Applied Soil Ecology 120 (2017) 62–69

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Characterization of dark septate endophyte fungi associated with cultivated soybean at two growth stages

C. Rothen[a,*], V. Miranda[a], A. Aranda-Rickert[a], S. Fracchia[a], M.A. Rodríguez[b]

[a] Centro Regional de Investigaciones Científicas y Transferencia Tecnológica de La Rioja (CRILAR), Provincia de La Rioja, UNLAR, SEGEMAR, UNCa, CONICET, Entre Ríos y Mendoza, 530 Anillaco, La Rioja, Argentina

[b] Laboratorio de Microbiología del Suelo, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IBBEA, CONICET, Argentina

ARTICLE INFO

Keywords:
Soybean
Dark septate endophyte
Crop
Argentina
Root endophytes

ABSTRACT

Dark Septate Endophytes (DSE) is a diverse group of Ascomycetes that colonize the roots of a wide range of plants species. They can be found in all ecosystems, from deserts to the tropics, but also in agroecosystems associated to the crops. Despite the ubiquitous of these fungi, only a few major crops were assessed for this association in field conditions. In general terms, a complex consortium of DSE fungi were observed to colonize the roots of some crops. In this work we study the DSE community associated to soybean in the most productive area of this crop in Argentina. We hypothesized that DSE root colonization level, and the identity/frequency of the isolates switch according to the growth stage of the crop. A total of 34 dark fungal colonies were isolated from two growth stages, being only six isolates asyntomatic in the resynthesis assay. Some taxa as *Boeremia* sp., *Cadophora* sp., *Coniothyrium* sp., *Corynespora cassicola, Peyronellaea* sp. and *Phaeosphaeria* sp. were exclusively isolated from seedlings, supporting the hypothesis of DSE fungal consortium switching. The soybean pathogens *Corynespora cassicola* and *Macrophomina phaseolina* were isolated from young and mature plants respectively. Additional studies should focus on a fine analysis of the dynamics of these fungi, considering also the driving factors that could determine these changes. To understand these mechanisms may be fundamental for a better sustainable management of the crop.

1. Introduction

The dark septate endophytes (DSEs) are a polyphyletic group of fungi including species from phylogenetically distant phyla. They are characterized by their darkly pigmented hyphae due to the presence of melanin, and colonize inter and intracellular parenchymatic root tissue, often forming microsclerotia (Jumpponen and Trappe, 1998). DSE fungi are widely distributed and registered in more than 600 phylogenetically diverse host species (Addy et al., 2005; Mandyam and Jumpponen, 2005). Several studies related to the mycorrhizal status of plant species on diverse biomes have included these endophytic fungi within their surveys, evidencing their abundance in plant communities worldwide (Fracchia et al., 2009; Rodriguez et al., 2009). Particularly, DSEs were predominantly observed in roots of plants growing in stressful and nutrient-limited environments (Lugo et al., 2009; Newsham et al., 2009; Silvani et al., 2013). This suggests a possible mitigating and relieving effect of DSEs in such extreme conditions, functionally behaving as a mycorrhizal symbiosis in some plants species (Porras-Alfaro and Bayman, 2011). Moreover, the broad geographic distribution of DSE fungi would be indicating low host specificity. For instance, the most studied DSE taxon, the *Phialocephala fortinii–Acephala applanata* complex, has been found in diverse hosts and biomes of the northern hemisphere (Grünig and Sieber, 2005).

When considering crop plants, the information related to their association with DSEs is scarce, and limited to descriptive analyses of the colonization of DSE on root samples from the field (Fernandes et al., 2015; Muthukumar and Tamilselvi, 2010), or to genomic analyses where sequences related to DSE taxa were detected (Bokati et al., 2016; Detheridge et al., 2016; Likar et al., 2008). Several studies have also focused on the interaction effects of isolated DSE strains from diverse origins on crop plants mainly as biotic/abiotic stress relievers (Narisawa et al., 2004; Su et al., 2013) as well as promoters of plant growth under greenhouse conditions (Della Monica et al., 2015; Yuan et al., 2010a). Although some isolates of DSE have demonstrated potential as bioinoculants in commercial crops, there are still many questions to be solved (Lugtenberg et al., 2016). One of them is related to the consortium concept of this fungal group, since commonly a number of species are associated simultaneously in the same root

* Corresponding author.
E-mail address: crothen@crilar-conicet.gob.ar (C. Rothen).

http://dx.doi.org/10.1016/j.apsoil.2017.07.033
Received 19 March 2017; Accepted 21 July 2017
Available online 10 August 2017

system, therefore the associated DSEs need to be addressed deeply for each crop and throughout the plant growth. On the other hand, as DSE fungi are established in root tissues, the nature of the association (i.e. biotrophic, necrotrophic) throughout the plant life cycle needs also to be taken into account (Rodriguez et al., 2009).

Soybean has emerged as one of the most important agricultural crop worldwide, being South America the main production area (Confalone et al., 2010). In Argentina, since 1970 the planted area destined for this crop have increased uninterruptedly, reaching more than 20 million hectares in 2016. This have made soybean in Argentina the first crop per cultivated area and tons of production (Ministerio de Agroindustria, 2017), with the genetically modified (GM) soybean (glyphosate herbicide tolerant) varieties representing over 90% of the planted area (Leguizamón, 2014). Currently, the standard culture system for soybean crops include commercial *Bradyrhizobium* spp. inoculation and no-tillage farming, thus increasing crop efficiency and grain yields (Díaz Zorita and Fernández Canigia, 1999). Despite the enormous economic importance of soybean, the knowledge regarding its associated fungal root endophyte community is almost limited to their symbiosis with arbuscular mycorrhizal (AM) fungi. Since the early works of Ross and Harper (1972), Safir et al. (1972) and Bethlenfalvay et al. (1983), the published studies have been focused mainly on the nutritional aspects of the AM symbiosis and the associated tolerance to stress conditions (Porcel and Ruiz-Lozano, 2004; Wang et al., 2011). When considering other root associated fungi, Russo et al. (2016) reported recently the isolation and identification of endophytic fungi in leaves, stems and roots of different soybean cultivars. Within them, the soybean pathogen *Macrophomina phaseolina* was identified as the only dark fungi associated with root tissues. However, it could be expected that a greater diversity of DSEs colonize soybean roots, interacting with their host throughout the plant life cycle.

The main objective of this work is to study the dynamics and composition of the DSE communities associated with soybean crop in the most productive area of this legume in Argentina. We set up the hypothesis that the DSE root colonization level as well as the identity/frequency of the isolates change with the growth stage of the plant. To test this hypothesis, we select a study area within the core region for soybean planting in Argentina, and assessed root colonization level and DSE community composition from plants sampled at two different growth stages (seedlings and mature plants). Additionally, we undertake a resynthesis assay under laboratory conditions with each of the fungal isolates and soybean plants, in order to attempt an approach of the nature (symptomatic/asymptomatic) of the dual association.

2. Materials and methods

2.1. Experimental site and sampling

The experimental site lies within an intensive soybean crop area located in the north of the Buenos Aires province (34° 08′ S–59° 16′ W). This area corresponds to the humid Pampa, the most productive region for soybean planting in Argentina. The soil is a Typical Argiudol pH 6.7 and 4–5% organic matter content; (see Etcheverry and Génova (2015) for general edaphic characteristics). The climate is temperate, with temperatures varying between 5 °C and 32 °C and mean annual precipitation of 980 mm. The field is cropped under no-tillage management, since more than 15 yr, and the entire crop rotation is soybean followed by a double crop of wheat/soybean. The soybean planted is the GM cultivar RR (Roundup Ready)® resistant to glyphosate, and the sowing is carried out inoculating the grain (140 kg ha^{-1}) with a commercial formulation of *Bradihizobium* spp. Glyphosate is applied before sowing and along crop development.

To sample soybean roots for the DSE colonization assessment and fungal isolation, two growth stages were selected: 4 leaves seedlings (stage 1–45 days old) and mature plants with pods (stage 2–135 days old). The plants were collected in November 2010 for stage 1 and in February 2011 for stage 2. A total of 25 whole plants were collected for each sampling date, following a 500 m transect, digging up 5 individuals every 100 m. Only healthy plants without disease symptoms were chosen. The plant material was stored up for 24 h at 4 °C until processed in the laboratory.

Table 1
Fungal endophytes taxa isolated from soybean roots: phylum, order, close relative taxa and GenBank accession numbers.

Phylum	Order	Close relative	GenBank accession numbers
Ascomycota	Pleosporales	*Alternaria alternata*	KX784250–KX784254, KX784256, KX784257–KX784261
Ascomycota	Pleosporales	*Alternaria arborecens*	KX784249, KX784255
Ascomycota	Pleosporales	*Boeremia* sp.	KX784233, KX784237
Ascomycota	Pleosporales	*Coniothyrium* sp.	KX784247
Ascomycota	Pleosporales	*Corynespora cassicola*	KX784231, KX784232
Ascomycota	Pleosporales	*Curvularia trifolii*	KX784248
Ascomycota	Pleosporales	*Paraphoma radicina*	KX784239–KX784246
Ascomycota	Pleosporales	*Peyronellae* sp.	KX784234–KX784236
Ascomycota	Pleosporales	*Phaeosphaeria* sp.	KX784238
Ascomycota	Capnodiales	*Cladosporium* sp.	KX784262
Ascomycota	Botryosphaeriales	*Macrophomina phaseolina*	KX784263
Ascomycota	Helotiales	*Cadophora* sp.	KX784264

2.2. Root colonization

Root samples were separated carefully from the rest of the plant, and cleaned with running tap water to remove adhering soil particles. A root subsample of each individual was separated to dye and quantify the DSE colonization level. These roots were cleared and stained as described by Phillips and Hayman (1970). Briefly, samples were cleared in 10% KOH at 90 °C for 30 min, followed by staining with trypan blue, distained with distilled H_2O and stored in lactic acid. In order to quantify the proportion of colonized root relative to the total root lenght, ten stained fragments from each plant were observed under binocular microscope at 200× magnifications, according to the method of McGonigle et al. (1990).

2.3. Fungal isolation

For DSE isolation we used the remaining root subsamples from the samples described above. Roots were surface sterilized by washing with 70% ethanol for 2 min, sodium hypochlorite solution 10% for 15 min and in an antibiotic solution (0.05% w/v Penicillin, 0.05% w/v Ampicillin, 0.05% w/v Streptomycin, 0.05% w/v Tetracycline) during 15 min, and then rinsed in abundant sterile distilled water. Under laminar flow, the sterilized fragments (2–3 cm) were cut in 2 mm pieces, discarding the ends, and inoculated in droplets of 0.35% w/v Gel-Gro® and 0.02% $MgSO_4$, in Petri dishes (Silvani et al., 2008). Twenty-five root fragments of each plant were incubated in the dark at 28 °C and checked every day, using a binocular microscope. The emerging fungi from the ends of the fragments were transferred individually to Petri plates with malt extract agar (MEA) medium at pH 6.5, and incubated in the dark at 25 °C. The dark pigmented colonies were finally transferred to glass tubes with the same medium and stored at 4 °C.

2.4. Molecular identification

To perform the molecular identification, DNA was extracted from all isolates, amplified, and sequenced. The fungal isolates were cultured in