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Antifungal activity of epibiotic bacteria isolated from Kenyan Coastal marine cyanobacterium, *Lyngbya majuscula* against phytopathogenic *Rhizoctonia solani*

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Abstract: *Lyngya majuscula is a filamentous marine cyanobacteria is known to be a source of wide array of natural products. Some of these products exhibit anti-microbial, anti-HIV, anti-proliferative, ant-cancer and photo-protective activity. It has also been reported to be associated with epibiotic bacteria on its surfaces. However the antifungal activity of the epibiotic bacteria associated with the Kenyan Coastal marine Lyngbya majuscula is not well documented. This study intended to assess the antifungal activity of epibiotic bacteria isolated from the surface of the Kenyan Coastal marine cyanobacterium Lyngbya majuscula. Eleven isolates were tested against Rhizoctonia solani. Some of these isolates showed antagonistic activity against the fungi, an indication that some these bacteria could be able to produce metabolites with antifungal activity. These epibiotic bacteria could find application as seed dressers to offer critical protection to the delicate seedlings before they get established in the field. This is the first report on the antifungal activity of epibiotic bacteria associated with the Kenyan Coastal marine cyanobacteria, Lyngbya majuscula.*

Key words: *Lyngbya majuscula, Cyanobacteria, Antifungal activity, damping-off disease*

1. Introduction

Fungal diseases of crop plants have increasingly become a major concern for agricultural production. The convectional practices to overcome this problem have been through the use of chemical fungicides, which may have several adverse effects on the environment, besides being a major health hazard to humans and other non target biota [1]. Thus there exists a pressing need to reduce the losses caused by these fungal diseases by developing environmental friendly practices.

The use of naturally occurring organisms such as bacteria, actinomycetes and fungi as a means of biological control has been addressed globally [1]. Fluorescent *Pseudomonads* have also been reported in the control of several soil borne and wilt diseases in important crops such as wheat, rice and commercial fruits and vegetables [2]. Among cyanobacteria, *Nostoc muscorum* is known to be effective against "damping off" disease caused by fungi [1] and several *Anabaena* and *Calothrix* strains exhibit fungicidal activity against species of *Pythium, Fusarium* and *Rhizoctonia*[1].

Rhizoctonia solani is a plant pathogenic fungus with a wide host range and worldwide distribution [3]. It is the major fungus responsible for damping-off, black spot and root rot diseases [3]. With most vegetables, few effective fungicides are available against *Rhizoctonia* diseases. The intensified use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and environment besides the buildup of resistance in pathogens [2]. These national and global problems can be solved by seeking alternatives to chemical control by investigating the use of antagonistic microbes [3].

Lyngbya majuscula is a marine filamentous cyanobacterium that has been reported to produce a wide array of natural products. More than three hundred (300) compounds have been isolated from this species alone [4]. Some of these products have been reported to exhibit bioactivities including anticancer, anti-inflammatory, antibacterial, antifungal and anti-infective therapeutic agents [5] and photoprotective agents such as scytonemin and mycosporine-like amino acids (MAAS) [6]. This species has also been reported to be associated with brightly coloured epibiotic bacteria (EB) that are reported to be a major source of phenazine compounds that have antimicrobial, antitumor and anti-malarial [7]. However the exact role played by the EB is not well established. This study aimedat establishing the antifungal activity of epibiotic bacteria associated with the Kenyan Coastal marine cyanobacterium, *L. majuscula* against the phytopathogen *R. solani.*

2. Materials and methods

2.1. Test organisms

The bacterial strains used in this study were previously isolated from the Kenyan marine *L. majuscula* collected from four different sites along the Kenyan Coast namely Mida (039.99505º to 039.96600ºE) and Kilifi (039.785˚E to 039.835˚E) in the North Coast and Shimoni (039.36565ºE to 039.36696ºE) and Wasini (039.35906ºE to 039.35942ºE).

The fungi *Rhizoctania solani* isolate C54 belonging to AG1-IC originally isolated from sugarbeet was used in this study.

2.2. Screening of the antagonistic effect of Epibiotic Bacteria against *Rhizoctonia solani* (isolateC54)

Screening of bacterial antagonist was carried out using dual culture assay (Dikin et al 2006). One 6-mm diameter plug of *R. solani* mycelia cultured for 3-5 days on PDA was placed at the centre of fresh PDA medium in a 9 cm diameter Petri dish. Bacterial isolate from a 24 hour old colony was introduced with sterile pin. A distance of 3 cm was maintained between the *R. solani* agar plug and bacterial isolate. Plates were sealed with parafilm and incubated for 5 days at 28 ± 2°C and examined for any evidence of growth inhibition.
Inhibition values were estimated using the formula described by [8]

$$\text{Inhibition Index} = \left[1 - \left(\frac{A}{B}\right)\right] X100\%$$

Where:
A = Distance between fungi in the center of Petri dish to epibioitic bacterial isolate
B = Distance between fungi in center of Petri dish and blank area without epibioitic bacterial isolate.
The treatments were replicated 3 times

2.3. Identification of the epibiotic with Antifungal Activity

The bacterial strains were identified using molecular methods by amplification and sequencing of the 16S rRNA using the primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT -3'). The PCR conditions were an initial denaturing temperature at 94ºC for 4 minutes, 30 cycles of 1 minute at 94ºC , 1 minute at 55ºC , 1 minute at 72ºC, and a final extension 72ºC for 20 minutes. PCR products of the amplified DNA were purified using Zymo Research DNA Clean & Concentrator^{TM} -5 kit (Zymo Research Corporation) using the manufacturer's instructions. The PCR products were then sequenced bi-directionally by automated sequencing using a 3130xl sequencer (Applied Biosystems).
Editing and alignment of the DNA sequences was performed using Bioedit software version 7.2.5.0. The obtained nucleotide sequences were compared to known sequences of the NCBI database using the BLAST tool and aligned with the closest relatives in terms of nucleotide sequence similarity. 16S rRNA gene sequences were deposited in the GenBank database under the accession numbers KY646114, KY646123, KY646127, KY646128 andKY646137.

3. Results

A few of the isolated epibiotic bacteria showed antagonistic activity against *Rhizoctonia solani,* with the highest zone of inhibition being 13.5mm. Isolate SHM175-1 had the highest growth inhibition effect against R. solani C54 while SHM172-4 had the lowest activity of the tested bacteria isolates with activity. Isolate MD587-2 had no antifungal among the tested isolates. There was no significant difference between the colors of epibiotic bacterial isolates and their zones of inhibition (Chi-square value=28.11, df=24, p=0.26).

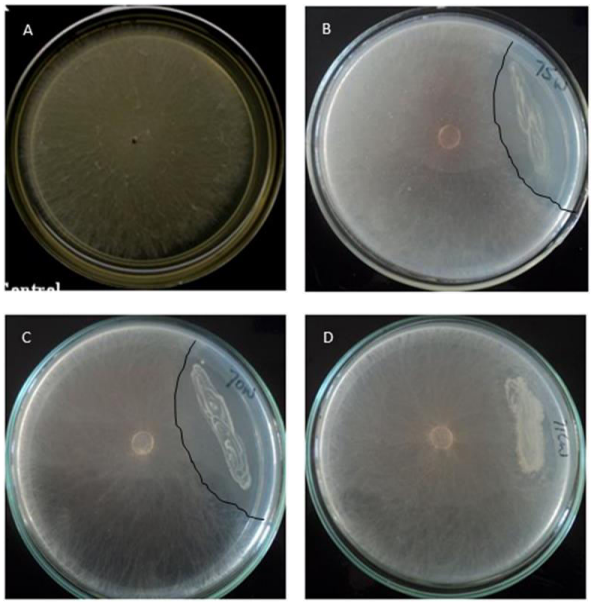

Figure 1: Antagonistic activity of epibiotic bacteria against *Rhizoctonia solani.* A: control, B: isolate SHM175-1, C: isolate SHM170-3 and D isolate SHM171-3.

Table 1: Antagonistic assay of epibiotic bacteria against *R. solani*

Isolate	Colour	Morphology	Inhibition zone (mm)	% inhibition	Antagonistic activity
SHM175-1	White Milky	Bacilli	13.5	45	+++
WAS184-1	Cream yellow	Cocci	12	40	+++
MD505-3	Yellow	Cocci	10	33	+++
SHM171-3	White milky	Bacilli	10	33	+++
SHM74-3	Cream white	Bacilli	10	33	+++
SHM170-3	White milky	Bacilli	9	30	+++
WAS182-1	White milky	Bacilli	9	30	+++
SHM176-5	Cream white	Bacilli	5	17	++
WAS180-1	White milky	Bacilli	5	17	++
SHM172-4	White milky	Bacilli	2	7	+
MD587-2	Pink	Cocci	0	0	-

Notes; - No inhibition, + low (1 to 5mm), ++ moderate (6 to 10mm), +++ high (11-15mm), SHM- Shimoni, WAS-Wasini, MD-Mida

Table 2: Identity of epibiotic bacteria co-existing with the Kenyan Coastal Marine ecosystem *Lyngbya majuscula*

Isolate	Accession number	Closest relative	% Similarity	Group
SHM175-1	KY646114	Bacillus aerius strain 24K	94	Bacilli
SHM171-3	KY646123	Bacillus aryabhattai strain B8W22	98	Bacilli
SHM170-3	KY646128	Alcaligenes faecalis strain NBRC 13111	91	β-proteobacteria
WAS180-1	KY646127	Exiguobacterium profundum strain 10C	94	Bacilli
SHM172-4	KY646137	Bacillus aerius strain 24K	96	Bacilli

4. Discussion

This is the first study to describe the antifungal activity of bacterial isolates co-exisiting with the Kenyan Coast marine cyanobacterium *L. majuscula*. The increased concern over the impact of agrochemicals to the environment has resulted into increased popularity on the use of biological agents as an option for the control of pests and diseases [9].

In recent years, *fluorescent pseudomonads* have drawn attention worldwide because of their production of secondary metabolites such as siderophores, antibiotics like 2, 4-diacetylphloroglucinol, volatile compounds (HCN), enzymes and phytohormones [2]. The work in this study has demonstrated the ability of epibiotic bacterial isolates to control the fungus *R. solani*. Dual culture studies have shown that most of the tested isolates inhibited fungal growth on agar. The inhibitory zones exhibited by these epibiotic bacterial isolates suggest the presence of fungistatic metabolites secreted by the bacteria.

Eight (8) of the tested bacterial isolates used in this study were morphologically bacilli, out of which four (4) were phylogenetically identified to be closely related to the *Bacilli* group while one belonged to the *β-proteobacteria* group.
The finding that the *Bacilli* had an antagonistic effect against the fungi *R. solani* corroborates that of [8]Wahyudi et al. (2011), where some genera including the *Bacillus* were determined as Plant Growth Promoting Rhizobacteria (PGPR). These findings suggest that the *Bacilli* used in this study acted as PGPR whose indirect effects are related to production of metabolites, such as antibiotics, siderophores, or HCN, that decrease the growth of phytopathogens and other deleterious microorganisms [8].

Although the epibiotic bacterial isolates used in this present study exhibited varied colors, there was no significant difference between their color and the corresponding zones of inhibition. This may suggest that ability to exhibit antifungal activity was not dependent on the presence or absence of color but could have been contributed by the presence of other factors such as metabolites of the bacteria.

5. Conclusion and Recommendation

We conclude that epibiotic bacteria co-existing with Kenyan Coast cyanobacterium *Lyngbya majuscula* are capable of inhibiting the growth of *R. solani in vitro*. These isolates can be suitable candidates for application to control damping-off caused by *R. solani*. There is need for further research to establish the kind of metabolites produced by these epibiotic bacteria.

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