Journal of Biotechnology 214 (2015) 158–168

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Site-specific distribution and competitive ability of indigenous bean-nodulating rhizobia isolated from organic fields in Minnesota

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ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form 2 September 2015
Accepted 11 September 2015
Available online 25 September 2015

Keywords:
Rhizobia
Dry beans
Organic farms
Genetic diversity
N_2-Fixation efficiency
Phaseolus vulgaris

ABSTRACT

Organic dry bean production systems have received increasing interest in many regions of the US, including Minnesota. Thus, improving biological N_2 fixation would be highly beneficial for organic crop production. To date, only limited work has been done to select efficient N_2-fixing rhizobia for organic dry bean production. In this study, soil samples from 25 organic fields in Minnesota, with a previous cropping history of dry beans, soybeans or both, were collected during May to July 2012. Genetic diversity of indigenous dry bean-rhizobia (511 isolates) was determined by using horizontal, fluorophore-enhanced, repetitive, extragenic, and palindromic-PCR (HFERP) DNA fingerprinting and isolates were classified as belonging to 58 different genotypes. The more abundant rhizobia isolated from bean nodules comprised 35.6% of the population. None of the isolates were identical to commonly-used commercial strains used in the U.S., including *Rhizobium tropici* CIAT899. Seventeen predominant genotypes were shown to represent two main species, *Rhizobium leguminosarum bv. phaseoli* (67.1%) and *Rhizobium etli* (30.2%). One of the indigenous strains, orgK9, displayed efficient N_2-fixation and competitive ability relative to the commercial strains tested. The lack of large numbers of indigenous dry bean-rhizobia at most study sites will be useful to avoid competition problems between inoculant strains and indigenous rhizobia. This will allow inoculation with highly effective N_2-fixing rhizobia, thus resulting in improved crop productivity. Our results highlight the existence of site-specific rhizobial genotypes in different organic fields and identify strains that may prove useful as novel inoculants for organic dry bean production systems.

1. Introduction

Dry beans (*Phaseolus vulgaris* L.) are among the most important legume crops grown worldwide (FAO, 2013; Graham and Vance, 2003). In the U.S., production of all dry beans was estimated at 1.52 million metric tons per year, from 728,434 harvested hectares (USDA, 2014). However, intensive conventional agricultural practices for dry bean production have posed severe environmental problems, including soil erosion and nitrate contamination of groundwater, due to the use of synthetic fertilizer and pesticides to achieve enhanced crop productivity (Comly, 1987; Elmi et al., 2002; Pimentel et al., 1995; Randall and Mulla, 2001; Smith et al., 1987; WHO, 2011). Organic production systems have received increasing interest in many countries including the U.S. Organic systems focus on the health of the environment, animals, and humans, but still produce good crop yields free of synthetic chemicals. Any crop or livestock labeled as "organic" must be produced according to the Nation Organic Program (NOP) rules (NOP, 2013). Organic management practices are different from conventional systems and are generally characterized as ecological management systems, in large part due to the use of organic fertilizers and more diverse crop rotations (Moncada and Sheaffer, 2010). These practices are proposed to enhance biodiversity, biogeochemical cycles and soil biological activity, relative to conventional systems (Mader et al., 2002; Van Diepeningen et al., 2006). However, generally, crop yields are lower in organic systems compared to conventional farms (Katungi et al., 2010; Mader et al., 2002; Miklas et al., 2013).

Organic dry beans are currently produced on limited acreage in Minnesota and in the U.S. However, based on the information of USDA-accredited state and private organic certifiers, the number of organic dry bean acres in Minnesota has dramatically increased from about 800 acres (324 hectares) in 2004–2006 to 2500 acres (1012 hectares) in 2011. Minnesota is now ranked third in cer-

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http://dx.doi.org/10.1016/j.jbiotec.2015.09.009
0168-1656/

tified dry bean acres in the U.S., after Michigan and California (USDA, 2013). Dry beans are considered to be relatively inefficient in symbiotic N_2 fixation (Graham, 1981; Moxley et al., 1986). Unlike soybean, these legumes typically require good soil quality and supplemental N (Hardman et al., 1990). The most challenging problem in organic systems, aside from disease and climate change, is the limitation of N and the use of synthetic N fertilizers is prohibited according to regulations of USDA's National Organic Program (NOP, 2013). Organic N fertilizer sources, such as livestock manures, are typically in limited availability or prohibitively costly. Consequently, organic dry bean production would benefit greatly by improving biological N_2 fixation.

An effective symbiosis between specific N_2-fixing bacteria (rhizobia) and legume plants is essential for efficient N_2 fixation. While crop yields have been shown to be increased by use of inocula, even in the presence of some local rhizobial communities in soils (Park et al., 2010), even greater production of organic dry beans requires selection of highly competitive and efficient N_2-fixing rhizobia for use in organic systems.

Despite this great need, there is a paucity of information regarding the genetic diversity of indigenous dry bean-rhizobia in organic farms in Minnesota. Here we report on the isolation and characterization of dry bean-rhizobia from Minnesota organic fields using molecular tools. We also describe the N_2 fixation efficiency and competitive ability of representative rhizobial strains for organic bean production. These findings can help inform the design of field experiments to better characterize novel inocula to improve yields of organic dry beans in Midwestern organic farms.

2. Materials and methods

2.1. Reference rhizobial strains used in this study

The reference *Rhizobium* strains, TAL 182 (USDA 2683), TAL 1383 (USDA 2739), TAL 634 (USDA 2428), and TAL 640 (USDA 2429) were originally obtained from the NiFTAL culture collection, Paia, HI and have been deposited in the *Rhizobium* culture collection, United States Department of Agriculture, USDA–ARS, Beltsville, MD. Strains isolated in this study, orgK1 (L), 1K440; orgK2 (S), 2-K6; orgK3 (E), 3K630; orgK4 (L), 4K270; orgK5 (E), 5K624; orgK6 (L), 6K471; orgK7 (L), 7K215; orgK8 (L), 8K140; and orgK9 (L), 9-K609 have been deposited in the *Rhizobium* culture collection, USDA–ARS, Beltsville, MD under accession numbers USDA 9047-USDA 9055, respectively. The reference strains, USDA 2048, USDA 2370, and CIAT 899 (USDA 2744) were kindly provided by Patrick Elia, USDA–ARS, Beltsville, MD.

2.2. Soil samples, trapping and isolation of indigenous dry bean-rhizobia

Twenty-five soil samples were collected from organic fields located in 13 counties in Minnesota, and 1 county in Wisconsin (near Washington County, Minnesota) during May to July, 2012 (Fig. 1 and Table 1). Fields had a previous cropping history of dry beans, soybeans or both. Kidney bean, *Phaseolus vulgaris* cv. Red Hawk, was used as the trap host to isolate indigenous dry bean-rhizobia from each soil sample. Seeds were surface sterilized as previously described (Wongphatcharachai et al., 2015) and each soil sample was decimally diluted with sterile-distilled water (10^{-1} through 10^{-6}). A 1.0 ml aliquot of each dilution was inoculated to seedlings. There were four replications per dilution. Plants were grown at 23 °C, 70% humidity, with an 18 h photoperiod. Root nodules were harvested after 4 weeks and the concentration of indigenous dry bean-rhizobia in each soil was calculated based on the most probable number (MPN) of cells g^{-1} soil. Nodules were surface-sterilized and placed into individual wells of a 96-well plate containing 100 μl of 0.85% NaCl (Somasegaran and Hoben, 1994; Wongphatcharachai et al., 2015). Each nodule was crushed with a sterile loop and streaked onto a tryptone-yeast extract (TY) agar plate (Beringer, 1974). Bacteria were purified by streaking several times on the same medium.

2.3. HFERP DNA fingerprinting and genetic diversity of indigenous dry bean-rhizobia isolated from organic farms

Seven reference strains and approximately 30 indigenous *Rhizobium* isolates obtained from each sample were further characterized via HFERP DNA fingerprinting as described previously (Johnson et al., 2004; Wongphatcharachai et al., 2015). Genetic diversity was evaluated based on HFERP DNA fingerprint profiles. Strain richness (the abundance-based coverage estimator, ACE) and the Shannon diversity index (H′) were analyzed by using SPADE (Species Prediction And Diversity Estimation) software (Chao and Shen, 2010).

2.4. 16S rRNA gene sequencing

A representative of each predominant *Rhizobium* genotype was selected for 16S rRNA gene sequencing by using primers; Bac27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and Univ1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′) (Lane, 1991). PCR amplicons were purified by using ExoSAP-IT® for PCR product cleanup (Affymetrix, Santa Clara, CA) and were submitted to the University of Minnesota Genomics Center (St. Paul, MN) for DNA sequencing using the classical Sanger method (ABI PRISMTM 3730xl DNA Analyzer). DNA sequences were analyzed and phylogenetic trees were constructed by using MEGA4 (Tamura et al., 2007). Nucleotide sequences of 16S rRNA genes of the indigenous dry bean-rhizobia were submitted to the GenBank database under accession numbers KR336588–KR336604.

2.5. N_2 fixation efficacy of the selected rhizobia strains

Nine strains from organic fields (orgK1–orgK9, Fig. 2) were selected to examine their ability to establish effective symbiotic relationships with two dry bean (*P. vulgaris* L.) varieties (kidney bean cv. Red Hawk and black bean cv. Eclipse) compared to the commercial strains (*R. tropici* CIAT 899 and *R. etli* TAL 182). Modified Leonard jar assemblies containing sterile vermiculite and perlite (3:1, v/v) were used for nodulation assays. Each Leonard jar assembly was planted with two seedlings of kidney bean and black bean and each seedling was inoculated with 1.0 ml (about 1×10^7 colony forming units (cfu) ml^{-1}) of a TY-grown, from stationary-phase culture, *Rhizobium* strain (Cregan and Keyser, 1986; Sadowsky et al., 1987). Seeds were covered with vermiculite:perlite potting mix and 1 cm of sterilized paraffin-coated sand (Sadowsky et al., 1987). Keyser's N-free nutrient solution was supplied as necessary (Cregan and Keyser, 1988). Plants were grown in growth chamber at 23 °C, with 70% humidity and a 16 h photoperiod. All experiments were done in triplicate and non-inoculated plants served as negative controls. Plants were harvested four weeks after inoculation. All fresh nodules were used for nitrogenase activity measurements, estimated using the acetylene reduction assay (ARA) as described previously (Sugawara et al., 2013). The concentration of ethylene produced in each bottle was quantified by gas chromatography using a Hewlett Packard gas chromatograph (HP 6890 series, USA). Acetylene reduction activity was calculated based on the production of ethylene per gram of nodule dry weight (Hardy et al., 1973) and nodule mass was examined as previously described (Cregan and Keyser, 1986).