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SHORT COMMUNICATION

Communities of endophytic microorganisms in different developmental stages from a local variety as well as transgenic and conventional isogenic hybrids of maize

Kelly Justin da Silva[1] · Rafael Dutra de Armas[2] · Cláudio Roberto F. S. Soares[2] · Juliana Bernardi Ogliari[1]

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Abstract The diversity of endophytic microorganisms may change due to the genotype of the host plant and its phenological stage. In this study we evaluated the effect of phenological stage, transgenes and genetic composition of maize on endophytic bacterial and fungal communities. The maize populations were composed of a local variety named Rosado (*RS*) and three isogenic hybrids. One isogenic hybrid was not genetically modified (*NGM*). Another hybrid (*Hx*) contained the transgenes *cry1F* and *pat* (T1507 event), which provide resistance to insects of the order *Lepidoptera* and tolerance to the glufosinate-ammonium herbicide, respectively. The third hybrid (*Hxrr*) contained the transgene *cp4 epsps* (NK603 event) combined with the transgenes *cry1F* and *pat* (T1507 event), which allow tolerance to the Roundup Ready herbicide, besides the characteristics of *Hx*. Evaluation of the foliar tissue was done through PCR-DGGE analysis, with specific primers for bacteria and fungi within four phenological stages of maize. The endophytic bacteria were only clustered by phenological stages; the structure of the fungal community was clustered by maize genotypes in each phenological stage. The fungal community from the local variety *RS* was different from the three hybrids (*NGM*, *Hx* and *Hxrr*) within the four evaluated stages. In the reproductive stage, the fungal community from the two transgenic hybrids (*Hx* and *Hxrr*) were separated, and the *Hxrr* was different from *NGM*, in the two field experiments. This research study showed that the genetic composition of the maize populations, especially the presence of transgenes, is the determining factor for the changes detected in the endophytic fungal community of maize leaves.

Keywords PCR-DGGE · Plant genetic resource · rDNA 16S/18S · *Zea mays* L. · Bacteria · Fungi

Introduction

Maize (*Zea mays* L.) is the third most important food crop in the world. It is a direct and indirect human food, a source of energy and protein, and a crop of great economic and social importance (Kirino 2003; FAO 2013). In Brazil, particularly in the Far West of the State of Santa Catarina (FWSC), many farmers still conserve in situ/on farm local and traditional varieties (landraces) of maize. Within two municipalities in this region (Anchieta and Guaraciaba), Costa et al. (2016) identified 1513 populations of maize landraces, comprising 1078 of popcorn, 337 of common maize, 61 of sweet maize and 37 of flour maize, and some constituted new races not yet described in the Americas. Additionally, hundreds of populations of its wild relatives were identified, and some belong to the species *Zea luxurians* (Silva et al. 2015). The earliest records of wild relatives of maize present in Brazil were initially described in a study from the 1930s (Pio-Corrêa 1984), but there is no information on the origin of the species.

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✉ Juliana Bernardi Ogliari
juliana.bernardi@ufsc.br

[1] Departamento de Fitotecnia, Universidade Federal de Santa Catarina, CP 476, Florianópolis, SC 88034-001, Brazil

[2] Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil

The in situ/*on farm* conservation of landraces is essential to maintain the genetic diversity of a crop (Brush 1995; Mercer and Perales 2010), since they have potential or real value for maize breeding programs. The local maize variety named Rosado from the municipality of Anchieta (FWSC) was chosen for this study since it has elevated functional levels of carotenoids and above all lutein (Kuhnen et al. 2012), and is one of the most productive of this municipality (Ogliari et al. 2013), besides being one of the oldest in FWSC (Costa et al. 2016).

In the same region, the improved hybrid maize crops are the most cultivated, especially the genetically modified (*GM*) hybrid maize. The expansion of the cultivated areas with transgenic maize crops has increased exponentially in Brazil (Céleres 2014) and in the rest of the world (James 2013). Among the transgenic hybrid crops recommended for the southern region of Brazil, are the carriers of the TC1507 and NK603 events. The TC1507 event contains two transgenes: *cry1F* and *pat*. The transgene *cry1F* encodes a delta-endotoxin (Cry1F) lethal to insects of the order *Lepidoptera*; the transgene *pat* encodes the phosphinothricin-N-acetyltransferase enzyme (PAT), which allows tolerance to the glufosinate-ammonium herbicide. The NK603 event contains the transgene *cp4 epsps*, responsible for the CP4-EPSPS protein expression, which allows tolerance to the Roundup Ready herbicide (Agbios 2008).

The evaluation of the ecological and biological impacts of this biotechnology is essential, since that there is a risk of contaminating local varieties with pollen from transgenic and non-transgenic crops (Langhof et al. 2008; Schmeller and Henle 2008), especially in the eminent threat on agrobiodiversity. Leaf endophytes have been used as non-target organisms of the transgenes to evaluate their indirect effects (Andreote et al. 2008, 2009a, b), due to the strict symbiotic relationship these microorganisms have developed with their host plant (Rasche et al. 2006; Stuart et al. 2010; Cheeke et al. 2012; Martín et al. 2013; Truter et al. 2014). Endophytic microorganisms are important for their hosts because they provide numerous beneficial properties for plant's defense mechanisms to biotic factors such as diseases and insect infestations (Azevedo et al. 2000; Hardoim et al. 2008). Furthermore, endophytes also provide adaptation of the plants when exposed to the adverse abiotic conditions, provided by high temperature, salt or drought stress (Redman et al. 2011; Naveed et al. 2014).

However, some studies have shown that the host genotype might affect the community of microorganisms that establish a symbiotic relationship with the plant, and that any alteration in the microbial community diversity or activity might have significant effects on the plant's ability to grow and adapt (Azevedo et al. 2000; Kiers and van der Heijden 2006; Andreote et al. 2008; Hardoim et al. 2008; Redman et al. 2011; Naveed et al. 2014; Peñuelas and Terradas 2014; Vandenkoornhuyse et al. 2015).

Therefore, the present study aims to characterize the structure of the endophytic bacterial and fungal communities of maize leaves, in different phenological stages from a local maize variety, two transgenic hybrids, and one non-transgenic hybrid, in field conditions. The hypotheses are that the maize genetic background (local variety × hybrids) and transgenes (*GM* hybrids × *NGM* hybrid) may generate changes in bacterial and fungal structure, and that the changes may be influenced by the phenological plant stage and the presence of transgenes.

Materials and methods

Plant material

The local variety Rosado (*RS*) was provided by a farmer from the municipality of Anchieta/SC, in the FWSC, southern Brazil. The isogenic hybrids belonged to the Biogene BG7060® series. The transgenic hybrid BG7060H (*Hx*) contained the transgenes *cry1F* and *pat* (TC1507 event, Herculex *GM* maize); the transgenic BG7060HR (*Hxrr*) contained the transgene *cp4 epsps* (NK603 event, Roundup Ready *GM* maize) combined with TC1507 event; and the conventional isogenic hybrid BG7060 (*NGM*) was used as a control since it did not contain transgenes.

Field conditions and sampling—Experiment I

The first experiment was performed in September 2012 on the Ressacada Experimental Farm at the University of Santa Catarina, in Florianopolis, SC, Brazil (27° 41′ 04.85″S; 48° 32′ 43.04″W). It was a preliminary experiment to be able to define leaf sampling in the field (stage) and which microbial group (bacteria, fungi or both) responded better to different treatments. The soil was sandy with pH 6.0, classified as Hydromorphic Quartzarenic Type, according to the Brazilian System of Soil Classification (Embrapa 2006). During the evaluation period, the mean temperature was 24.2 °C, and the accumulated precipitation was 175.6 mm, distributed adequately during the Experiment I (Source: Weather station Epagri/Ciram). The climate conditions in this experiment provided good growing conditions to the maize, which showed good development and had no symptoms of drought stress.

Based on results of soil chemical analysis, the soil was fertilized with nitrogen, phosphorous, and potassium, where 60 N kg ha^{-1}, 125 P_2O_5 kg ha^{-1}, and 110 K_2O kg ha^{-1} were used at the moment of sowing, and 20 N kg ha^{-1} was used during the stage near eight leaves. No pesticides were applied during the experiment.

The four treatments (*RS*, *NGM*, *Hx* and *Hxrr*) were evaluated in a homogeneous area, and each area was analyzed in a unique plot composed of three rows 5.0 m in length and 1.0-m spacing between the rows, with a plant density of 60,000 plants ha^{-1}. Three random plants from the central area of each plot were used as samples for leaf collection, and from each plant we collected one leave for the analyses of maize leaf endophytes. The same procedure was adopted for the four different stages of plant development.

The evaluations of maize leaf endophytes were done at 28, 50, 73, and 91 days after seeding (DAS), corresponding with the stages called V1, V2, R1, and R2, respectively. In V1, the maize plants were in the vegetative stage with six expanded leaves. In V2, the plants were still in the vegetative stage and contained eight expanded leaves. In R1, the plants were starting the reproductive period, with ten expanded leaves and developing tassel and ear. In R2, the plants contained twelve expanded leaves, with well-formed tassel and ear, and were in the pollination period. Healthy leaves were collected, in the median session including the midribs. At the time of collection, the leaf samples were packed in sterile plastic bags, maintained in coolers and stored at −80 °C until processing.

Field conditions and sampling—Experiment II

A new evaluation was performed in the following year (March 2013) in a field near Experiment I (27° 41′ 07.86″S, 48° 32′ 37.60″W), with similar fertility and pH conditions. The experimental unit was composed of four rows 5.0 m in length and 1.0-m spacing between rows, and the final stand was 60,000 plants ha^{-1}. The cropping practices were made in accordance with Experiment I. The mean temperature was 20.9 °C and the accumulated precipitation was 273 mm, during the growing season (source: weather station Epagri/Ciram). The climate conditions in this second experiment also provided good growing conditions to the maize, which showed good development and had no symptoms of drought stress.

The treatments were the same (*RS*, *NGM*, *Hx* and *Hxrr*), but leaf collection was only performed on the R1 stage (74 DAS), because it was capable of differentiating the treatments, according to the preliminary results from Experiment I. Ten random plants from the central area of each plot were defined as the sample for collecting healthy leaves. DNA extraction from each plant (one leaf per plant) contributed equally to the composite sample, and so bulk DNA was obtained from ten plants. After collection, the leaf samples were stored in sterile plastic bags, maintained in coolers and stored at −80 °C until processing.

DNA extraction

The superficial disinfection of the maize leaves was carried out to avoid epiphytes. Thus, the leaves were immersed for 2 min in 70 % alcohol, 3 min in sodium hypochlorite (2.5 % active chloride) (V/V), 1 min in 70 % alcohol, and four times for 1 min in distilled sterilized water (Hardoim et al. 2011). The last water wash was used in PCR with specific primers for bacteria and fungi, and confirmed the elimination of epiphytes on the leaves (data not shown). In order to extract DNA, each sample was pulverized in liquid nitrogen with a mortar and pestle. An aliquot (100 mg) of each sample was added to 1.5 mL microtubules, and the extraction was done in CTAB 2 % (Doyle and Doylle 1990). DNA concentration was determined using Nanodrop (Thermo Scientific, Wilmington, EUA), and each sample was adjusted to a 100 ng μL^{-1} concentration.

Transgenic detection

The presence of transgenes in the transgenic hybrids *Hx* and *Hxrr*, and the absence of them in the conventional hybrid (*NGM*) and in the local variety *RS*, was conducted by detecting the presence of the TC1507 event, since both the transgenic hybrids were carriers of this event. The *pat* transgene region was amplified with the primers *TC1507f* (5′ CTT GTG GTG TTT GTG GCT CT 3′), and *TC1507r* (5′ TGG CTC CTC CTT CGT ATG T 3′) (Li et al. 2009), in the following conditions: buffer solution 1×, containing 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 U DNA Taq polymerase (Life Technologies, São Paulo, Brazil), 10 mM of the primers and 100 ng of the DNA. The PCR amplification conditions were: 8 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 50 s at 72 °C, and final extension of 8 min at 72 °C.

PCR-DGGE analysis

The V3 region of the rDNA 16S gene of bacteria was amplified with the primers *BAC338FGC* (5′ GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGA CTC CTA CGG GAG GCA GCA G 3′), and *UN518R* (5′ ATT ACC GCG GCT GCT GG 3′) (Øvreås et al. 1997). Amplification was performed in 1× Taq Platinum DNA polymerase buffer containing 0.2 mM dNTPs, 3 mM MgCl2, 1 U Taq Platinum DNA polymerase (Life Technologies, São Paulo, Brazil), 5 pmol of each primer, and 100 ng of metagenomic DNA. PCR amplification conditions were: 5 min at 95 °C, 30 cycles 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, and final extension for 10 min at 72 °C.

The fungal community was evaluated with the partial amplification of the small-subunit (ssu) 18S rDNA gene

with fungal primers *FR1GC* (5′ GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG AIC CAT TCA ATC GGT AIT 3′), and *FF390* (CGA TAA CGA ACG AGA CCT) (Vainio and Hantula 2000). Amplification was performed in 1× Taq Platinum DNA polymerase buffer containing 0.2 mM dNTPs, 3 mM MgCl2, 1 U Taq Platinum DNA polymerase (Life Technologies, São Paulo, Brazil), 5 pmol of each primer, and 100 ng of metagenomic DNA. PCR amplification conditions were: 8 min at 95 °C, 30 cycles 30 s at 95 °C, 45 s at 50 °C, 2 min at 72 °C, and final extension for 10 min at 72 °C.

Amplicons of 300 ng were analyzed by denaturing gradient gel electrophoresis (DGGE) through the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The amplified products were analyzed with gel electrophoresis with 8 % (m/V) acrylamide: bisacrylamide (37.5:1, m:m), containing a gradient of 25–65 % of formamide and urea (Øvreås et al. 1997). This gradient main separates the amplification product length of bacteria (236 pb) or fungi (390 pb) in the gel. Fragments with different G≡C content migrate differently through gel, and thus, fragments with more G≡C migrate to the lower part in the gel. The electrophoresis was carried out at 200 V and 60 °C constant, for 4 h and 30 min, in 1× Tris-acetate-EDTA buffer. The DNA was stained with Sybr Safe (Life Technologies, São Paulo, Brazil) and images of the gels were made with a Gel Logic 2200 Pro gel documentation system (Carestream Health, New York, USA).

Statistical analysis

The profiles of the gels of DGGE were converted into binary matrices (presence vs. absence of bands for each plant replicate), and the hierarchical grouping analysis was performed using the *Jaccard* coefficient and the UPGMA grouping model, using the software *Gel Compar II version 6.5*. For the comparisons of the pairs, we used an ANOSIM ($p \leq 0.001$) similarity test, using the *Jaccard* coefficient, by the PAST 3.04 software (Hammer et al. 2001).

Results

Plant material confirmation

We confirmed the presence of the *pat* transgene in the transgenic hybrids BG7060H (*Hx*) and BG7060HR (*Hxrr*), and the absence of the same transgene in the conventional hybrid BG7060 (*NGM*) and in the local variety Rosado (*RS*) (Fig. S1, supplementary material). Based on these analyses, it was possible to verify that all of the treatments had the indicated genetic specifications. It was also possible to confirm that the local variety Rosado (*RS*) had not been accidentally contaminated in previous crops by genetically modified maize carrying the TC1507 event.

Endophytic bacterial community in maize

Experiment I The endophytic bacterial community from maize leaf was present in the DGGE gels (Fig. S2, supplementary material). The samples were only grouped by maize phenological stages, without distinguishing between the treatments evaluated (Table S1, supplementary material). The analysis of the hierarchical grouping showed the formation of two dominant groups, one within the two vegetative stages (V1 and V2), and the other group with the reproductive stages (R1 and R2). The structure of the bacterial community was more homogeneous in the reproductive stages (Fig. 1).

The insertion of transgenes did not significantly affect the endophytic bacterial maize leaf community, as there was no distinction between the samples *NGM*, *Hx* and *Hxrr* (Table S1, supplementary material). At the same phenological stage, the endophytic bacterial community structure was similar amongst all samples, although we observed small differences when the maize was in the phase of vegetative growth (Fig. 1). These results indicate that the endophytic bacterial community was not significantly affected by the presence of the T1507 event isolated in the *Hx* hybrid or T1507 combined with NK603 event in the hybrid *Hxrr*. Based on these results, no analysis was performed for the endophytic bacterial community of the maize leaf in Experiment II.

Endophytic fungal community in the maize

Experiment I The endophytic fungal community in maize leaf was present in the image of the DGGE gels (Fig. S3, supplementary material). The samples were classified into four groups according to genetic makeup of the maize in each phenological stage (Fig. 2).

The endophytic fungi in the local maize variety *RS* were different from the three hybrids (*NGM*, *Hx* and *Hxrr*), in the four phenological stages. In the vegetative stages (V1 and V2), there was also a distinction between the two transgenic maize hybrids (*Hx* and *Hxrr*) and the conventional hybrid (*NGM*). Only in the R1 stage, the fungal community was different from the transgenic hybrid *Hx* (carrier of one event with the transgenes cry1F and pat) and the transgenic hybrid *Hxrr* (carrier of two events with the transgene cp4 epsps combined with cry1F and pat), while the conventional hybrid (*NGM*) was similar to the *Hx*.

In the R2 stage, the fungal community differed only between the variety *RS* and the three hybrids; no difference was observed due to transgene insertion (Table S2, supplementary material). Consequently, the fungi from the

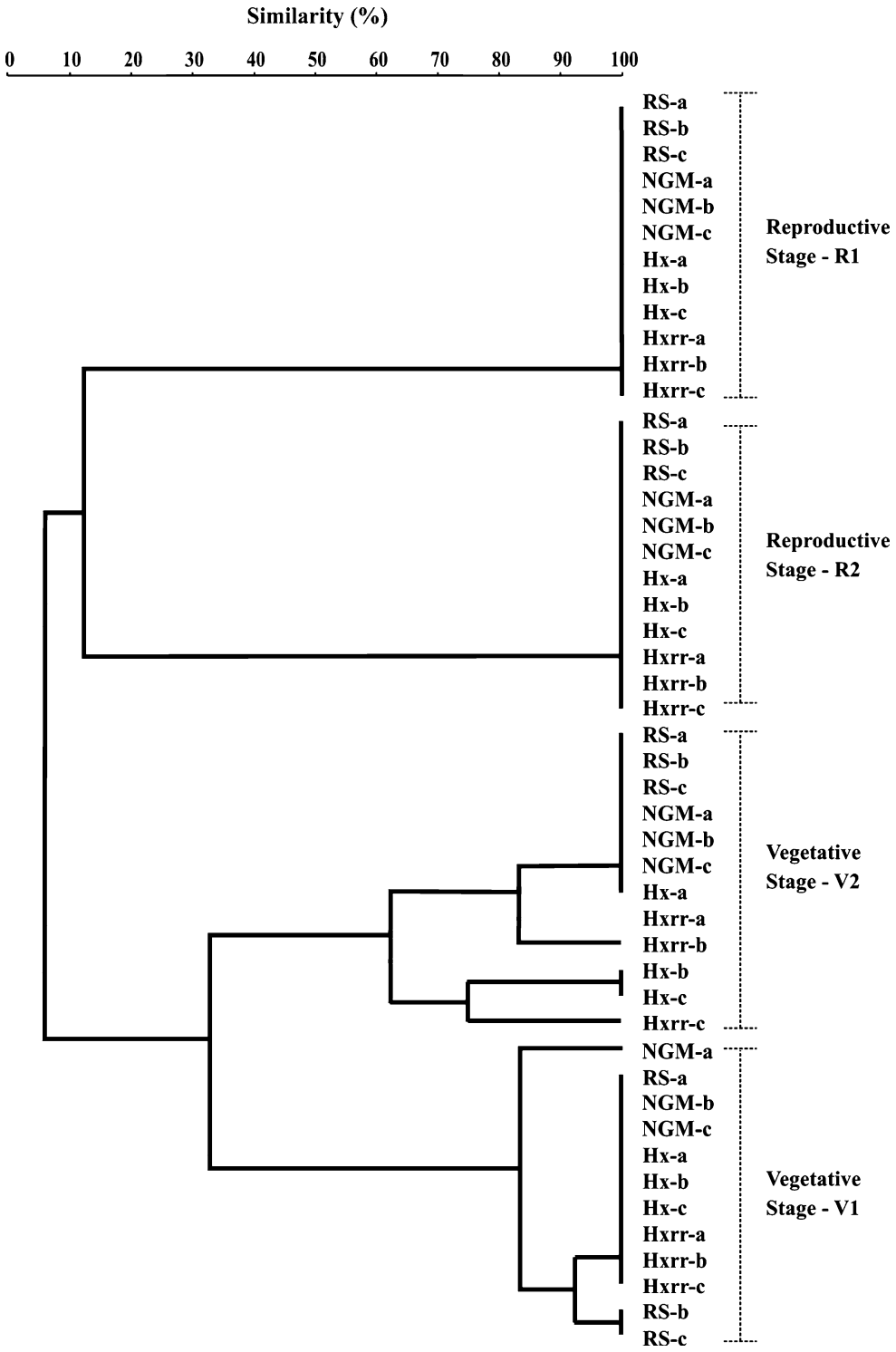

Fig. 1 Dendrogram from UPGMA cluster analysis based on the *Jaccard* coefficient of endophytic bacteria, DGGE patterns of the 16S rDNA gene of bacterial community. V1, V2, R1 and R2 correspond to the four growth stages when the leaves were collected at 28, 50, 73, and 91 days after seeding, respectively. *RS* (Rosado), *NGM* (conventional hybrid), *Hx* (transgenic hybrid with TC1507 event) and *Hxrr* (transgenic hybrid with TC1507 + NK603 events) are treatments of the Experiment I; the letters *a*, *b* and *c* represent the individual plant samples of each treatment

maize leaves were the endophytes that showed alterations due to the genetic constitution of the plants (Fig. 2), differing because of the: (1) absence and presence of transgenes in the isogenic hybrids with the T1507 event isolated or combined with the NK603, in the stages V1 and V2; (2) genetic differences between the local variety (*RS*) and the hybrids (*NGM*, *Hx* and *Hxrr*), in the stages V1, V2, R1 and R2; and (3) type of transgene inserted, if T1507 event isolated or T1507 combined with NK603, in the R1 stage.

In the R1 stage, both the genetic background (local variety × isogenic hybrids) and the highest number of inserted events in the hybrids (*Hx* × *Hxrr*) were able to

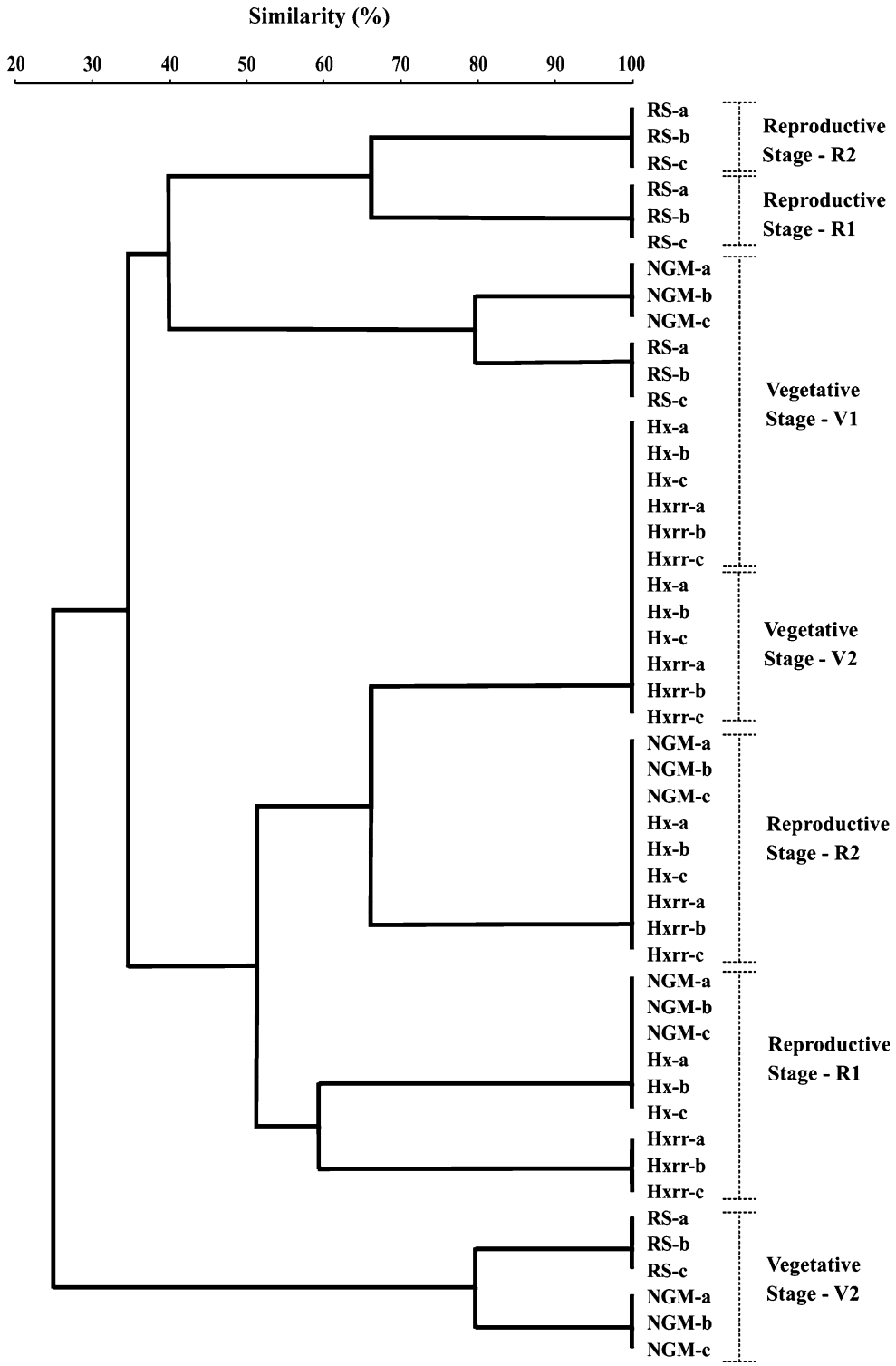

Fig. 2 Dendrogram from UPGMA cluster analysis based on the *Jaccard* coefficient of endophytic fungi, DGGE patterns of the ssu 18S rDNA gene of fungal community. V1, V2, R1 and R2 correspond to the four growth stages when the leaves were collected at 28, 50, 73, and 91 days after seeding, respectively. *RS* (Rosado), *NGM* (conventional hybrid), *Hx* (transgenic hybrid with TC1507 event) and *Hxrr* (transgenic hybrid with TC1507 + NK603 events) are treatments of the Experiment I; the letters *a*, *b* and *c* represent the individual plant samples of each treatment

differentiate the structural diversity of endophytic fungal community. Therefore, the R1 stage seems to be the stage that is most appropriate for following changes in the structural diversity of endophytic fungal community in maize. Based on the ability of discrimination between treatments, the reproductive stage R1 was chosen to confirm the reproducibility of the main results from the Experiment I through a technical experiment (Experiment II).

Experiment II In this experiment, where only the fungal community in the R1 stage was evaluated, the samples were separated by the genetic constitution of the maize

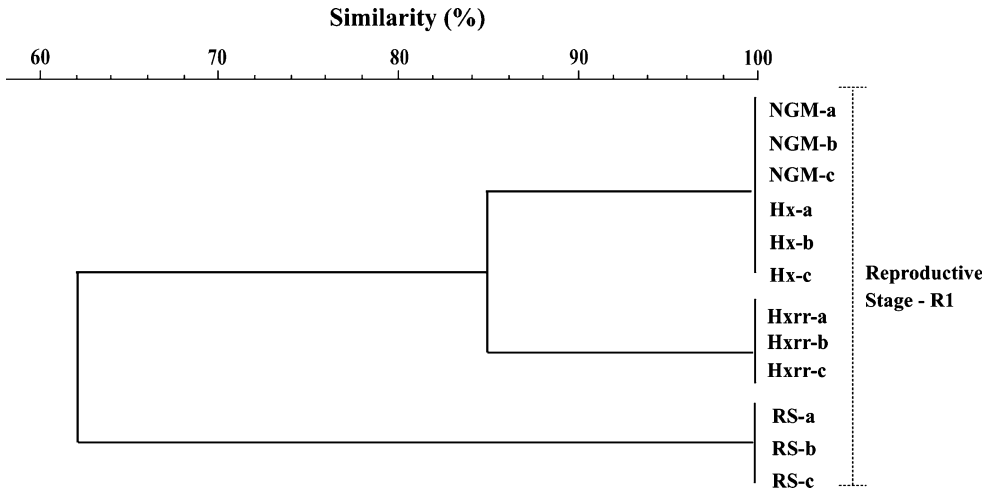

Fig. 3 Dendrogram from UPGMA cluster analysis based on the *Jaccard* coefficient of endophytic fungi, DGGE patterns of the ssu 18S rDNA gene of fungal community. R1 correspond to the reproductive stage when the leaves were collected at 73 days after seeding. *RS* (Rosado), *NGM* (conventional hybrid), *Hx* (transgenic hybrid with TC1507 event) and *Hxrr* (transgenic hybrid with TC1507 + NK603 events) are treatments of the Experiment II; the letters *a*, *b* and *c* represent the composite samples from ten plants of each treatment

(Fig. 3). The results of this second experiment were similar to those obtained in Experiment I. The endophytic fungi in the local maize variety *RS* differed from the three hybrids (*NGM*, *Hx* and *Hxrr*) in the R1 stage. They were also different between the transgenic hybrid *Hx* and the hybrid *Hxrr*, but the conventional hybrid (*NGM*) was similar to the hybrid *Hx* (Table S3, supplementary material).

Discussion

Studies of plant-endophyte interactions are commonly based on controlled conditions of host plant growth, and rarely based on field-realistic conditions (Hardoim et al. 2015). In this study, the bacterial and fungal endophytic communities in the local variety, transgenic, and non-transgenic hybrids of maize were evaluated in two experiments with field-realistic conditions. We observed that differences in plant metabolism may affect symbiosis with endophytic bacteria and may select specific groups for each stage of plant development. In regards to the developmental steps of maize in the vegetative stages, the plant metabolites are mainly translocated to biomass, especially for the growth and development of roots, stems and leaves, furthermore in this period there is a high demand for water and nitrogen. On the other hand, during the reproductive stage, maize metabolism is directed to seed formation, which also demands high phosphorus and potassium (Ritchie et al. 1992).

Similar data were found in endophytic bacteria of maize, potato and rice where significant differences did not occur between the crops or applied management, but occurred between the different stages of plant development (Roesch et al. 2006; van Overbeek and van Elsas 2008; Prakamhang et al. 2009; Rangjaroen et al. 2014). Heuer and Smalla (1999) evaluated endophytic bacteria of transgenic potato leaves (T4—lisoenzima) and verified changes according to the phenological stage of the plant, and no difference was observed with the conventional potato. However, another study presented contradictory data when evaluating endophytic bacteria, fungi, and archaea of transgenic and conventional maize roots (*cry 1F*, *pat* and *cry 1Ab*). This study showed that the bacteria community changed due to the genotypes of the maize plants (Silva et al. 2014).

In the present study, the community structure of the endophytic fungi of maize leaves varied similarly among the maize populations in the two experiments performed in different growing seasons. The most significant changes among the transgenic and non-transgenic hybrids of maize occurred in the V1 and V2 periods; however, the endophytic fungi from the local variety *RS* were different from those of the three isogenic hybrids in all stages. These results corroborate Pan et al. (2008), who concluded that fungal diversity is associated with the genotype of maize plants. Piano et al. (2005) also concluded that the fungi isolated from tall fescue plants varied according to cultivars studied. While, Unterseher et al. (2013) verified that the diversity of isolated fungi changes more between the two genera plant-host than between the different altitudes evaluated in Peru.

In the R1 stage of both experiments, the fungal endophytes did not show changes between hybrids *NGM* and *Hx*. However, the fungal endophytes of the transgenic hybrid *Hxrr*, which has two combined events, were different when

compared with those of the conventional hybrid *NGM*. In fact, other studies have also shown that changes in endophytic fungal communities may occur due to the locations and quantities of transgenes inserted into the plant genome, modifying or silencing other genes in maize (Dietz-Pfeilstetter 2010; Rajeevkumar et al. 2015).

Indirect effects of the transgenes in plants and in their endophytic symbionts are still not well known. The structural diversity of endophytic bacteria of the maize leaves was different only between the different phenological stages. Therefore, DNA sequencing technique should be used to analyze the endophytic bacterial diversity associated to the different maize genotypes.

On the other hand, the endophytic fungal community of maize leaves was distinguished in accordance with the genetic background (local variety × isogenic hybrids) and the presence of NK603 combined with T1507 event (*Hx* × *Hxrr*). Therefore, we found that the reproductive stage R1 was appropriate to analyze changes in the structural diversity of endophytic fungi according to any maize genotype using the PCR-DGGE technique.

This research study is the first to evaluate the symbiosis of microbial foliar communities between maize hybrids or landraces. It showed that the genetic composition of the maize populations is the determining factor for the changes detected in the endophytic fungal community of maize leaves. Further studies are required to verify if such changes can affect the plant response to biotic and abiotic factors of the crop environment, and consequently, compromise agrobiodiversity conservation.

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Compliance with ethical standards

Conflicts of interest No conflict exists for any author and disclosure of potential conflicts of interest.

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