Diversity of fungal endophytes associated with Rwandan *Brachiaria* ecotypes (*Brachiaria spp*)

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Abstract

Brachiaria species are important tropical forage grasses of East Africa origin. It is known to produce palatable and nutritious biomass for livestock, enhances nitrogen use efficiency, sequesters carbon and reduces greenhouse gas emissions. Despite significant potential of this grass; no information available on endophytic fungal associated with Rwandan *Brachiaria* ecotypes which can have impacts on plant protection under extreme environment. This study identified and characterized the *Brachiaria* associated fungal endophytes from the stems and leaves of 36 Rwandan *Brachiaria* ecotypes collected from three agro- ecological zone of Rwanda in 2014. DNA was extracted from the pure isolated fungal using PrepMan Kit and PCR amplification using 2ITS. Fungal endophytes were characterized *in vitro* by conducting bio-chemical tests for Phosphate solubilization, Indole-3-Acetic Acid (IAA) production, antagonism test, pathogenicity test and siderophores. The numbers of fungal species identified were 21species isolated from stems and leaves of 36 local Brachiaria. The most frequent species were *Epicoccum nigrum* followed by *Cladosporium cladosporioides* and *Coprinopsis atramentaria.*30.1% of total isolate fungal were positive for IAA (Auxins) , 47.3 % of the total isolates fungal were not pathogen to the plant host ;10.1% were antagonistic to *Nigrospora sphaerica* , 8.3% to *Phoma herbarum* and 5% to*the Aspergillus flavus*; 49 % of isolates fungal were able to produce siderophore, No of the ffungal isolates specieswas be able to solubilize Phosphate compound. Results showed that Brachiaria with associated fungal are diverse and contain significant number of endophytes, which need to be explored/conserved and applied to others forage crops in Rwanda.

Key words: Diversity, Fungal, endophytes, Brachiaria ecotypes, ITS,Rwanda

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Introduction

The genus *Brachiaria* consists of over 100 documented species of which a few are grown as improved pastures in the tropics (Miles et al., 1996). *Brachiaria* is is native to East Africa and it is an important flora of Savannah grassland ecosystems. Adaptation of *Brachiaria* grass to drought and low fertility soils make it perfect forage for drought prone semi-arid regions of Africa. It produces palatable and nutritious biomass(Yuseika et al., 2006)resulting in an increase livestock productivity. *Brachiaria* grass enhances nitrogen use efficiency, sequesters carbon and reduces greenhouse gas emissions**(**Djikeng et al., 2014). These desirable attributes make *Brachiaria*, an ideal forage option which currently ranks as the world's most extensively cultivated tropical forages in monoculture. Despite high agricultural and environmental utility ,the cultivation of this native flora is extremely limited in Africa. In this context, a native *Brachiaria* grasses is the perfect candidate as it is known for high yield, drought tolerance, resistance to pest and diseases, adapted to different soil types and climatic conditions of tropics and subtropics areas. East Africa harbor wider genetic diversity among and within *Brachiaria* species. This part of the world (centre of host origin) should also represent the highest diversity in the microbes communities associated with this grass. Plant-microbe interactions both above- and belowground have been recognized as keys to unlocking plant productivity, with transferable symbioses providing access to novel plant traits such as enhanced drought resistance, decreases in toxicity, increases in thermo-tolerance, and improved nutrient content(Ghimire, 2014).

All plants in natural ecosystems appear to be symbiotic with fungal endophytes.The fossil record indicates that plants have been associated with endophytic and mychorrhizal (Redecker et al., 2000; Krings et al., 2007) fungi forover 400 million years and were likely associatedwhen plants first colonized the land. Highly diverse group of fungi can have impacts on plant communities through increasing fitness by conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption or decreasing fitness by altering resource allocation (Rodriguez, (2008); Lugtenberg et al.,(2016)). , Endophytes are as microorganisms (bacteria/fungi) living inside plant tissues (leaves, stems, roots, and fruits) without causing any symptoms of disease in the host(Saikkonen et al., 2004; Schulz & Boyle, 2005). "Endophyte" means "within the plant".

Plants benefit extensively by harboring these endophytic microbes because they promote plant growth and confer enhanced resistance to various pathogens by producing antibiotics . Endophytes also produce unusual secondary metabolites of plant importance under stress conditions together with some valuable pharmaceutical substances of biotechnological interest(Neha Chadha et al.,2015). On the basis of host-microbe evolution, taxonomy, and ecological functions, endophytes are divided into two major groups: (i) calvicipitaceous (C-endophytes), or class I endophytes which inhabit some grasses and (ii) non--calvicipitaceous (NC-endophytes), or class II endophytes which are associated in the asymptomatic tissues of non-vascular plants, ferns and allies, conifers and angiosperms (Harman, 2011; Rodriguez et al.,2009). There are several types of endosymbiotic fungi that grow within roots and shoots .Certain fungal endophytes permit adaptation and survival of plants to high salt stress habitats .But, under severe water stress, endophyte infected plants maintained better leaf expansion and produced significantly more leaf biomass than did the clean plants(Kelemu and Rao,1998). Endophytic fungi have been known to play an essential role in plant growth, especially grasses; however, few reports have elucidated their symbiosis with crops. Therefore, this study aims at analyzing fungal endophytes community inhabiting in the aerial

tissues of local *Brachiaria* grasses from Rwanda representing place of origin for most of the *Brachiaria* species. And generate information on the available endophytic fungi associated with these ecotypes that may have important role on plant host productivity under extreme environments.

Materials and Methods

Brachiaria plant materials

The plant materials used in this study were36 Rwandan Brachiaria ecotypes collection in 2014 from Bugesera, Nyamagabe and Rwamagana districts of Rwanda The whole plant samples were collected and transported to the College of Agriculture, Animal Sciences and Veterinary Medicine of University of Rwanda, and maintained individually in plots with regular watering in a greenhouse (Table.1). After three months, young stems and leaves of 36 Brachiaria ecotypes were collected in separate zip lock bags, then transported on ice chest from collection sites to BecA-ILRI, Hubin, Nairobi, Kenya for analysis.

Plant sample processing

Plant materials (stems and leaves samples) were thoroughly washed with running tap water, rinsed and cut under sterile conditions into small pieces of 3–4 cm prior to rigorous surface sterilization with (95% ethanol for 30s, 70% ethanol for 5 min followed by 0.6% and 1.2% sodium hypochlorite (JIK) and a drop of TTween 20 for 25–30 min for stems and leaves respectively. Surface sterilized tissues were rinsed three times with sterile water, blot dried, cut into small pieces (1–1.5 cm) and plated on PDA plates amended with three antibiotics (100µg/ml Ampicillin, 50µg/ml Streptomycin Sulfate and 50µg/ml Chloramphenicol). The plates were then incubated in the dark for up to 1 month at 24°C and examined regularly for emerging fungal colonies. Emerging fungal colonies(fig.2) were sub cultured twice to ensure attainment of pure cultures prior to preparing agar slants for long-term storage and collecting fungal materials for DNA extraction.

DNA extraction

Fungal material for DNA extraction was harvested from 1 to 2 week-old on pure fungal cultures grown on potato dextrose agar (PDA) amended with 100µg/ml Ampicillin. Colonized agar blocks of approximately 1.5 cm3 were cut and placed in a 1.5 ml micro-tube with a single 4.5 mm stainless steel bead. These micro-tubes were arranged in a rack and covered with an Air Pore filter, stored at −80°C overnight and lyophilized for 24 hrs. DNA of the fungal isolates was extracted from lyophilized tissue using QIAGEN MagAttract 96 DNA Plant Core Kit according to the manufacturers' instructions. The quality and quantity of extracted DNA were checked using 1.0% agarose gel stained by 2.5 µl GelRed and quantified using NanoDrop 2000C Spectrophotometer.

Polymerase chain reaction, sequencing and database search

The DNA has been diluted to 1µl of DNA/10 µl of distilled water for PCR. The universal fungal primers-The internal transcribed spacer (ITSIF & ITS4) regions of fungal ribosomal DNA (rDNA) were used in this study for PCR amplification because they are highly variable in sequence, and thus of great importance in distinguishing fungal species (White et al. 1990).The fungal specific primers ITS1F and ITS4, amplifying the highly variable ITS1 and ITS4 sequences surrounding the 5.8S-coding sequence. Then, the PCR master mix was calculated in 50µl reaction volume consisting;2500 µl of bulk premix(2.5 µl 10x pfu buffer with MgSO4, 0.5 µl of 10Mm dNTPs , 0.5 µl pfu DNA polymerase and 17.5 µl of RNAse free water), 200 µl of ITSIF ,200 µl of ITS4 ,1900 µl of ddH20 and 2 µl of DNA and distributed into a plate of 96wells for PCRPCR program(4 min at 94 oC for initial denaturation followed by 40 cycles of 45 sec at 94 oC for denaturation, 45 sec at 50 oC for annealing temperature, 45 sec at 72oC for extension with 10 min for final extension). Quality of PCR was checked in agarose gel electrophoresis and then under UV light. Finally the ITS-amplified DNA was purified using QIAquickRMultiwell PCR Purification (QIAGEN) and submitted for DNA sequencing.

Gene sequences were manually inspected, edited and appended into contigs using CLC genomic workbench DNA sequence assembly software Sequencher® version 4.9 (Gene Code Corporation, Ann Arbor, Michigan). These sequences were then subjected to BLASTn searches against the NCBI non redundant database and the top three hits (with lowest e-value) were used to assign identities to test isolates at the deepest possible taxonomic resolution. A Phylogenetic tree was constructed using MEGA 6 inferred by using the maximum likelihood method, based on Kimura2-parameters at 1000 bootstrap replications.

Biochemical characterization of endophytic fungal

1. Phosphate solubilization

The National Botanical Research Institute's phosphate (NBRIP) media consisting of a mixture of 10g glucose, 5g Ca3(PO4)2 ,5g Mgcl2.6H2O, 0.25g MgSO4.7H2O, 0.2g KCl, 0.1g (NH4)2SO4 in a liter with de-ionized water. pHof the media was adjusted to 6.8 using 0.1M KOH solution(Kumar et al., 2012). 15g Agar was then added and autoclaved at 15 psi for 20-30 min .The poked NBRIP media was poured in the square plates and 3µl of fungal mycelia were cultured in the four corners of plate. A Phosphate-solubilising bacteria CSB_B039 was used as control cultured in centre of plate. Air dried inoculated plates were put into sealed containers and incubated upside down at 28oC. The plates were then monitored for the presence of clearance zones around the inoculums.

2. Indole-3-Acetic Acid (IAA) production

The purpose of this test was to see whether the isolates endophytes fungi have ability to produce IAA, plant hormone known as auxins which contributes to the plant growth and biomass production. Assayed by colorimetric method using Ferric chloride–perchloric acid (FeCl3–HClO4) with modifications, Patel et al.(2012).The isolates fungi were grown in 1500 µl of Czapek solution (pH 6.5)containing 2g NaNO3, 1g KH2PO4, 0.5 g MgSO4.7H2O, 0.5 g KCl, 0.01g FeSO4.7H2O, 30g sucrose supplemented with 25g DL-tryptophan/L and incubated in a rotary shaker for 5 days at 100 RPM at 28oC. The pH of the media was adjusted to 6.8. The fungal supernatant (800µl of each sample) was harvested after centrifugation at 2500RPM for 25 minutes and transferred into transparent flat-bottom of 24 wells containing 1600 µl of Salkowski reagent(yellow in color) composed by HCl04, FeCl3 and ddH20 . After thorough mixing , the samples were kept in dark area for 20-45 minutes. After 45 minutes, the IAA production was quantified using Gen5-Optical density reading (OD) at 535 nm of absorbance and compared to the standard curve to determine the concentration of IAA production by each isolate fungal.

3. Fungal antagonistic test

To screen the identified endophytic fungi for biological control against phytopathogens, a modified dual culture method was used Companile et al, (2007). Six fungal pathogen (Nigrospora grisea (MGO1), Fusarium equisetii (F5), Nigrospora sphaerica(F10), Aspergillus flavus(F6), Phoma herbarum (F20) and Nigrospora oryzae(F25) were used against endophytic fungal to test if the isolates fungi had capability to protect plant against pathogens fungi. On PDA media supplemented with 100µg/ml ampicillin in Petri plates, the pathogen fungus was cultured in the centre of plate and surround by the cultures of four endophytic fungi (3cm apart) in one plate. The cultures were parafilmed and incubated at room temperature(25oc) for 21days. If any endophyte fungal played a role of plant protection against pathogen fungus it inhibits the growth of the pathogen. The identified fungi that suspected to give positive results were sub-cultured for test confirmation.

4. Pathogenicity test

The total of 169 fungal isolates were tested for their ability to inhibit/kill or promote growth of collard seeds grown in the lab. Each isolate was ground to make inoculants by grinding 5mm x 5mm agar block in 300µl sterile water. Each inoculant was spread on PDA media amended with 100µg/ml ampicillin, parafilmed and incubated at 25°C for three days in the dark to allow growth of the fungi. The sterilized vegetable collards seeds with 1.2 % of Sodium hypochloride were sown randomly on grown fungus in small plate (6 seeds/plate) as well as a control plate with PDA amended media only incubated at room temperature (24°C) and monitored for germination and persistence over a two week period.The number of seedlings per plate were

then recorded in comparison to number of seedlings in plates without inoculants (control).

5. Siderophores production

To test siderophores production, Fiss glucose minimal media was used as an iron-restricted media, because Siderophores are only produced under iron-limiting conditions. The Fiss glucose minimal media was prepared bydissolving 5.0 g K2HPO4 and 5.0 g L-asparaginein 950 ml of ddH20 and pH was adjusted to 6.8,then the media was autoclaved. After, the sterile media was amended with: 50%glucose, 0.005% ZnCl2, 0.001% MnSO4, and0.4% MgS04. The Chrome Azurol S (CAS Assay)media amended with 10ml of iron III and 40ml of Hexadecyl-trimethylammonium (HDTMA) solutions was also used to detect the presence of siderophores, because it has high affinity for ferric iron. CAS is blue in color. When Siderophoresare present, releases the orange dye.

The isolates fungi were grown in two variants of the Fiss-glucose media: one supplemented with 0.5 µM FeSO4 (low iron) and the other supplemented with 20 µM FeSO4 (high iron). Cultures were grown for 5 days on a rotary shaker at 100RPM and the supernatant from each was collected by centrifugation at 2500RPM for 35 minutes. Using No.2 a cork borer, wells were bored into a CAS media plate and 60 µl aliquots of each culture supernatant were distributed in separate well. Sterile CAS media was also added to a well as a control. The plates were then incubated at room temperature. Depending on the culture, color formation may take 30 minutes to 5 hours (protocol). Development of yellow or orange halos around the well indicated the ability of Siderophores production (Vallore, 2001).

Results and discussion

Isolation of endophytic fungi

In this study, ,morphologically a total number of 169 fungal emerged (additional file:Fig.10) and 21 of them were identified. The commonly isolated endophytes in the order of high to low occurrence were from the genus *Epicoccum nigrum*, *Cladosporium cladosporioides*, *Coprinopsis atramentaria, Mucor hiemalis, Alternaria alternate,Gibbella zeae, Mucor racemosus and Phoma ssp.* Other twelve genera were represented once, twice or three times in the population (Table2).

Table 2: Isolated endophytes, genus/species and percentage identity from NCBI (National Centerfor Biotechnology Information website)

Endophytes isolate	Fungal taxa	Number	Accession No.	% identity
EP_ITS	***Epicoccum nigrum***	**36**	**FJ904918.1**	**100**
Gl_ITS	***Glonium pusillum***	**1**	**EU552134.1**	**99**
Pe_ITS	***Peyronellaea glomerata***	**3**	**KC802087.1**	**98**
SY_ITS	***Syncephalostrum racemosum***	**2**	**JN689349.1**	**99**
Ph_ITS	***Phoma ssp***	**5**	**EU343168.1**	**99**
St_ITS	***Stagonosporospis cucurbitacearum***	**1**	**HQ684031.1**	**99**
Bo_ITS	***Boeremia exigua***	**1**	**KF428212.1**	**99**
Al-al_ITS	***Alternaria alternata***	**12**	**DQ023279.1**	**100**
Al-te_ITS	***Alternaria tenuissima***	**5**	**HG798721.1**	**100**

Ma_ITS	***Macrophomina phaseolina***	**1**	**DQ233665.1**	**99**
Mi_ITS	***Microsphaeropsis arundinis***	**1**	**HA607976.1**	**99**
Le_ITS	***Leptosphaerulina chartarum***	**1**	**HQ607815.1**	**99**
Pa_ITS	***Paraphaeosphaeria michotii***	**1**	**JX496079.1**	**99**
Cla_ITS	***Cladosporium cladosporioides***	**13**	**AY251074.2**	**99**
Fu_ITS	***Fusarium cortaderiae***	**2**	**KF576625.1**	**99**
Gi_ITS	***Gibberella zeae***	**7**	**AB250414.1**	**100**
Fu-Eq_ITS	***Fusarium equiseti***	**3**	**KM246255.1**	**99**
Co_ITS	***Coprinopsis atramentaria***	**1**	**FJ478115.1**	**97**
Mu-hi_ITS	***Mucor hiemalis***	**13**	**GU566266.1**	**100**
M-ra_ITS	***Mucor racemosus***	**7**	**AJ271061.1**	**97**
Ap_ITS	***Apodus deciduus***	**1**	**AY681199.1**	**89**

- Identification and phylogenetic analysis, to determine the relatedness among fungi populations revealed a wide range of fungal genera associated with local *Brachiaria* grasses. The phylogenetic tree showed three clusters of fungal species from 21 taxa represented by different colours (fig. 1).Rodriguez, (2008), Lugtenberg et al.,(2016) reported the highly diverse group of fungi in plant host which can have great impacts on plant communities through increasing fitness by conferring abiotic and biotic stress. This is the first study that documents the endophytic fungal community from the aerial tissues of *Brachiaria* grasses from Rwanda and revealed that the isolated fungal are diverse.

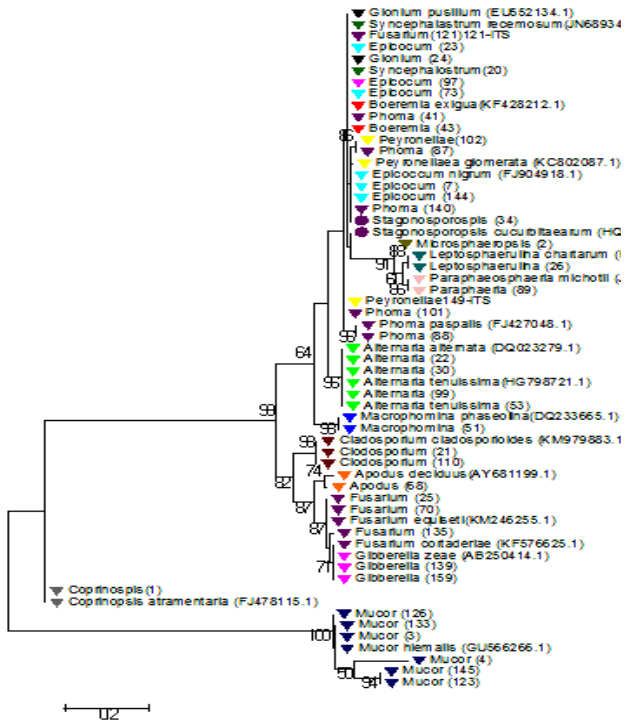


Fig.1 Phylogenetic tree of endophytic fungal communities isolated from the stems leaves of local Rwandan *Brachiaria*.

The taxonomic affiliations of these endophytes clearly showed them all as Class1 clavicipitaceous endophytes (Rodriguez,2008) and all are included in ascomyceta group .These Class 1 endophytes occur exclusively in the above-ground tissues (Ghimire, 2015) . Many authors reported that *Brachiaria* grasses harbor diverse group of endophytic fungi in above ground aerial tissues. It was very surprising that *Acremonium implicatum*, a common endophyte of Brachiaria species in South America(Ghimire,2015) was not detected at all in African *Brachiaria* including ecotypes from Rwanda.

- **Biochemical –characterization of endophytic fungal**

The isolates fungi from stems and leaves of 36 Rwandan *Brachiaria* ecotypes were tested for Phosphate Solubilisation,Indole-3- Acetic Acid(IAA)production, Antagonistic test, Pathogenicity test and Siderophores testto check some novels traits that could be beneficial to plant growth and/or suppression of plant pathogens .

Phosphate Solubilization test

This test was carried out on 169 isolates fungi, to see if fungi are capable of hydrolyzing organic and inorganic phosphorus from insoluble compounds, to make it available in soil for plant uptake. The result showed a clear zone surrounding bacteria(CSB_B039), compared with the 169 isolates fungi in which no clear zone identified (Fig.2).This may be due to all fungi used in this test were not located in under-ground ofthe plant (Ghimire, 2015).

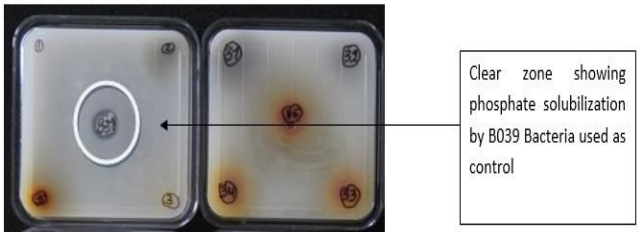


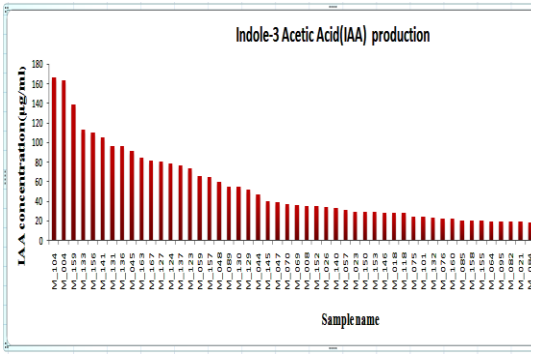
Fig.2: Phosphate-solubilizing bacteria-fungal test cultured in square Petri dish.

Indole-3- Acetic Acid (IAA) production

Indole-3-acetic acid (IAA) is the most common, naturally-occurring, plant hormone known as auxins which contributes to the plant grow and biomass production.

The purpose of this test was to see whether the isolates endophytes fungi from Rwandan ecotypes might produce auxins. The result showed that 30.1% of total isolate fungal were positive for IAA(fig.3)

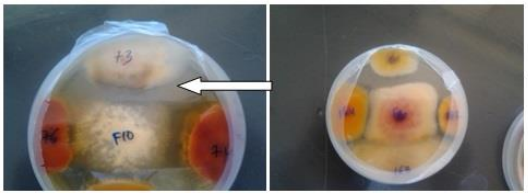
Fig.3: The IAA production indicated by Pink color



- Fig.4: IAA Concentration(µg/ml)
- As shown in above figure, 62 out of 169 isolates fungal tested were able to produce significant quantity of indole compounds. According to Abdul at al., (2015 endophytic fungi showed their role in improving crop productivity under abiotic stresses such as salinity, drought, temperature and heavy metal pollution while producing gibberellins.

Fungal-antagonistic test

- This test was carried out on 169 isolates of fungi, and fungal plant pathogen (*Nigrospora grisea* (MGO1), *Fusarium equisetii* (F5), *Nigrospora sphaerica*(F10), *Aspergillus flavus*(F6), *Phoma herbarum* (F20) and *Nigrospora oryzae*(F25) were used against endophytic fungi to test if the isolates fungi had capability to protect plant against pathogens by inhibiting the growth of the pathogen. After 20 days of incubation, 10.1% were antagonistic to *Nigrospora sphaerica*(F10), 8.3% to *Phoma herbarum*(F20)and 5% to *the Aspergillus flavus*(F6)(fig. 4)



- Fig.5: Antagonism between isolates and plant pathogen fungal (F10, right and F20, left)

This antagonism test *in vitro* was in concordance with reports of several microbiologists (White and Cole, 1986; Siegel and Latch, 1991; Christensen, 1996) which confirmed the antagonism existing between endophytic fungi and grass pathogens. Biological control of pathogens and pests through micro-organisms that can inhibit or antagonize them could be promoted, not only to reduces the costs of bio-chemical pesticides but also saves the environment against pollution due to the wide spread use of pesticides. Few studies have shown that Endophytes confer stress tolerance to host species and have a significant role in the survival of some crop plants under high-stress environments (Rodriguez et al., 2004).

Pathogenicity test

The purpose of this test was to confirm whether the isolates fungi had capability to induce germination of the seeds and promote also the grow of the plant, or kill the seeds by producing toxic substances. 47.3 % of the total isolates fungal were not pathogen to the plant host. This was confirmed by good germination of collards seeds in some plates after two weeks of seedling (fig. 5)

Fig.6: Seedling

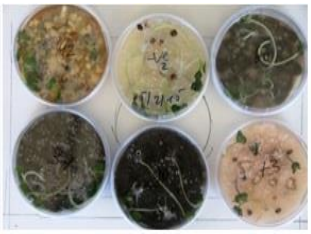


Fig. 7: Seeds germination (7days old)

In this test, 80 out of 169 isolates fungal showed good symbiosis with collards plant when compared with the control(-Ve_2).According to Neha Chadha et al.,(2015) plants benefit extensively by harboring these endophytic microbes because they promote plant growth and confer enhanced resistance to various pathogens by producing antibiotics. This author reported that Endophytes also produce unusual secondary metabolites of plant importance under stress conditions.

Siderophores production

Siderophores are low molecular iron chelating compounds produced by micro-organisms in response to limited iron(Ahmed and Holmström (2014)).Through this pathway, micro-organisms are able to acquire iron and avail it to the plants for their survival under limited conditions. The result showed that49 % of isolates fungal were able to produce Siderophores which was highlighted by orange colour (fig.9)

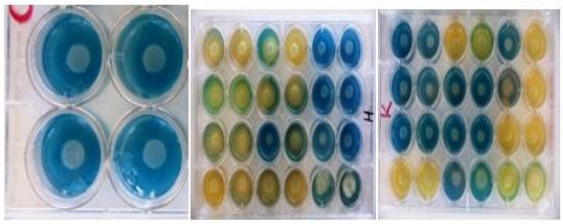


Fig.8: The Control (uncultured media) Fig.9: The Siderophores production (orange colour)

Fe is an essential micronutrient for plant growth (Kobayashi and Nishizawa, 2012). According to Kraemer et al., (2006), under conditions of Fe deficiency, graminaceous plants such as barley and wheat have developed an efficient strategy for acquiring Fe from insoluble sources. This is in the same case of our study *Brachiaria* species .These plants secrete Fe(III)-chelating compounds called phytosiderophores that form specific strong complexes with Fe(III) (Ma, 2005).Furthermore, Siderophores has an important role in weathering soil minerals and enhancing plant growth(Crowley,2006). In additional, Siderophores has also important function in biocontrol, biosensors, and bioremediation as chelation agents (Ahmed and Holmström, 2014).

Conclusion

Brachiaria harbor complex and diverse fungal community possibly with varied roles to host plant ranging from plant pathogen to beneficial organism. This study presents the preliminary data of the aerial endophytes community of *Brachiaria* grasses and opens opportunities for further research on the utilization of these microbes for agricultural and industrial applications. This symbiotic technology may be helpful in mitigating impacts of climate change in crops and expanding agriculture production into marginal lands. In this case, we are simply evaluated the natural symbiotic populations of a wild grass for their incorporation into the same species.

Table 1. *Brachiaria* ecotypes used in genetic diversity study and collection details

Ecotype ID	Genus	Species	Status	Collection date	Country	Altitude (m asl.)	Lat. (S)	Long. (E)
RW_EcoBug01 (rw1)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1387	-2.2776	30.2599
RW_EcoBug02(rw2)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1384	-2.2776	30.2588
RW_EcoBug03 (rw3)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1387	-2.2776	30.2577
RW_EcoBug04 (rw4)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1465	-2.2704	30.2043
RW_EcoBug05 (rw5)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1407	-2.2546	30.0552
RW_EcoBug06 (rw6)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1407	-2.2545	30.0554
RW_EcoBug07 (rw7)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1395	-2.2545	30.0561
RW_EcoBug08 (rw8)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1576	-2.1854	30.0901
RW_EcoBug09 (rw9)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1574	-2.1855	30.0901
RW_EcoBug10 (rw10)	Brachiaria	Not known	Wild	6/6/2014	Rwanda	1387	-2.2777	30.2596
RW_EcoNya01 (rw11)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1712	-2.4964	29.5942
RW_EcoNya02 (rw12)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1914	-2.4662	29.5752
RW_EcoNya03 (rw13)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1903	-2.4652	29.5753
RW_EcoNya04 (rw14)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	2009	-2.4814	29.5564
RW_EcoNya05 (rw15)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	2009	-2.4814	29.5564
RW_EcoNya06 (rw16)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	2079	-2.4817	29.5365
RW_EcoNya07 (rw17)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	2080	-2.4817	29.5367

Ecotype ID	Genus	Species	Status	Collection date	Country	Altitude (m asl.)	Lat. (S)	Long. (E)
RW_EcoNya09 (rw18)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	2082	-2.4817	29.5356
RW_EcoNya11 (rw19)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1719	-2.4969	29.5933
RW_EcoNya12 (rw20)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1737	-2.4971	29.5923
RW_EcoNya13 (rw21)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1880	-2.4783	29.5875
RW_EcoNya14 (rw22)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1880	-2.478	29.5874
RW_EcoNya15 (rw23)	Brachiaria	Not known	Wild	6/11/2014	Rwanda	1883	-2.4776	29.5874
RW_EcoRwa01 (rw24)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1430	-1.8976	30.2746
RW_EcoRwa02 (rw25)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1446	-1.8924	30.2983
RW_EcoRwa03 (rw26)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1444	-1.8922	30.2983
RW_EcoRwa04 (rw27)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1443	-1.8925	30.2984
RW_EcoRwa05 (rw28)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1445	-1.8928	30.2988
RW_EcoRwa06 (rw29)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1436	-1.8925	30.2989
RW_EcoRwa07 (rw30)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1462	-1.9109	30.312
RW_EcoRwa08 (rw31)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1462	-1.9108	30.3119
RW_EcoRwa09 (rw32)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1600	-1.9411	30.3311
RW_EcoRwa10 (rw33)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1428	-1.8975	30.2747
RW_EcoRwa11 (rw34)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1430	-1.8973	30.2749
RW_EcoRwa12 (rw35)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1430	-1.8969	30.2754
RW_EcoRwa13 (rw36)	Brachiaria	Not known	Wild	6/12/2014	Rwanda	1433	-1.8966	30.2753

RW_Eco: Rwandan ecotype; Bug=Bugesera, Nya= Nyamagabe, Rwa=Rwamagana
[1]Numbers in parentheses are sample ID used for Phylogenetic analysis

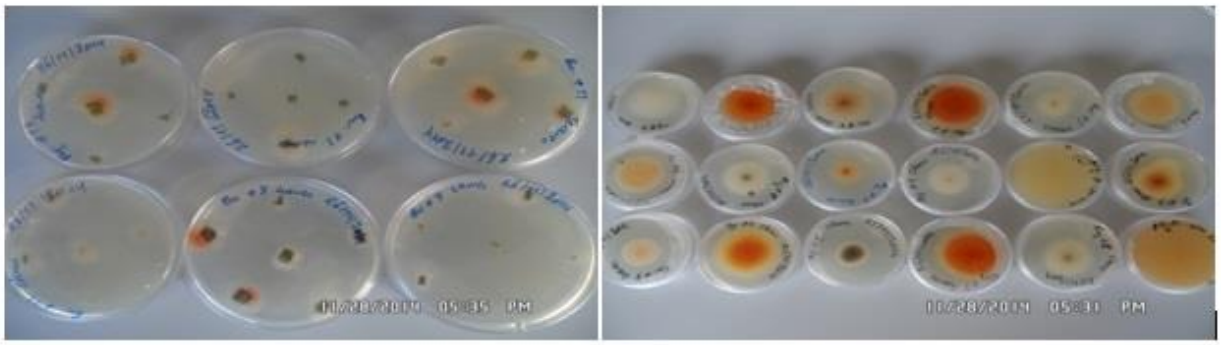


Fig.10 Diversity of emerging fungal colonies from 36 local *Brachiaria* ecotypes

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