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

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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

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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.