Agriculture, Ecosystems and Environment 192 (2014) 115–121

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

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

Occurrence of root endophytic fungi in organic *versus* conventional vineyards on the Croatian coast

T. Radić[a,*], M. Likar[b], K. Hančević[a], I. Bogdanović[a], I. Pasković[a]

[a] Institute for Adriatic Crops, Put Duilova 11, 21 000 Split, Croatia
[b] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 1 April 2014
Accepted 3 April 2014
Available online 30 April 2014

Keywords:
Root endophytic fungi
Grapevine
Weeds
Organic practice
Adriatic coast

ABSTRACT

The aim of this study was to determine the abundance and diversity of root endophytic fungi in organic *versus* conventional vineyards on the eastern Adriatic coast (Croatia). Samples were collected from four locations in the karst region along the coast. Each of these locations included both organically and conventionally managed vineyards of cv. 'Plavac mali' on *Vitis berlandieri* × *Vitis rupestris* rootstock. The total root colonization ranged from 39% to 78% for the grapevines, and from 9% to 99% for the dominant weeds, with generally significantly higher fungal colonization in the organic than the conventional vineyards. Increased abundance of the smallest class of fungal spore (diameter, 40–100 μm) was observed in organic vineyards. There was a similar pattern for fungal diversity, with the overall dominance of *Ascomycota*, and an average of 8.5% of all sequences belonging to *Glomeromycota*. Our study confirms the initial hypothesis that the change in vineyard soil management toward more sustainable practices improves the abundance and diversity of endophytic fungi, thus suggesting their greater ecological importance in environments with significantly reduced inputs.

1. Introduction

Biological soil indicators are becoming increasingly used in soil quality differentiation of areas with contrasting agricultural management (Bending et al., 2004; Marinari et al., 2006). These indicators are sensitive to changes in the soil, and they can provide information that integrates many different environmental factors (Mijangos et al., 2005). In the case of endophytic arbuscular mycorrhizal fungi (AMF), organic crop management is known to increase spore abundance and species diversity, when compared to conventional systems (Lee and Eom, 2009; Oehl et al., 2004). Under conditions of low-input agricultural systems, the plants are expected to be particularly dependent on effective AMF symbiosis (Oehl et al., 2002; Scullion et al., 1998). Low-input organic practices provide better conditions to support high AMF diversity by preventing the selection of the few AMF taxa that can tolerate high nutrient levels (Hijri et al., 2006). In addition, some crops, such as grapevines, are characterized by coarse roots and are almost fully dependent on symbiosis with AMF (Menge et al., 1983). Organic agriculture also increases the diversity and activity of other fungal endophytes (Schmid et al., 2011), which often show remarkable chemical diversity of their secondary metabolites, with positive effects on plant growth and resistance to biotic and abiotic stress (Faeth and Fagan, 2002; Nunez-Trujillo et al., 2012)

In conventional agriculture, fungicide (Sigler and Turco, 2002) acidification of the soil due to excessive fertilizer input (Munoz-Leoz et al., 2012; O'Donnell et al., 2001) or tillage practices (Mijangos et al., 2005) can have severely negative effects on soil microbial communities. It has been indicated that in such high-input conventional agriculture, fungal endophytes are not important for crop growth and development (Ryan and Graham, 2002).

The grapevine is one of the most important crops in the Mediterranean climate, including the area along the Croatian Adriatic coast. Many of the vineyard soils are considered highly degraded due to the predominance of conventional viticulture. Organic practices increase the importance of the biological function of the soil in vineyards (Probst et al., 2008), and grapevines are known to be positively affected by AMF in terms of their growth and mineral uptake, especially under drought conditions (Schreiner, 2005) and for stimulation of bud sprouting, and setting and ripening of the berries (Usha et al., 2005). However, scientific knowledge of the effects of organic viticulture on soil functioning is scarce, especially in the Mediterranean region.

* Corresponding author. Tel.: +385 21 434469; fax: +385 21 316584.
E-mail addresses: tomislav.radic@krs.hr (T. Radić), matevz.likar@bf.uni-lj.si (M. Likar), katarina@krs.hr (K. Hančević), irena.bogdanovic@krs.hr (I. Bogdanović), igor.paskovic@krs.hr (I. Pasković).

http://dx.doi.org/10.1016/j.agee.2014.04.008
0167-8809/

The aim of the present study was to determine the differences in abundance and diversity of root endophytic fungi between organic and conventional vineyards on the eastern Adriatic coast of the Mediterranean Sea. We hypothesized that these different agricultural methods will affect the fungal endophyte communities of the grapevines and herbaceous weeds, which will be reflected in their colonization levels and diversity.

2. Materials and methods

2.1. Site description and sampling strategy

Samples were collected from eight vineyards along the central and southern Croatian Adriatic coast (see Supplementary Table S1). Four locations were chosen where organic and conventional vineyards share the same climatic and pedological conditions. This is a region of intensive viticulture under Mediterranean dry and hot summers (mean maximum temperature, 26–30 °C in the sampled year) and mild and rainy winters (mean minimum temperature, 6–10 °C). The maximum precipitation was in the autumn/winter period (up to 190 mm/month in Ivan Dolac, Milna and Posedarje, and 440 mm/month in Prizdrina), and the minimum precipitation in the June–August period (20–30 mm/month). All of the four organic vineyards sampled are part of the system of certified organic producers (according to the Law on organic production and labeling of organic products, Croatia).

The rhizosphere soil and young grapevine roots were collected from four grapevines per vineyard at a depth of 0–30 cm in March 2010, in the phase of winter resting. All of the sampled grapevines were 3–5-year-old plants of cv. 'Plavac mali' on *Vitis berlandieri* × *Vitis rupestris* rootstock, a rootstock that can tolerate drought, high temperatures, and rocky and sandy soil. The five dominant weed species from each vineyard were also collected for root colonization estimates (five plants per species per vineyard, for a total of 25 plants per vineyard). Only one vineyard (Milna organic) had a permanent green cover crop, which consisted of native wild plants that were periodically mowed. For all of the other organic vineyards, the weeds were controlled mechanically and they covered less than 10% of the vineyard during the sampling period. This mechanical weed control was accompanied by the use of herbicides in the conventional vineyards.

2.2. Soil chemical parameters

Soil samples were air dried, sieved to 2 mm, and ground to a fine powder in an agate mortar. The pH of the dried samples was measured as a soil suspension in deionized water (1:2.5; w/v) according to ISO 10,390:2005. The Cu concentrations in the soils were determined after digestion with *aqua regia* in a microwave at 1000 W (Perkin Elmer Multiwave 6MF100), following the ISO 11,466:1994 procedure. The total Cu was determined by flame atomic absorption spectrometry (Varian 220), following the ISO 11,047:1998 procedure. The total organic matter was measured by wet combustion, according to Kandeler (1995). Plant-available phosphorous was extracted using 0.5 M $NaHCO_3$, and determined photometrically, according to Olsen and Sommers (1982).

2.3. Root endophytic fungal colonization and AMF spore abundance

For the fungal root colonization, root samples from the grapevines and weeds were washed in tap water, cleared with KOH, acidified, stained with Trypan blue, and stored in glycerol. After the staining, the root fragments were examined under the light microscope (Phillips and Hayman, 1970). The total colonization, and the arbuscular, vesicular and hyphal colonization, were determined according to the magnified intersections method (McGonigle et al., 1990), with 150–200 intersections analyzed per plant root.

AMF spores were isolated from 100 g soil samples using the wet sieving and decanting method, followed by sucrose gradient centrifugation (Gerdeman and Nicholson, 1963). They were counted under the stereomicroscope at 50× magnification.

2.4. DNA extraction from grapevine roots, PCR amplification, cloning and sequencing

Collected young grapevines roots (four grapevines per vineyard) were washed, and 150 mg fresh weight per sample was crushed in liquid nitrogen and used for the DNA extraction, following the procedure of the DNeasy Plant Mini kits (Invitrogen, San Diego, USA) (total, 32 DNA extracts). Two series of PCR amplifications were carried out for each sample, using the primers designed for two rDNA regions. For amplification of the internal transcribed spacer (ITS) rDNA region, we used the ITS1F and ITS4 primers (Gardes and Bruns, 1993; White et al., 1990). The PCR reaction mixture (25 μl) contained: 2.5 μl 10 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each nucleotide, 500 nM of each primer, 0.75 U DNA polymerase, and 12.5 μl of the 100-fold diluted template. The PCR amplification was performed as follows: 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 35 s, annealing at 55 °C for 35 s, and elongation at 72 °C for 30 s. The elongation step was increased by 5 s every cycle. The final extension was at 72 °C for 10 min.

Another target region was a 550-bp-long stretch of the 18S rRNA gene. The forward primer in the PCR amplifications was the universal eukaryotic primer NS31 (Simon et al., 1992) and the reverse primer was a mixture of the AM1 (Helgason et al., 1998), AM2 and AM3 (Santos-Gonzalez et al., 2007) primers. The AM1 primer was originally designed for specific amplification of *Glomeromycota* DNA, but the additional primers AM2 and AM3 have shown better efficiency for detection of *Glomus* group B and *Glomus* group C (*Diversisporaceae*) (Santos-Gonzalez et al., 2007). 1 μl of the fungal DNA template was used in a final volume of 25 μl PCR mix, using 2.5 μl 10 × PCR buffer, 1.5 μM $MgCl_2$, 200 μM of each nucleotide, 400 nM of each primer, and 1.25 U Taq polymerase (all Fermentas). The PCR thermocycling program was as follows: 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min. The program ended with 72 °C for 8 min.

For identification of the fungal endophytes, the 18S rDNA and ITS rDNA regions were amplified as described and cloned, prior to sequencing. TOPO TA cloning kits (Invitrogen, San Diego, USA) were used for the cloning, according to the manufacturer instructions. A minimum of 10 positive clones per sample (40 per vineyard; 320 in total) was re-amplified using the M13 forward and reverse primers, to verify successful ligation. The clones giving the correct size products were amplified using the M13 primers, purified with NucleoSpin kits (Macherey-Nagel, Duren, Germany), and cycle-sequenced with the M13 primers, using BigDye terminator Ready Reaction Cycle Sequencing kits on an ABI 3730xl DNA analyzer (Applied Biosystems), as provided by the Macrogen Company (Korea).

2.5. Phylogenetic analysis

The sequences were subjected to GenBank searches using the default option of gapped-BLAST (Altschul et al., 1997), and aligned with the closest matches and additional representatives of the groups from GenBank. Neighbor-joining analysis was performed using MEGA4 (Tamura et al., 2007). The robustness of the internal branches was assayed by bootstrap analysis (1000 runs).