoculation under field conditions is complex and depends on several factors. This molecular traceability method should be further used in studies searching for better inoculation methods. Even if the markers used in the present were specific enough, specificity of the markers could be further enhanced by sequencing whole mitochondrial genome to find more specific regions (Formey et al., 2012; Beaudet et al., 2013; Badri et al., 2016). Intergenic regions of mitochondrial genomes are highly polymorphic and they are suitable for designing accurate markers targeting AMF isolates (Formey et al., 2012). In addition, this study showed that mtLSU-based Real-Time qPCR assays are adapted to quantify selected AMF strains in potato roots in order to compare different potato cultivars and find the most colonized cultivars by AMF (*e.g.* Nicola > Charlotte > Bintje).

Acknowledgments

This research was sponsored by the DGA (Direction générale opérationnelle de l'Agriculture, des Ressources naturelles et de l'Environnement du service public de wallonie) and by the host laboratory of mycology. We would like to thank Catherine Rasse of «Support en Méthodologie et Calcul Statistique» (Université catholique de Louvain) for her help in statistical analyses. We also thank Hugues Seutin (Walloon Agricultural Research Centre) for his technical support in the field trials.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.03.007.

References

Alkan, N., Gadkar, V., Coburn, J., Yarden, O., Kapulnik, Y., 2004. Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. New Phytol. 161, 877–885. doi:http://dx.doi.org/10.1046/j. 1469-8137.2004.00975.x.

Borriello, R., Bianciotto, V., Orgiazzi, A., Lumini, E., Bergero, R., 2014. Sequencing and comparison of the mitochondrial COI gene from isolates of Arbuscular Mycorrhizal Fungi belonging to Gigasporaceae and Glomeraceae families. Mol. Phylogenet. Evol. 75, 1–10. doi:http://dx.doi.org/10.1016/j.ympev.2014.02.012.

Börstler, B., Raab, P.A., Thiéry, O., Morton, J.B., Redecker, D., 2008. Genetic diversity of the arbuscular mycorrhizal fungus *Glomus intraradices* as determined by mitochondrial large subunit rRNA gene sequences is considerably higher than previously expected. New Phytol. 180, 452–465. doi:http://dx.doi.org/10.1111/j.1469-8137.2008.02574.x.

Börstler, B., Thiéry, O., Sýkorová, Z., Berner, A., Redecker, D., 2010. Diversity of mitochondrial large subunit rDNA haplotypes of *Glomus intraradices* in two agricultural field experiments and two semi-natural grasslands. Mol. Ecol. 19, 1497–1511. doi:http://dx.doi.org/10.1111/j.1365-294X.2010.04590.x.

Badri, A., Stefani, F.O.P., Lachance, G., Roy-Arcand, L., Beaudet, D., Vialle, A., Hijri, M., 2016. Molecular diagnostic toolkit for *Rhizophagus irregularis* isolate DAOM-197198 using quantitative PCR assay targeting the mitochondrial genome. Mycorrhiza 26, 721–733. doi:http://dx.doi.org/10.1007/s00572-016-0708-1.

Bayrami, S., Mirshekari, B., Farahvash, F., 2012. Response of potato (*Solanum tuberosum* cv. Agria) to seed inoculation with mycorrhiza strains in different phosphorus fertilization Journal of Food. Agric. Environ. 10, 726–728.

Beaudet, D., Nadimi, M., Iffis, B., Hijri, M., 2013. Rapid mitochondrial genome evolution through invasion of mobile elements in two closely related species of arbuscular mycorrhizal fungi. PLoS One 18 (8 (4)), e60768. doi:http://dx.doi.org/10.1371/journal.pone.0060768 Print 2013.

Berruti, A., Lumini, E., Balestrini, R., Bianciotto, V., 2016. Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. Front. Microbiol. 6, 1559. doi:http://dx.doi.org/10.3389/fmicb.2015.01559.

Bhattarai, I., Mishra, R.R., 1984. Study on the vesicular-arbuscular mycorrhiza of three cultivars of potato (*Solanum tuberosum* L.). Plant Soil 79, 299–303. doi:http://dx.doi.org/10.1007/BF02182354.

Buysens, C., César, V., Ferrais, F., Dupré de Boulois, H., Declerck, S., 2016. Inoculation of *Medicago sativa* cover crop with *Rhizophagus irregularis* and *Trichoderma harzianum* increases the yield of subsequently-grown potato under low nutrient conditions. Appl. Soil Ecol. 105, 137–143. doi:http://dx.doi.org/10.1016/j.apsoil.2016.04.011.

Chagnon, P.L., Bradley, R.L., Maherali, H., Klironomos, J.N., 2013. A trait-based framework to understand life history of mycorrhizal fungi. Trends Plant Sci. 18, 484–491. doi:http://dx.doi.org/10.1016/j.tplants.2013.05.001.

Cranenbrouck, S., Voets, L., Bivort, C., Renard, L., Corbisier, A.M., Declerck, S., 2005. Methodologies for *in vitro* cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck, S., Strullu, D.G., Fortin, J.A. (Eds.), Root-Organ Culture of Mycorrhizal Fungi. Spinger Verlag.

Croll, D., Wille, L., Gamper, H.A., Mathimaran, N., Lammers, P.J., Corradi, N., Sanders, I.R., 2008. Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol. 178, 672–687. doi:http://dx.doi.org/10.1111/j.1469-8137.2008.02381.x.

D'Souza, J., Rodrigues, K.M., Rodrigues, B.F., 2013. Modified strullu and romand (MSR) medium devoid of sucrose promotes higher *in vitro* germination in *Rhizophagus irregularis*. J. Mycol. Plant Pathol. 43 (2) .

De Jaeger, N., de la Providencia, I.E., Rouhier, H., Declerck, S., 2011. Co-entrapment of *Trichoderma harzianum* and *Glomus sp*. within alginate beads: impact on the arbuscular mycorrhizal fungi life cycle. J. Appl. Microbiol. 111, 125–135. doi:http://dx.doi.org/10.1111/j.1365-2672.2011.05035.x.

Declerck, S., Strullu, D.G., Plenchette, C., 1996. In vitro mass-production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol. Res. 100, 1237–1242.

Declerck, S., Strullu, D.G., Plenchette, C., 1998. Monoxenic culture of the intraradical forms of *Glomus sp*. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90, 579–585.

Dickie, I.A., Davis, M., Carswell, F.E., 2012. Quantification of mycorrhizal limitation in beech spread New Zealand. J. Ecol. 36, 210–215.

Doner, L.W., Bécard, G., 1991. Solubilization of gellan gels by chelation of cations. Biotechnol. Tech. 5, 25–28. doi:http://dx.doi.org/10.1007/BF00152749.

Douds, D.D., Nagahashi, G., Reider, C., Hepperly, P.R., 2007. Inoculation with arbuscular mycorrhizal fungi increases the yield of potatoes in a high P soil. Biol. Agric. Hortic. 25, 67–78.

FAO, 2013. FAOSTAT. Agriculture. FAO, Rome. http://faostat3. fao.org/home/E.

Filion, M., St-Arnaud, M., Jabaji-Hare, S.H., 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. J. Microbiol. Methods 53, 67–76. doi:http://dx.doi.org/10.1016/S0167-7012(02)00225-7.

Formey, D., et al., 2012. Comparative analysis of mitochondrial genomes of Rhizophagus irregularis −syn. Glomus irregulare −reveals a polymorphism induced by variability generating elements. New Phytol. 196 (4), 1217–1227.

Gallou, A., De Jaeger, N., Cranenbrouck, S., Declerck, S., 2010. Fast track *in vitro* mycorrhization of potato plantlets allow studies on gene expression dynamics. Mycorrhiza 20, 201–207. doi:http://dx.doi.org/10.1007/s00572-009-0270-1.

Gamper, H.A., Young, J.P.W., Jones, D.L., Hodge, A., 2008. Real-time PCR and microscopy: are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance? Fungal Genet. Biol. 45, 581–596. doi:http://dx.doi.org/10.1016/j.fgb.2007.09.007.

Gianinazzi, S., Gollotte, A., Binet, M.N., van Tuinen, D., Redecker, D., Wipf, D., 2010. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20, 519–530. doi:http://dx.doi.org/10.1007/s00572-010-0333-3.

Gollotte, A., van Tuinen, D., Atkinson, D., 2004. Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. Mycorrhiza 14, 111–117. doi:http://dx.doi.org/10.1007/s00572-003-0244-7.

Gosling, P., Hodge, A., Goodlass, G., Bending, G.D., 2006. Arbuscular mycorrhizal fungi and organic farming. Agric. Ecosyst. Environ. 113, 17–35.

Helgason, T., Fitter, A.H., Young, J.P.W., 1999. Molecular diversity of arbuscular mycorrhizal fungi colonising Hyacinthoides non-scripta (bluebell) in a seminatural woodland. Mol. Ecol. 8, 659–666. doi:http://dx.doi.org/10.1046/j.1365-294x.1999.00604.x.

Hijri, M., 2016. Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. Mycorrhiza 26 (3), 209–214.

Hodge, A., 2001. Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine patches in soil. New Phytol. 151, 725–734. doi:http://dx.doi.org/10.1046/j.0028-646x.2001.00200.x.

Ijdo, M., Schtickzelle, N., Cranenbrouck, S., Declerck, S., 2010. Do arbuscular mycorrhizal fungi with contrasting life-history strategies differ in their responses to repeated defoliation? FEMS Microbiol. Ecol. 72, 114–122. doi:http://dx.doi.org/10.1111/j.1574-6941.2009.00829.x.

Johnson, N.C., Pfleger, F., 1992. Vesicular-arbuscular mycorrhizae and cultural stresses. Mycorrhizae Sustain. Agric. 1, 71–99.

Koch, A.M., Kuhn, G., Fontanillas, P., Fumagalli, L., Goudet, J., Sanders, I.R., 2004. High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proc. Natl. Acad. Sci. U. S. A. 101, 2369–2374. doi:http://dx.doi.org/10.1073/pnas.0306441101.

Kohout, P., Sudová, R., Janoušková, M., Čtvrtlíková, M., Hejda, M., Pánková, H., Slavíková, R., Štajerová, K., Vosátka, M., Sýkorová, Z., 2014. Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: is there a universal solution? Soil Biol. Biochem. 68, 482–493.

Krüger, M., Stockinger, H., Krüger, C., Schüßler, A., 2009. DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol. 183, 212–223. doi:http://dx.doi.org/10.1111/j.1469-8137.2009.02835.x.

Krak, K., Janoušková, M., Caklová, P., Vosátka, M., Štorchová, H., 2012. Intraradical dynamics of two coexisting isolates of the arbuscular mycorrhizal fungus *Glomus intraradices* sensu lato as estimated by real-time PCR of Mitochondrial DNA. Appl. Environ. Microbiol. 78, 3630–3637. doi:http://dx.doi.org/10.1128/AEM.00035-12.

Lehman, R.M., Taheri, W.I., Osborne, S.L., Buyer, J.S., Douds, D.D., 2012. Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. Appl. Soil Ecol. 61, 300–304.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J.A., 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115, 495–501.

Öpik, M., Moora, M., 2012. Missing nodes and links in mycorrhizal networks. New Phytol. 194, 304–306. doi:http://dx.doi.org/10.1111/j.1469-8137.2012.04121.x.

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., Sieverding, E., 2010. Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. Soil Biol. Biochem. 42, 724–738. doi:http://dx.doi.org/10.1016/j.soilbio.2010.01.006.

Raab, P.A., Brennwald, A., Redecker, D., 2005. Mitochondrial large ribosomal subunit sequences are homogeneous within isolates of *Glomus* (arbuscular mycorrhizal fungi, Glomeromycota). Mycol. Res. 109, 1315–1322. doi:http://dx.doi.org/10.1017/S0953756205003977.

Redecker, D., 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10, 73–80. doi:http://dx.doi.org/10.1007/s005720000061.

Rengel, Z., 2002. Breeding for better symbiosis. Plant Soil 245, 147–162. doi:http://dx.doi.org/10.1023/A:1020692715291.

Sokolski, S., Dalpe, Y., Seguin, S., Khasa, D., Levesque, A.C., Piche, Y., 2010. Conspecificity of DAOM 197198, the model arbuscular mycorrhizal fungus, with Glomus irregulare: molecular evidence with three protein-encoding genes. Botany 88, 829–838.

Sýkorová, Z., Börstler, B., Zvolenská, S., Fehrer, J., Gryndler, M., Vosátka, M., Redecker, D., 2012. Long-term tracing of *Rhizophagus irregularis* isolate BEG140 inoculated on *Phalaris arundinacea* in a coal mine spoil bank, using mitochondrial large subunit rDNA markers. Mycorrhiza 22, 69–80. doi:http://dx.doi.org/10.1007/s00572-011-0375-1.

Schläppi, K., Bender, S.F., Mascher, F., Russo, G., Patrignani, A., Camenzind, T., Hempel, S., Rillig, M.C., van der Heijden, M.G.A., 2016. High-resolution community profiling of arbuscular mycorrhizal fungi. New Phytol. 212, 780–791. doi:http://dx.doi.org/10.1111/nph.14070.

Smith, S., Read, D., 2008. Mycorrhizal Symbiosis. Elsevier Ltd..

Verbruggen, E., van der Heijden, M.G.A., Rillig, M.C., Kiers, E.T., 2013. Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. New Phytol. 197, 1104–1109. doi:http://dx.doi.org/10.1111/j.1469-8137.2012.04348.x.

Voets, L., De La Providencia, I.E., Fernandez, K., Ijdo, M., Cranenbrouck, S., Declerck, S., 2009. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. Mycorrhiza 19, 347–356. doi:http://dx.doi.org/10.1007/s00572-009-0233-6.

Walker, C., 2005. A simple blue staining technique for arbuscular mycorrhizal and other root inhabiting fungi. Inoculum 56 (4), 68–69.