BACTERIAL ENDOPHYTES OF CLIMATE-SMART *BRACHIARIA* SPECIES IN ETHIOPIA

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Abstract
Brachiaria species are vital grass species that have revolutionized dairy and beef production of the western world. Brachiaria is adapted to arid and marginalized environments and is native to east and central Africa. The present study was aimed to dissect and explore endophytic bacteria associated with Brachiaria. Fresh seeds, leaves, stems and roots of Brachiaria were considered and a total of 215 endophytic bacteria were isolated from seeds (26), roots (31), leaves (77), leaf sheath (35) and stem (46) on nutrient agar medium. The strains were identified using 16S rRNA gene sequence and were characterized for Auxin production, phosphate solubilization, siderophores production, ACC-deaminase synthesis and antifungal properties against Aspergillus flavus 006 and A. flavus 023. A significant number of endophytes were able to produce IAA (56%), solubilize phosphate (38%), siderophores production (64%), ACC-deaminase synthesis (33.5%), and 96 (43%) strains inhibited A.flavus 006 and 70 strains (32.5%) inhibited A.flavus 023. Based on 16S rRNA gene sequence they were grouped into 4 phyla, 5 classes, 7 orders, 13 families and 42 taxa. Two most frequently isolated endophytes were Pseudomonas (60 or 27.9%), Pantoea (38 or 17.6%). Characterization of these strains showed that 55 out of 215 strains had a minimum of four plant growth promotion properties.
In conclusion, endophytes isolated from Brachiaria showed wider genetic and functional diversity that would contribute for plant growth promotion and development which might increase fitness and survival of Brachiaria in arid and marginalized environments.

Key words:Brachiaria;Endophytes;Brachiaria endophyte;plant growth promotion

1. Introduction

Brachiaria (syn. Urochloa) species have been extensively useful for increasing livestock productivity in tropical and sub-tropical regions of the world (Mutai, Njuguna and Ghimire, 2017). It is native to east and central Africa including in Ethiopia (Maass et al., 2015). Despite Brachiaria is native to the region its use for fodder production remains to be fully realized . It has high nutritive value, is well adapted to semi-arid climatic conditions and has proven to be a climate-smart grass species that can provide forage for livestock in drought affected regions of tropics with economic viability for smallholder farmer's (Kelemu et al., 2002; Maass et al., 2015). The reason that makes Brachiaria grasses to be drought resistant and climate compliant is its C4 photosynthetic pathway, high nitrogen use efficiency, deep root system enabling acquisition of water from soils, ability to sequester atmospheric cardon-di-oxide into soils and extensive root system contributing to high amount of organic carbon that can in turn improve soil fertility (Krause et al., 2006; Maass et al., 2015; Shen et al., 2019).
Studies on plant-microbe interactions have been on-going for centuries mostly focusing on rhizobium species and legumes. Recent studies indicate that monocot plants species could also be associated with beneficial mutualistic microorganisms called endophytes that play a pivotal role in the survival and reproduction of host plants. These microbes have been found to be promising for sustainable agriculture with economic viability to smallholder farmers. The approach is confirmed to be environmentally friendly and highly promising for sustainable environment and agriculture. Currently, the yield of agriculturally important crops has been largely increased through the application of inorganic fertilizers and modern breeding technologies (Borlaug, 2014) with negative effects on the environment and human health. Application of biofertilizers developed from endophytes of plant origin are getting high attention and are promising alternatives to replace agrochemicals whilst ensuring sustainable agriculture and biodiversity.

Ethiopia lies within the region that represents a centre of the genus Brachiaria (Keller-Grein et al., 1996). In Ethiopia Brachiaira grass grows naturally in different parts of country and we anticipated high level of morphological and genetic diversity. However, existing knowledge about nutritive values of Brachiria species in Ethiopia and beneficial microbes (endophytes) associated with them are very limited. Therefore, this study was conducted to isolate, identify and characterize bacteria endophytes associated with Brachiaria grass ecotypes from natural habitat and field genebank accessions from Ethiopia.
Table 1: The Brachiaria grass sample collection sites and their geographical coordinates, altitude, temperature, and precipitation; types of genetic material; and plant part collected for microbe isolation.

2. Objectives

- To explore the diversity of endophytes associated with Brachiaria
- To investigate the functional role of endophytes to the host plant
- To evaluate possible beneficial properties of endophytes for agricultural applications

3. Material and methods

3.1. Sample collection

A total of 56 above- and below- ground samples of Brachiaria leaves, roots, stems and seeds were collected in Ethiopia during the dry season (May- June 2014) shown below (table 1).

Table 1: The Brachiaria grass sample collection sites and their geographical coordinates, altitude, temperature, and precipitation; types of genetic material; and plant part collected for microbe isolation.

Sites	GPS coordinate (Lat. & Lon.)	Altitude (m asl)	Temperature (°C)	Precipitation (ml)	Type of genetic material	Plant part collected
Wondo Genet College of Forestry (WGCF)	7°192N & 38°382E	1827	Min. 18°C Max. 21°C	Min. 800 ml Max.1600 ml	Ecotype	Seed, root, leaf, stem
Wondo Genet site, Ethiopian Institute of Agricultural Research (EIARW)	7°05' N & 38°37' E	1815	(min, max) 12.5°C, 28.4°C	1121.8	Accessions	Seed, root, stem, leaf
Hawassa Airport (HAP)	NR	1699	NR	NR	Ecotype	Seed, root, leaf, stem
Hawassa Saint Geberel Church (HSGC)	NR	1715	NR	NR	Ecotype	Root, leaf, stem
Hawassa University College of Agriculture (HUCA)	NR	NR	NR	NR	Ecotype	Root, leaf, stem, seed
ILRI-Ziway (ILRIW)	NR	NR	NR	NR	Accessions	Leaf, leaf sheath

Note: NR=note recorded.

2.5. Sequence analysis

The raw sequences were processed and analysed using the QIAGEN CLC Genomics Workbench V7.0.3 (http://www.clcgenomics), and molecular identities of the bacterial strains obtained using the BLAST tool on the NCBI database and the nucleotide sequences were aligned, after adjusting gaps and missing data with MEGA version 6 and unique taxa were identified by sequence homology search.

2.2. Process of surface sterilization

Brachiaria leaves, roots, stem and seeds samples were processed and surface disinfected according to established protocols (Taghavi et al., 2009). Briefly, samples were thoroughly washed in running tap water and then cut into small (3–4 cm) pieces; surface sterilized in 70% ethanol for 1 min, and subsequently in 1.2% sodium hypochlorite (NaOCl), amended with two drops of Tween-20 per 100 ml solution, for 10 min for leaf and stems, 20 min for root and seed samples. The samples were rinsed three times in sterile distilled water and blot dried in between with sterile paper towels.

2.3. Isolation of bacterial endophytes

One gram of surface-disinfected plant samples, cut into 2–3 mm pieces, was macerated in 9 ml of 10 mM/L magnesium Sulphate (MgSO4) solution using a sterile mortar and pestle and the suspension diluted serially. Then a total volume of 100 µl samples from serial dilutions was plated on nutrient agar and 869 media in 90 mm petri-dish, incubated at 28°C for up to 3 days and the emergent colonies were picked and purified through repeated streaking to get single-colony bacteria subculture. A total of 215 putative bacterial endophytic colonies were recovered and stored at 4°C cold rooms for further analysis.

2.4. Genomic DNA extraction, 16S rRNA gene PCR amplification and sequencing

The genomic DNA was extracted from pure culture isolates by a freeze-thaw method of DNA extraction with a modification of a rapid boiling method designed for preparation of bacterial plasmids by (Holmes and Quigley, 1981). Pure bacterial colonies (2-5) were incubated in boiling water for 5 min and were transferred immediately to -20°C and were incubated for 15 min and the process was repeated twice. The 16S rRNA gene was amplified using primer pairs 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Frank et al., 2008). The PCR was performed using AccuPower® PCR PreMix (Bioneer) in 25µl reactions under cycling conditions consisting of an initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s and extension at 72°C for 45 s; and a final extension at 72°C for 10 min. Amplification products were run on 1% agarose-0.5XTris-Borate-EDTA gels and PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Purified PCR products were Sanger sequenced using primers used in sequencing at COMPANY NAME, CITY, and COUNTY).

2.5 Functional characterization of endophytes

Bacterial endophytes were tested for the following functional properties (Table 3) with modification of procedures followed.

Table 3: Functional tests of bacterial endophytes

Tests	Medium	Method	Remarks
Auxin (IAA)	NB-L trypt.	Gordon 1951	Colorimetric
Phosphate	NBRIP	(Mehta and Nautiyal, 2001).	Halozone formation around test colony
Siderophres	CAS	(Vellore et al., 2001)	Blue and orange selection
ACC-deaminase	DF+ACC and DF+N	(Kiani et al., 2015)	Colound formation
Antagonism	PDA	(Liu et al., 2001)	Inhibition zone
Plant growth promotion	Soil less		CFU at 108 cells/ml
Nested PCR	-		Nif gene detection
Box-PCR	-		Repetititve extra genic PCR

2.6. Data analysis

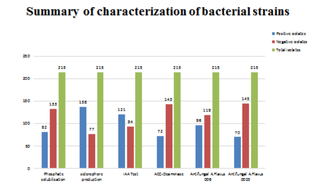
Sequence of the bacterial endophytes were trimmed, assembled using CLCbiomainworkbench and their phylogeny was analyzed and constructed using Mega v6 software.

3. Results and Discussion

3.1. Brachiaria grass harbours distinct bacterial endophytes

Endophytic bacteria were isolated from Brachiaria grass species collected at different sites in Ethiopia during May- June 2014. The sites included Wondo Genet College of Forestry, Hawassa University College of Agriculture, Hawassa Saint Gebrel Church compound, Hawassa Airport, Wondo Genet centre of Ethiopian Institute of Agricultural Research , and genbank collections from ILRI-Zeway.

Seven Brachiaria species from genbank collections and four ecotypes were considered for endophytes isolation. Leaf, leaf sheath, stem, and root tissue of Brachiaria grass were processed to isolate endophytes s. The study confirmed that Brachiaria hosts diverse bacteria with multiple beneficial traits helpful for plant growth promotion and development. A total of 215 endophytic isolates were recovered from the 6 sites and few strains were found site specific. The endophytes also showed phenotypic and functional diversity. Through 16S rRNA gene sequencing most of the bacterial isolates were identified and were analysed to the lowest taxonomic level (from phylum to genus). The strains were classified into 4 Phyla, 4 classes, 5 orders, 11 families, and 13 genera and 38 taxa. The most dominant bacterial phyla were Proteobacteria and Firmicutes. Phylum Actinobacteria and Bacteroidetes were found less dominant. Pseudomonas were most frequently isolated from all Brachiaria growing sites followed by Pantoea species, both from phylum Proteobacteria known to commonly associated with plants (Cho et al., 2007; Rybakova et al., 2017).



4. Conclusion

The adaptability of Brachiaria grasses could be ascribed to the presence of beneficial endosymbiotic bacteria that are helpful for the plant to thrive and survive in arid and marginalized environments. Endophytes associated with Brachiaria grasses shown multiple plant-beneficial traits like phytohormone production, siderophores synthesis, ACC-deaminase production, anti-fungal activity, and inorganic phosphate solubilization which are important for growth and development of the grass.