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Diazotrophic bacteria isolated from wild rice *Oryza glumaepatula* (Poaceae) in the Brazilian Amazon

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Abstract: Bacterias diazotróficas aisladas de arroz silvestre *Oryza glumaepatula* (Poaceae) en la Amazonia brasileña. The association of wild grasses with diazotrophic bacteria in Brazilian biomes is poorly understood. The isolation and characterization of bacteria associated with wild grasses can contribute to understand the diazotrophic ecology as well as to identify bacteria with biotechnological applications. In this study, we isolated and characterized diazotrophic bacterial isolates from *Oryza glumaepatula* collected in Cerrado and Forest areas of the Amazon in Roraima State, Brazil. Healthy *O. glumaepatula* plants were collected at five sampling sites at Forest and seven at Cerrado, respectively. The plants were collected at the Cerrado areas in September 2008 while the Forest plants were collected in June/2008 and April/2009. The plants and the soil adhering to the roots were transferred to pots and grown for 35 days in greenhouse conditions. During the harvest, the shoots and the roots were crushed separately in a saline solution; the suspension was diluted serially and inoculated in Petri dishes containing D yg’s medium. All distinct bacterial colonies were purified in the same medium. The diazotrophic capacity of each bacterium in microaerophilic conditions was assessed in semisolid BMGM medium. In addition, the pellicles forming bacterial isolates were also evaluated by PCR amplification for *nif*H gene. The diversity of *nif*H+ bacteria was analyzed by Box-PCR fingerprinting. For selected strains, the growth promoting capacity of *O. sativa* as a model plant was also evaluated. A total of 992 bacterial isolates were obtained. Fifty-one bacteria were able to form pellicles in the semisolid medium and 38 also positively amplified the 360bp *nif*H gene fragment. Among the 38 *nif*H+ isolates, 24 were obtained from the shoots, while 14 originated from the roots. The Box-PCR profiles showed that the bacterial isolates obtained in this study presented a low similarity with the reference strains belonging to the *Herbaspirillum*, *Azospirillum* and *Burkholderia* genus. The growth-promoting ability was confirmed for at least five isolates. For these bacteria, the root and shoot growing results showed higher increases when compared to those observed in plants inoculated with the evaluated reference strains. These results indicate that *O. glumaepatula* is colonized by a high diverse diazotrophic community in the Amazon.
Interaction among diazotrophic bacteria and non-legume plants is widely described in scientific literature. The association of several non-legume species, especially members of the Poaceae family, and diazotrophic bacteria have already been well studied and characterized (Baldani et al. 2009). In Brazil, the studies regarding the interactions among the associative diazotrophs and grasses is well established to crop plants such as rice, maize, sugar-cane and wheat (Baldani & Döbereiner 1980, Oliveira et al. 2006, Hungria et al. 2010). As a result of the information acquired in this field, the use of inoculants has recently been recommended for several grasses like maize, rice, wheat and sugar-cane (Silva et al. 2009, Hungria et al. 2010). Despite the fact that research on grasses and diazotrophic bacteria association has increased, there is a lack of knowledge regarding these associations for wild plant species in Brazil. Few recent studies have shown large diversity of cultivated bacteria isolated from wild grasses in Brazil (Brasil et al. 2005, Fernandes Júnior et al. 2011, 2012).

In Brazil four species of wild rice have been identified and among them Oryza glumaepatula Steud (Poaceae) is now indicated as a potential gene source to transfer to rice cultivars by crop breeding, due their similarity in genomic structure (Rangel et al. 2006, Rosa et al. 2006, Veasey et al. 2011). Although the potential of O. glumaepatula as a genetic resource was shown, the interaction of this species with soil microorganisms remains largely unknown and must be investigated. Recent studies have shown that the Amazonian wild rice O. glumaepatula is able to associate with dark septate endophytic (DSE) fungi that are able to colonize the roots of O. glumaepatula and O. sativa (Pereira et al. 2011, Ribeiro et al. 2011).

Regarding the diazotrophic community, other studies have also shown that screening of diazotrophic bacteria isolated from wild rice species O. alta Swallen and O. rufipogon Griff revealed a diverse and efficient diazotrophic community in Asia (Peng et al. 2008, Zhang et al. 2008, Tan et al. 2009). These results indicate the wild rice species as sources of several microorganisms with biotechnological potential. The search for efficient diazotrophic bacteria associated with wild grasses may also result in the obtainment of bacterial strains adapted to harsh conditions and applicable to the inoculation of crop plants.

In spite of the diazotrophic ability of the microbial community associated with Amazonian O. glumaepatula (Prast & Esteves 1998), there is no information regarding its bacteria isolation and characterization. The aim of this study was to evaluate the diversity and the growth promotion ability of diazotrophic bacteria isolated from O. glumaepatula obtained in Forest and Cerrado areas in the Amazon forest, in Roraima State, Brazil.

**MATERIALS AND METHODS**

**Sample sites, plant cultivation and bacteria isolation:** Healthy plants of Oryza glumaepatula were collected at the Cerrado and Forest areas in Roraima State, Brazil. At the Cerrado, seven sites (02°59'6.8" N - 60°21'7.4" W; 02°59'17.5" N; 60°23'26.9" W; 02°58'51.0" N - 60°23'20.8" W; 02°57'58.7" N - 60°21'39.9" W; 02°57'39.1" N; 60°21'20.8" W; 02°55'43.1" N; 60°24'02.0" W and 03°02'03.0" N - 60°19'12.9 W) were sampled in September/2008. For forest sampling, five sites (01°25'44.2" N - 60°59'0.3" W; 01°25'10.2" N - 60°59'1.7" W; 01°24'1.1"

**Key words:** biological nitrogen fixation, plant growing promoting bacteria, non-symbiotic associations, microbial ecology, inoculant.

the Brazilian Amazon. Further investigations are now being carried out to determine the taxonomic positions of these isolates and their growth promoting mechanisms. Rev. Biol. Trop. 61 (2): 991-999. Epub 2013 June 01.
N - 60°59'1.7" W; 01°24'2.4" N - 60°59'9.2" W and 01°24'56.7" N - 60°59'13.8" W) were sampled in June/2008 and April/2009. At both sites at least three clumps of plants were collected for each sample. These samples were packed into plastic bags with the soil adhering to the roots and transported to the laboratory. The plants were transplanted to pots with the soil adhering to the roots and were maintained in a greenhouse for 35 days. During this period, the plants received weekly 200mL of nitrogen free Hoagland solution (Hoagland & Arnon 1950) and aiming to increase the vegetative growth of these plants, 20mM ammonium sulfate was applied on the 7th and 21st day after the transplantation. On the 35th day after transplanting, the plants were collected and cut into shoots and roots. The roots and shoots were then washed separately with tap water and finally washed with sterile distilled water.

Ten grams of each sample (root or shoot) per pot were crushed in a common blender containing 90mL of saline solution NaCl 8.5g/L. The suspensions were serially diluted until 10⁻⁶ and aliquots of 100μL were, in duplicate, were spread on Petri dishes containing Dyg’s medium (Rodrigues Neto et al. 1986). The Petri dishes were incubated in the dark at 28°C for ten days and, after this period, the colonies, which presented distinct phenotypical characteristics on the same plate, were considered as a single isolate and purified also in the Dyg’s medium.

Assessment of diazotrophic capacity: To assess qualitatively the biological nitrogen fixation (BNF) capacity of the bacteria in microaerophilic conditions (Döbereiner et al. 1995, Mantilla-Paredes et al. 2009, Sgroy et al. 2009, Silva & Melloni 2011), the purified isolates were inoculated, in duplicate, in assay tubes containing 5mL of BMGM semisolid medium (Estrada de los Santos et al. 2001) and incubated for ten days at 28°C. After this period, the pellicle formation was analyzed below the medium surface and the isolates that presented this characteristic were inoculated twice in the same medium to confirm the diazotrophic ability. The identified pellicle-forming isolates were considered as potential diazotrophs and were selected for further evaluation.

Molecular fingerprinting of the bacterial isolates: The pellicle-forming isolates where grown in liquid Dyg’s medium with constant shaking of 100 revolutions per minute (rpm) during 24 hours. The culture broth was used for the DNA extraction using the DNA extraction kit “RTP Bacteria DNA” (Invitek, Berlin, Germany) according to the manufacturer’s instructions. To amplify the nifH gene the primers PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA) were used, and PCR conditions described by Poly et al. (2001) adapting the annealing temperature to 55, 59 or 62°C. The PCR to nifH was carried out for all 51 pellicule-forming isolates at three different annealing temperatures as described above. The PCR products were submitted to electrophoresis in agarose gel (12g/L; 120V by 30min) followed by ethidium bromide (0.5g/L) staining and observation in a UV chamber. The amplifications were considered positive to the nifH gene (nifH⁺) when it was possible to observe the fragment with around 360bp in at least one annealing temperature tested. As a positive control, the BR 11001 strain (Azospirillum brasilense) was also evaluated.

The nifH⁺ isolates were again evaluated according to their capacity to fix nitrogen in micro-aerophilic conditions now in three semi-selective semisolid media: NFb; LGI and JMV (Döbereiner et al. 1995). This assay was performed in triplicate and it was considered as a positive strain whenever the characteristic pellicle occurred in all replications.

The nifH⁺ isolates were also analyzed by Box-PCR profiles. For PCR amplification, the A1 primer (CTACGGCAAGGGCGACGCTGGACG) (Versalovic et al. 1994) was used. The PCR and electrophoresis conditions were adapted from those proposed by Hungria et al. (2008). The gel image was analyzed through the Bionumerics software (Applied Maths, Kortrijk, Belgium) following the clustering by unweighted pair group method with
Plant growth assay: To evaluate the ability of selected bacterial isolates to promote the plant growth, an assay was performed using the rice (O. sativa) as a model plant. The growth promotion ability of seven selected bacterial isolates, obtained from O. glumaepatula (ERR 1019, ERR 1021, ERR 1028, ERR 1029, ERR 1030, ERR 1038 and ERR 1042), one Herbaspirillum seropedicae (BR 11175) and an A. brasilense (BR 11001), were evaluated. An uninoculated treatment was used additionally as absolute control. The bacteria were grown in Dyg’s liquid medium during 48h with constant shaking at 100rpm and 500μL of each bacterial broth was used as an inoculant.

The rice seeds (cv. BRS Roraima) were superficially disinfected with ethanol, hydrogen peroxide followed by ten washings with sterile distilled water and germinated previously in Petri dishes containing agar-water medium. The seedlings were transferred to assay tubes (one plant per tube) containing 50mL of solid Hoagland medium (Hoagland & Arnon 1950) and were inoculated with the corresponding bacteria. The tubes were kept in an incubation room with a 12h photoperiod and the temperature ranged from 26 to 28°C. The experiment was conducted with a completely random design with four replicates.

Thirty days after inoculation, the plants were collected and the shoots were separated from the roots. The roots and shoots were washed and dried at 65°C for 72h. The shoot, root and total dry mass were evaluated. The data were analyzed by variance analysis and the averages separated by the Scott-Knott (p<0.05).

RESULTS

Bacterial isolation and BNF assessment: In order to assess the higher bacterial diversity inhabiting the tissues of Oryza glumaepatula, we previously isolated the bacteria in the solid medium. This approach allowed to obtain a large number of different bacterial phenotypes reaching a total of 992 isolates (Table 1) of which fifty one were pellicle-forming bacteria, i.e., possessed the capacity to fix nitrogen in micro-aerophilic conditions assessed in semi-solid BMGM medium. This amount, therefore, indicated that around 5% of the bacteria obtained are pellicle-forming. The percentage (5%) of diazotrophic bacteria found in this study agrees with other studies that have shown similar ranges from wild or cultivated rice genotypes with low breeding improvement, applying the same strategy adopted in this study (the isolation and purification of bacteria previously in the solid medium) (Barraquio et al. 1997, Stoltzfus et al. 1997).

Molecular fingerprinting: The PCR reaction showed that 38 bacteria presented positive amplification to the fragment of around 360bp, the size expected for the nifH gene (Fig. 1). The bacterial isolates that did not present positive amplification of nifH gene were considered nifH<sup>-</sup> while the isolates with positive amplification to the 360bp fragment were considered nifH positive (nifH<sup>+</sup>).

The analysis of the capacity of the bacterial isolates to grow and fix nitrogen on three different media showed that, in the NFb and LGI media, only one bacterial isolate was not able to grow and fix nitrogen in microaerophilic conditions, while in the JMV medium, three of the 38 isolates were not able to grow and presented positive nitrogen fixation. The ability of the bacterial strains to grow and fix N in different media indicates the metabolic versatility
of these bacteria that were able to grow with positive N fixation in media with different pH and carbon sources (Mantilla-Paredes et al. 2009, Silva & Melloni 2011).

The Box-PCR fingerprinting obtained from \( nif^+ \) isolates showed that most of the bacteria and also the reference strains present different profiles (Fig. 2), indicating a large genetic diversity of these bacteria. It was possible to observe 13 clusters with 60% of similarity. Some of these clusters were formed exclusively with isolates from Cerrado (clusters IV and XII), either from the roots and shoots, while groups I, III and VIII covered only isolates from the shoots. Other peculiarities observed referred to the four groups with
a single isolate (from Cerrado) with a high
discrepancy to others (Fig. 2). All of the iso-
lates obtained from the *O. glumaepatula* also
showed low similarity to the reference strains
indicating the genetic distance between the
reference strains and the new bacteria.

**Growth promoting assay:** The ability of
the bacterial strains to promote plant growth
was evaluated and we observed that among
the eight bacterial strains evaluated, four of
them were able to increase the root dry mass
compared to the absolute control and the posi-
tive controls inoculated with the BR 11001 (*A.
brasilense*) and BR 11175 (*H. seropedicae*)
(Table 2). Three of the isolates were also able
to increase the root dry mass of *O. sativa* more

**TABLE 2**

<table>
<thead>
<tr>
<th>Inoculation treatment</th>
<th>Root dry weight (mg/plant)</th>
<th>Shoot dry weight (mg/plant)</th>
<th>Plant dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERR1029</td>
<td>180 a*</td>
<td>107 b</td>
<td>287 a</td>
</tr>
<tr>
<td>ERR1028</td>
<td>153 a</td>
<td>117 a</td>
<td>270 a</td>
</tr>
<tr>
<td>ERR1019</td>
<td>115 b</td>
<td>72 b</td>
<td>187 b</td>
</tr>
<tr>
<td>ERR1021</td>
<td>118 b</td>
<td>115 a</td>
<td>233 a</td>
</tr>
<tr>
<td>ERR1030</td>
<td>127 b</td>
<td>123 a</td>
<td>250 a</td>
</tr>
<tr>
<td>ERR1038</td>
<td>103 b</td>
<td>103 b</td>
<td>207 b</td>
</tr>
<tr>
<td>ERR1042</td>
<td>162 a</td>
<td>102 b</td>
<td>263 a</td>
</tr>
<tr>
<td><em>H. seropedicae</em></td>
<td>148 a</td>
<td>108 b</td>
<td>257 a</td>
</tr>
<tr>
<td><em>A. brasilense</em></td>
<td>155 a</td>
<td>97 b</td>
<td>252 a</td>
</tr>
<tr>
<td>Absolute control</td>
<td>125 b</td>
<td>98 b</td>
<td>223 b</td>
</tr>
</tbody>
</table>

* Means followed by the same letter in the column do not
differ from the Scott-Knott means test (p<0.05).

than BR 11001 and BR 11175. It must be
pointed out that the isolate ERR 1028 was the
only one able to increase both root and shoot
biomass of the *O. sativa* plants, compared to
the absolute and the inoculated controls. None
of the evaluated reference strains achieved this
capacity, which indicates the biotechnological
potential of these bacteria.

**DISCUSSION**

Although only around 5% of the bacterial
isolates are able to fix nitrogen in micro-aero-
philic conditions, some of these bacteria may
not be isolated if the generally used strategy
was applied to obtain the diazotrophic bacte-
rial strains from crop species, during the first
isolation step in semisolid medium (Stoltzfus
*et al.* 1997); because the semisolid culture
media normally used during these isolations
is indicated to obtain the most studied and
characterized diazotrophic bacteria such as
*Azospirillum* (Baldani & Döbereiner 1980);
*Herbaspirillum* (Brasil *et al.* 2005); *Burkholde-
ria* (Guimarães *et al.*, 2007) and *Sphingomonas*
(Videira *et al.* 2009).

Among all *nifH*⁺ isolates, only 10 bacteria
originated from the Forest area (ERR1001;  
ERR1002; ERR1013; ERR1014; ERR1015;  
ERR1021; ERR1003; ERR1020; ERR1050 and  
ERR1019), while 28 were obtained from Cerr-
ado areas (ERR1005; ERR1040; ERR1004;  
ERR1012; ERR1022; ERR1023; ERR1033;  
ERR1037; ERR1045; ERR1049; ERR1007;  
ERR1009; ERR1011; ERR1027; ERR1032;  
ERR1046; ERR1047; ERR1034; ERR1036;  
ERR1025; ERR1038; ERR1048; ERR1029;  
ERR1028; ERR1042 and ERR1030). In addi-
tion, most of the bacteria, 24 isolates, were
obtained from plant shoots, whereas only 14
from roots. This higher abundance of *nifH*⁺
bacteria in Cerrado areas compared to the For-
est samples, indicates an ecological adaptation
of the wild rice to the environmental condi-
tions. In Cerrado areas, the *O. glumaepatula*
normally presents an annual cycle and the
plants originate from seeds that are spread in
the soil at the end of the rainy season. The plant
growth during this period follows the water
availability, and when the water level decreas-
es, the plants reach physiological maturity and
disperse the seeds into the environment, which
will germinate at the beginning of the next
rainy season (Rosa *et al.* 2006). Instead, in
Forest areas, during the larger part of the year,
the plants are still in the water because of the
drought and flood, which are less prominent.
It is likely that in these conditions, the well-established Forest plants are less dependent on the associative bacteria than the younger Cerrado plants.

Normally, the studies that evaluate the isolation and characterization of nifH+ bacteria from non-legume plants indicate that the majority of the isolates are obtained from root tissues (Videira et al. 2009, Vendant et al. 2010, Venieraki et al. 2011). However, there is evidence that during the vegetative development of grasses in plots where the water availability ranges from flood to drought, as in the Amazon, more diazotrophs must be isolated from shoots rather than from plant roots (Brasil et al. 2005, Koemnok et al. 2007).

*Oryza* species are widely colonized by bacteria belonging to the *Herbaspirillum*, *Burkholderia*, *Azospirillum* and other species (Bal-dani et al. 2009). Genetic evaluations have shown that wild rice species are colonized by a higher diazotrophic community compared to *O. sativa* (Hurek & Reinhold-Hurek 2005). As we observed in this study, previous evaluations have also shown the low similarity among the strains from wild *Oryza*, and the reference strains belonging to *Herbaspirillum*, *Burkholderia* and *Azospirillum* (Tan et al. 2009) indicating the presence of new bacteria. The characterization of bacteria from wild *Oryza* species has also been performed over the last few years and new species (Cheng-Hui & Yokota 2005, Peng et al. 2008) and genera (Zhang et al. 2008) have been described.

DSE fungi were already isolated from Amazonian *O. glumaepatula*, which are able to colonize and promote the growth of *O. sativa* (Pereira et al. 2011, Ribeiro et al. 2011). In this study, we found that this species of wild rice is also colonized by diazotrophic plant growth-promoting bacteria with biotechnological potential. The *O. glumaepatula* has already been indicated as a source of genes for rice breeding programs (Rangel et al. 2006). The recently published studies evaluated the diversity of DSE fungi and the results found in the present study also indicate this species as a source of beneficial microorganisms that must be applied to produce inoculants for rice crops. Further studies are now being carried out to investigate the growth-promoting mechanisms of those bacteria and to evaluate their taxonomic position.

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**RESUMEN**

La asociación de gramíneas silvestres con bacterias diazotróficas en los biomas brasileños es poco conocida. El aislamiento y caracterización de las bacterias asociadas con gramíneas silvestres puede contribuir a entender la ecología de las diazotróficas y bacterias con aplicaciones biotecnológicas. En este estudio, caracterizamos aislamientos bacterianos de diazotróficas de *Oryza glumaepatula* recolectadas en Cerrado y zonas boscosas de la Amazonia en el estado de Roraima, Brasil. Plantas sanas de *O. glume-patula* fueron recolectadas en cinco zonas boscosas y siete en Cerrado. Las plantas de Cerrado fueron recolectadas en septiembre 2008, mientras que las del bosque en Junio 2008 y Abril 2009. Las plantas y el suelo adherido a las raíces se trituraron por separado en una solución salina, la suspensión se diluyó en serie y se inocularon en placas Petri que contenían medio Dyg. Todas las colonias de bacterias se purificaron en el mismo medio. Se evaluó la capacidad diazotrófica de cada bacteria en condiciones microaerofílicas en medio semisólido BMGM. Además, los aislamientos bacterianos que formaron películas se evaluaron también mediante amplificación por PCR para el gen *nifH*. La diversidad de bacterias *nifH*+ se analizó por Huella Genética utilizando la Reacción en Cadena de la Polimerasa. Para las cepas seleccionadas, la capacidad de promover el crecimiento de *O. sativa* como modelo de planta también se evaluó. Se obtuvo un total de 992 cepas bacterianas. Cincuenta y una bacterias fueron capaces de formar películas en el medio semisólido y 38 amplificaron positivamente el fragmento 360bp del gen *nifH*. De los 38 aislamientos de *nifH*+, 24 fueron obtenidos de los brotes, mientras que 14 se originaron a partir de las raíces. Los perfiles de PCR-Box mostraron que los aislamientos bacterianos obtenidos en este estudio presentaron una baja similitud con las cepas de referencia pertenecientes a *Herbaspirillum*, *Azospirillum* y el género *Burkholderia*. La capacidad promotora del crecimiento fue confirmada por al menos cinco aislamientos.
Para esta bacteria, la raíz y bróte mostraron resultados de crecimiento mayores en comparación con los observados en las plantas inoculadas con las cepas de referencia. Estos resultados indican que O. glumaepatula es colonizada por una muy diversa comunidad diazotrófica en la Amazonia brasileña. Se están llevando a cabo otras investigaciones para esclarecer la taxonomía de estas cepas y sus mecanismos para promover el crecimiento.

**Palabras clave:** fijación biológica de nitrógeno, bacterias promotoras de crecimiento de plantas, asociaciones no-simbioticas, ecología microbiana, inoculante.

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