European Journal of Soil Biology 45 (2009) 394–399

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Phenotypic and genotypic diversity of rhizobia in cropping areas under intensive and organic agriculture in Hungary

László Ködöböcz [a,*], András Halbritter [a], Tamás Mogyoróssy [b,c], Mihály L. Kecskés [d]

[a] Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Herman O. u. 15, Budapest H-1022, Hungary
[b] Eötvös Loránd University of Sciences, Budapest, Hungary
[c] DNS—IT Limited Partnership, Győr, Hungary
[d] SUNFix Centre for Nitrogen Fixation, Faculty of Agriculture, Food and Natural Resources, University of Sydney, NSW 2006, Australia

ARTICLE INFO

Article history:
Received 8 December 2008
Received in revised form
17 May 2009
Accepted 10 July 2009
Available online 25 July 2009
Handling editor: Kristina Lindström

Keywords:
BOX-PCR
Diversity
White lupin
Rhizobia

ABSTRACT

Nitrogen-fixing bacteria are important components of various soil–plant ecosystems and arable lands. Interrelations between the mono- or dicotyledonous crop hosts and the plant growth promoting (PGP) bacteria are highly influenced by biotic and abiotic environmental stress factors. Among the methods of community analysis available, the polymerase chain reaction (PCR)-based genetic investigations are especially important. In this study the phenotypic and genotypic diversity of rhizobia colonising white lupin (*Lupinus albus* L.) in cropping areas under 'intensive' and organic agriculture in Hungary was evaluated. Genetic diversity was assessed by DNA analyses using a BOX-PCR method. Rhizobia of the intensive agricultural practices were genetically more diverse and were also phenotypically different to the organic system. This reduced diversity can be attributed to selection pressures exerted by the re-circulation of seeds of the lupin variety harvested from the same area under organic agriculture. In addition, genetic and phenotypic differences between strains infecting the primary and secondary roots of lupin, suggest more than one initial infection event. Similar to earlier studies, our observations also highlight the importance of retaining genetic diversity in agricultural practice both of the host plants and of the rhizobial microbial populations.

1. Introduction

Legume inoculation by rhizobial strains to enhance naturally occurring biological nitrogen fixation (BNF) has been a common practice for more than a century. Nevertheless, while it can be an important issue in legume nodulation, neither change in the natural biodiversity nor the short and long-term impact on newly released strain is usually known [4,27]. Thies et al. [33] have shown that nodule occupancy by inoculant strains is inversely proportional to the number of indigenous rhizobia and when background population exceeds approx. 1000 cells g of soil^{-1} introduction of new strains becomes practically impossible.

Soil management practices can also readily influence rhizobial population numbers and biodiversity [6,25]. In 'organic' or in "no-tillage" compared to "intensive" agricultural systems, a larger and more varied population of rhizobia are expected to be present in soil, partly attributed to the effect of extra-organic matter retained in the system and the reduced use of chemicals [3,7,11,21]. However, within years of introduction of inoculant strains, spontaneous horizontal transfer of mobile symbiotic genetical elements can also add to the biodiversity of rhizobia [20,31]. Cultivation of the host plant also plays an important role in the plant–rhizobium symbiosis. Different soil chemical and physical factors can affect the microsymbiont directly or indirectly through the host plant. For example, for legumes grown in low pH soil, the amount of fixed nitrogen depends on plant persistence as well [34]. Soil water content can act through the plant water regime on the plant–microbe interaction [32] Low soil NO_3^- content supports the processes of nodule formation and nitrogen fixation while high concentrations of soil nitrogen reduce the infectivity of microbes, nodule formation and nitrogenase activity [1,10]. To comprehend the magnitude of changes due to interventions biodiversity studies of agricultural systems are required, but there have been serious technical limits in the past given the vast array of microorganisms present in soil.

Rapid improvements in molecular, and in especially PCR (polymerase chain reaction) linked techniques, have made such research possible. One of the best suited procedures for DNA-based typing of bacterial strains at the subspecies level proved to be the rep-PCR techniques [19,35].

* Corresponding author. Tel.: +36 1 2243648.
E-mail address: kodobocz@hotmail.com (L. Ködöböcz).

1164-5563/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejsobi.2009.07.005

This approach is based on the idea that chromosomal segments lying between two repetitive subsequences of different length in the bacterial genome can be amplified with PCR. Samples separated by electrophoresis give a strain specific pattern, where the information corresponds to the genomic distances of neighbouring repetitive elements. The BOX element [18] containing the box A, B and C subunits is one of the intergenic, repetitive DNA segments found in bacterial chromosomes. If the repetitive sequence targeted as a primer-binding site happens to be the boxA element, then the method is called BOX-PCR [15]. Other repetitive elements widely present in bacteria, and used in rep-PCR, are the REP and the ERIC sequences [35]. Rademaker et al. [23] concluded that out of the different rep-PCR techniques (BOX-, REP- and ERIC-), the most informative and highest resolution pattern (containing the most fragments) can be obtained with BOX-PCR using the BOXA1R primer [15,36].

In this study we compared the genotype relationships in rhizobial strains isolated from the root nodules of lupin (*Lupinus albus* L.) under intensive and organic cultivation in Hungary using the BOX rep-PCR method. Our aim was to establish links and determine variation between some genotypical and phenotypical characteristics of isolates under the two different agricultural techniques.

2. Materials and methods

Root nodular bacterial strains were isolated from the *Nelli* variety of white lupin (*L. albus* L.) grown in sandy soils receiving either organic (long-term crop rotation experiment established in 1929 by Vilmos Westsik in the region of Nyíregyháza, Hungary (Table 1)) [13] or control (intensive agricultural) treatment. For more detailed description of the long-term Westsik's crop rotation trials see Reeves [24] and Lazányi [14].

2.1. Soil characteristics

Soil characteristics, including pH, soil plasticity, $CaCO_3$, macro- and micro-nutrients were determined according to Buzás [5].

2.2. Isolation of rhizobial strains

For the isolation of root–nodule bacteria, lupin plants were collected at the beginning of flowering. Replicate treatment and control plants were chosen from different locations within the same block. Plants with soil were dug carefully and roots were thoroughly cleaned with a soft water jet. Bacterial strains were isolated from nodules of primary or lateral roots or even from the same nodules based on their colony morphology using the method of Vincent [37]. In all cases large sized randomly chosen active (pink coloured) nodules were selected and surface-sterilised with 3% hypochlorite solution followed by rinsing five times with sterile tap water. Nodules were dissected with a sterile scalpel and plant tissue that contained bacteroids was transferred onto YMA (yeast extract-mannitol agar) slants with an inoculation loop. A total of 140 isolates were obtained from the root nodules of about 60 plants. From the intensive field (area ~ 1 ha) and the organic field (area ~ 1, 5 ha) 56 strains from 24 plants and 84 strains from 36 plants were isolated, respectively.

Isolates were purified using Vincent's method (1970). After 24 h of incubation, one loopfull of each culture was transferred into 10 mL sterile tap water and suspended by vortex mixing. Serial dilutions of these suspensions were prepared by transferring 1 mL into 9 mL sterile tap water until 10^{-5} dilution. Twice for each dilution 100 μL of suspension was spread onto a yeast extract-mannitol agar (YMA) plate containing Congo red indicator followed by incubation at 28 °C for 24 h. From these plates, discrete, mucous and transparent colonies were transferred into pure cultures. Pure stock cultures, kept at 4 °C, were refreshed every 6–8 weeks.

The number of samples for the subsequent PCR was determined according to Kaschuk et al. [12]. Their results showed that 25 samples were representative for an experimental area of about 1 ha. For PCR reactions strains were randomly chosen from both experimental sites. Strains for the purpose of antibiotic resistance tests were selected based on morpho-physiological characteristics: colour, mucosity, transparency, borders, elevation and acid/alkaline reaction.

2.3. Reference strain

DSMZ 30134 (*Rhizobium lupini*) was provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

2.4. Antibiotic sensitivity test

Antibiotic sensitivity of bacterial strains was tested by 'Sensi-Disc' antibiotic discs (Becton Dickinson Microbiology Systems) (Table 2); 24-h cultures of strains were suspended in sterile YM broth and 100 μL of each was spread onto YMA plates using a sterile glass rod and three antibiotic discs were placed on each plate. Tests were prepared in three replicates, and after incubation at 28 °C for 24 h, the diameter of the inhibition zones was measured. Inhibition zones were circular in each case. Altogether 11 strains from the intensive and 19 from the organic treatment sites were tested against 10 antibiotics and the average inhibition zones were

Table 1
Summary of treatments of the Westsik's long-term experiment initiated in 1929. Bold displays legumes (table used by permission) [39].

Westsik-type code	Treatment, fertilisers	Crop rotation sequence
I.	Out of crop, weed ploughing, ––	S, R, S
II.	**Lupin green manure, P–K**	**L, R, S**
III.	**Lupin root manure, P–K**	**L, R, S**
IV.	Sand improvement with straw, N–P–K	S, R, R
V.	Straw manure fermented with NH_4NO_3, N–P–K	R, S, R
VI.	Straw manure fermented with water, N–P–K	S, R, R
VII.	Non-fermented straw manure, ––	R, S, R
VIII.	**Lupin root- and green manure, P–K**	**S, R, L**
IX.	**Leguminous stringy forage crops, P–K**	**S, R, L**
X.	**Dual forage crops, ––**	**S, V, R**
XI.	**Farmyard manure, P–K**	**V, S, R**
XII.	Autumnal forage crop mix, P–K	R, S, R
XIII.	Second-sowed lupin green manure, N–P–K	S, R, R
XIV.	Second-sowed lupin green manure, N–P–K	S, R, R
XV.	Second-sowed lupin green manure, ––	S, R, R

S: potato (*Solanum tuberosum* L.); R: rye; L: lupin; V: vetch with oats.

Table 2
List and concentration of applied antibiotics.

Antibiotics	Concentration/disc
Gentamycin	120 μg
Streptomycin	300 μg
Ampicillin	25 μg
Erythromycin	15 μg
Tetracycline	30 μg
Rifampicin	15 μg
Bacitracin	10 IU
Kanamycin	30 μg
Chloramphenicol	3 μg
Polymyxin-B	300 IU