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Original Article

Diversity of Ethiopian Kale *(Brassica carinata)* Endophytes and their Antagonism against *Colletotrichum higginsianum in vitro*

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Abstract

Brassica carinata is an important underutilized cruciferous vegetable that has strong potential for reducing food insecurity and boosting crop production due to its drought and pest resistant qualities. The vegetable supported a total of 5128 colonies with bacterial colonies higher at 4440 while fungal colonies were 688. Bacterial colonies were isolated in high amounts at 10^{-1} while fungal colonies were highly recovered at 10^{-5}. Bacterial results revealed that 37% were circular shaped while rhizoid form were lowest at 4.5% and the majority were gram positive. The Bacilli bacteria were more dominant over the cocci shaped bacteria. The roots harbored higher number of nitrogen fixing bacteria at 75% of the isolated endophytes as compared to roots and stem. Additionally, the roots contributed 60% of the endophytes that had the ability to solubilise phosphates. Antifungal results showed that four endophytes inhibited the growth of *Colletotrichum higginsianum.* Fungal isolates exhibited more *Fusarium* species isolated but *Aspergillus spp* had the highest phosphorous stabilization efficiency (PSE). One *Fusarium spp* had the capacity to inhibit the growth of *C. higginsianum*.

Keywords: Ethiopian kale; Antifungal; Anthracnose; Nitrogen fixation; Phophorous solubilization

INTRODUCTION

The vast majority, approximately 80% of Kenya's agricultural land is classified as arid or semi-arid, and Kenya's susceptibility to droughts and flooding is expected to increase due to climate change in coming decades. Ethiopian Kale's *(Brassica carinata),* drought hardiness makes it a fitting crop for the region's agricultural landscape. There is little written history on Ethiopian Kale's traditional uses and farming patterns, but modern uses range from medicinal values for fighting off certain cancers, to use for development of jet bio-fuels for aircraft [1]. The nutritious vegetable is grown particularly by poorer small scale farmers in rural communities but is not grown extensively despite being identified as particularly viable food crop for arid and semi-arid agricultural communities [2]. It is used by farming families for household consumption rather than commercial purposes and this has made the Kenyan government to promote research and higher production of African Indigenous Vegetables (AIVs) and other traditional Kenyan foods [3]

Ethiopian kale, like other brassicas, is susceptible to a range of pests and diseases, including aphids, diamondback moths, downy mildew, black rot and anthracnose. Anthracnose caused by the fungus *Colletotrichum higginsianum* is one of the major diseases for brassica family and can cause significant losses under favorable environmental conditions. It can lead to yield losses ranging from 10-30% in some cases, and can even cause complete crop failure in severe situations [4].

Endophytes are a type of non-pathogenic fungal and bacterial endosymbionts. They colonize plants and are able to enhance plant growth and nutrient gain without causing disease to the plant hosting it [5]. During establishment of plant tissue, endophytes have the ability to establish symbiotic relationship with the plant thus making them adequate biocontrol and medicinal agents [6]. Some of these benefits include increased tolerance to drought, or resistance to infection from pests and disease. Endophytes can appear in many parts of the plant such as the roots, stem, fruit, seeds, etc., but they a plant does not necessarily have endophytes on it, often inheriting endophytes and their endophytic activity from its parents, and their presence on plants is highly variable [5]. Endophytes are known to enhance plant growth, improve defense, increase their tolerance to environmental stress, and facilitate nutrient uptake [7]. Endophytes may positively influence host's biosynthesis pathways and gene expression systems to promote the production of particular secondary metabolite. The main aspect of endophytes is that they can be easily isolated, cultured, are amenable to genetic manipulations, and can be scaled up for bioactive compound production [8]. In view of great importance of endophytes to both plant and human health, there is an increased focus on developing endophytes into herbal remedies [9]. Therefore, there is need to incorporate the knowledge of endophytes in Kenya's forgotten crops, especially the Ethiopian kale that is of nutritional importance to small-scale farmers and the entire country at large.

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MATERIALS AND METHODS

Sample Collection and Preparation

The experiment was carried out at Egerton University Research Field 7 which is located at Njoro Sub-county in Nakuru County, Kenya. It is located at 0°22`S 35°56`E and lies at an altitude of about 2267 m above sea level. The site receives an average annual rainfall of 1800 mm annually with average temperature ranges of between 10.2-22.0°C. The distribution of rainfall is bimodal with long rains between April and August and short rains between October to December. The soils are clay loamy and well drained with a pH of 5.5. Primary and secondary tillage. were done to obtain fine tilth in April 2023. Well decomposed farm yard manure was applied at the rate of 10 t/ ha and mixed thoroughly with the soil before planting. Certified Ethiopian kale seeds were sown at a spacing of 30 cm by drill. Weeding was done manually by uprooting the weeds as they emerged. No pesticides were applied on the crops throughout the season. The plants were carefully uprooted at flowering stage and bagged in sterile bags and transported to Egerton Biotechnology lab.

Plant samples were cleaned with running tap water for 10-15 minutes to remove soil particles then air dried on blotting paper. The roots, stem and leaves were separated from the plant and cut up into small, one-gram pieces using a weighing scale. The samples were soaked in distilled water and drained on blotting paper. Surface sterilization was done by dipping the pieces in 70% ethanol for 30 seconds then dipped in 4% sodium hypochlorite for five minutes (stem and leaves) while the roots were dipped in 4% sodium hypochlorite for ten minutes then treated with 70% ethanol. The pieces were rinsed five times in sterilized distilled water and blot-dried with sterile filter paper.

Isolation of Endophytes

Isolation was done following the procedure described by Sharma and Roy [10]. Briefly, samples were macerated in sterile distilled water using a mortar and pestle. Each sample underwent a series of serial dilutions, up to 10^{-5}. One hundred microliters of each sample dilution were then placed separately into petri plates with nutrient agar medium (NA) for bacterial isolation and potato dextrose agar (PDA) medium, supplemented with Kanamycin antibiotic for fungal isolation. Plating was done in triplicate for each dilution. Bacterial endophyte plates were incubated at 37°C for 72-96 hours while fungal endophyte plates were incubated at 28°C for two weeks. The isolated bacterial endophytes were sub-cultured and maintained as pure cultures on nutrient agar medium, and fungal endophytes were maintained on potato dextrose agar medium.

Determination of bacterial population

Colony forming units (cfu) of bacterial endophytes were calculated after incubation at 37°C for four days, and 28°C for fourteen days for fungal endophytes. Colony counts are expressed in cfu/g. The colony forming units of fungal and bacterial isolates in roots, stems and leaves were calculated using the following formula:

Cfu/g = number colonies / (dilution factor * dilution plated)

The following categories of observational data were collected: colony type, margin of the colony, colony elevation, color of colony, and surface and opacity of colony.

Characterization of fungal endophytes was conducted using a reference manual on endophytes and based on different morphological features such as growth of fungi, color of colony (front and reverse), size and shape of colonies. Microscopic identification of endophytic fungi was done by lactophenol cotton blue staining technique.

Screening of Endophytes for Plant Growth Promotion (PGP)

The isolated endophytes were tested for plant growth promotion characteristics. The nitrogen-fixing ability of bacterial endophytes was detected by inoculating the isolated pure endophytic bacterial cultures on Jensen's media and incubated at 37°C for five days.

The phosphate solubilizing ability of bacterial and fungal endophytes was detected via spot inoculation of pure isolated endophytic bacterial and fungal cultures, separately, on Pikovskaya's medium, which is regularly used for cultivating phosphate solubilizing microorganisms. The endophytic bacterial cultures were incubated at 37°C for three days and the fungal cultures were incubated at 28°C for seven days, alongside control plates that were not inoculated. All inoculations were done in triplicate. The phosphate solubilization efficiency (PSE) was determined by the following formula.

$$PSE = \frac{diameter\ of\ halogenation\ zone\ -\ diameter\ of\ entire\ colony}{diameter\ of\ entire\ colony} \times 100$$

Isolation of Anthracnose Pathogen *(Colletotrichum higginsianum)*

Diseased Ethiopian Kale infected with anthracnose were collected from field seven at Egerton University in Kenya at the same time as healthy plants. Leaf cuttings of anthracnose were isolated from the diseased plant and sterilized in the same way as the healthy plant but were not macerated. The diseased part with a small lining of healthy leaf were cut using sterile scalpel then inoculated to PDA plates and incubated at 28°C for fourteen days. After culturing, to test which endophytes on healthy plants resisted anthracnose, the fungal growth on diseased plant plates was dual cultured with endophyte isolates for the same incubation period.

RESULTS

Endophyte isolation from Ethiopian kale

Brassica carinata had a total of 5128 bacterial and fungal endophyte colonies. Bacterial colonies were higher at 4440 (Table 1), while fungal colonies were 688. Bacterial isolation from the leaves were the highest at 1596 colonies followed by 1462 and 1383 colonies from the stem and roots, respectively. The highest recovery of bacterial colonies was at a dilution of 10^{-1} with 1113 colonies while the lowest was at 10^{-5} with 627 colonies.

Characterization of endophytic bacteria

Morphological characteristics of the isolated endophytes from Ethiopian kale showed that most of the bacteria were circular in form at 37% while rhizoid form were lowest at 4.5%. For bacterial color, yellow were the majority at 55% while cream white were the least at 5%. Gram staining results indicated that most of the bacteria were gram positive at 58% that gram negative at 42%. The bacilli shaped bacteria were more dominant that the cocci shaped bacteria (Table 1).

Table 1: Morphological traits of isolated bacteria from Ethiopian kale

Isolation	Color	Form	Gram Stain	Shape	Total colonies
RB1	White	Filiform	-	Bacilli	363
RB2	Yellow	Circular	-	Cocci	588
RB3	Cream White	Circular	-	Cocci	34
RB4	Cream Yellow	Filiform	+	Bacilli	397
SB1	White	Filamentous	-	Bacilli	18
SB2	Yellow	Circular	+	Cocci	1022
SB3	Cream White	Rhizoid	+	Bacilli	77
SB4	Cream Yellow	Irregular	+	Bacilli	345
LB1	White	Filamentous	-	Bacilli	193
LB2	Yellow	Irregular	+	Bacilli	817
LB3	Cream White	Rhizoid	+	Bacilli	94
LB4	Cream Yellow	Irregular	+	Bacilli	492

Table 2: Nitrogen fixation of isolated endophytes.

Source	White	Yellow	Cream White	Cream Yellow
Root	+	+	-	+
Stem	-	-	+	+
Leaf	-	+	-	+

Key: + indicates positive nirogen fixation

- indicates no nitrogen fixation

Nitrogen fixation by bacterial endophytes

A total of 7 (58%) endophytes out of the 12 isolated bacterial endophytes were able to fix nitrogen fix nitrogen. From the roots, 75% of the isolated bacteria were able to fix nitrogen while the root and leaf endophytes had 50% bacterial endophytes in each that were able to fix nitrogen. The root bacterial endophytes had the highest nitrogen fixing bacterial endophytes at 43% of the total bacterial able the fix nitrogen (Table 2).

Phosphorous solubilization of bacterial endophytes

Bacterial endophytes from the roots contributed 60% of the bacterial endophytes that solubilized phosphates. The highest solubilization was by the bacterial endophyte was from the stem with over 100 phosphorous soubilization efficiency (Figure 1).

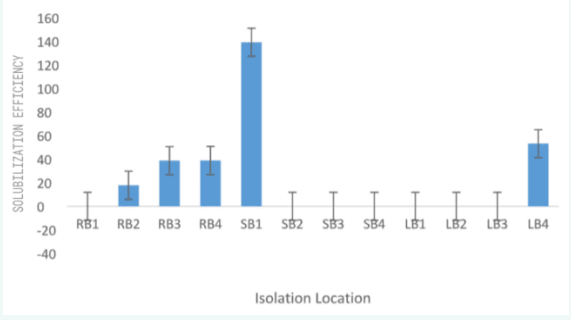


Figure 1: Phosphorous solubilization efficiency (PSE) of bacterial endophytes

Bacterial endophytes antagonism against *Colletotrichum higginsianum in vitro*

Colletotrichum higginsianum was visible as a cottony mycelium on PDA media. RB2 inhibited the growth of the *C. higginsianum* by over 50% where the fungi was seen growing away from the bacterial endophyte while LB4 had no inhibition effect as the fungi was seen growing over the bacteria (Figure 2).

Morphological characterization of fungal endophytes

Fusaium spp was the most isolated fungi at over 50% from the roots, stem and leaves. Others isolated include yeast from the roots, *Alternaria spp, Aspergillus spp* and *Botrytis spp* all from the leaves (Figure 3).

Phosphorous solubilzation of fungal endophytes

One *Fusarium spp* from the roots, *Aspergillus spp, Fusarium spp* and *Botrytis spp* from the leaves solubilised phosphates on pikovskayas media. *Aspergillus spp* had the highest PSE of over 100% while *Botrytis spp* had the lowest 15% (Plate1; Figure 4). No fungal endophyte from the stem solubilised the phosphates

Plate 1: Phosphorous solubilization of fungal endophytes on Pikovskayas media a) *Apergillus spp* b) *Fusarium spp* c) *Borytis spp*

Antifungal effect of fungal endophytes against *Colletotrichum higginsianum*

One *Fusarium spp* from the stem inhibited the growth of the pathogen as it was seen growing away from the endophyte (Plate 2).

DISCUSSION

Brassica carinata is one of the most important vegetables in East Africa but has been largely neglected in research. Although not as widely grown as the common kale, this vegetable has the potential to become an important substitute for other vegetables to improve food security, ensure production resilience and maintain the health of consumers. The isolation of endophytes in *B. carinata* is the first of its kind and it offers a foundation for future insights for enhanced production. Results revealed higher isolation of bacterial endophytes at 87% as compared to fungal endophytes at 13%. Similar results were reported by Jzar *et al.* [11], who reported bacterial isolation at 99% and fungal at 1% from chia plant where the most dominant bacteria were *Pseudomonas, Bacilli and Cocci.* While both bacterial and fungal endophytes are found in plants, research suggests that bacterial endophytes are often more abundant and diverse than fungal endophytes. Studies indicate that plant roots can harbor more diverse and widespread bacterial communities compared to fungal communities. Furthermore, bacterial endophytes have been shown to have a greater impact on plant growth and biomass compared to fungal endophytes in some cases [12].

This report observed that most of the bacterial endophytes were gram positive at 58% as the compared to the gram negative at 42%. This result is in agreement with the investigation by Chauhan and Singh [6], who found out that bacterial endophytes isolated from periwinkle medicinal plant were 60% and 40%, gram positive and gram negative, respectively. Sgroy *et al.* [13], reported 68.9% Gram positive bacteria and 31.1% Gram negative in the root of *Prosopis strombulifera,* while Panchal

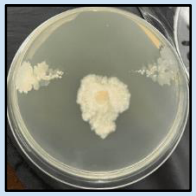
Isolation	Dual culture	Anti-fungal +/-
Control		
RB2		+
SB1		+
		+
LB4		-

Figure 2: Dual culture of bacterial endophytes with *Colletotrichum higginsianum* + indicates bacterial endophyte has anti-fungal effect while (-) no anti fungal effect.

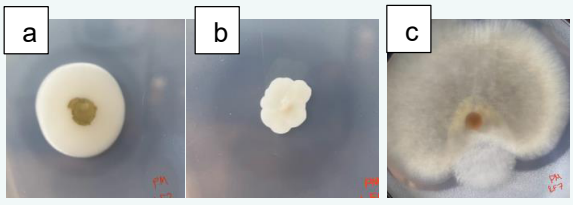


Plate 1: Phosphorous solubilization of fungal endophytes on Pikovskayas media a) *Apergillus spp* b) *Fusarium spp* c) *Borytis spp*

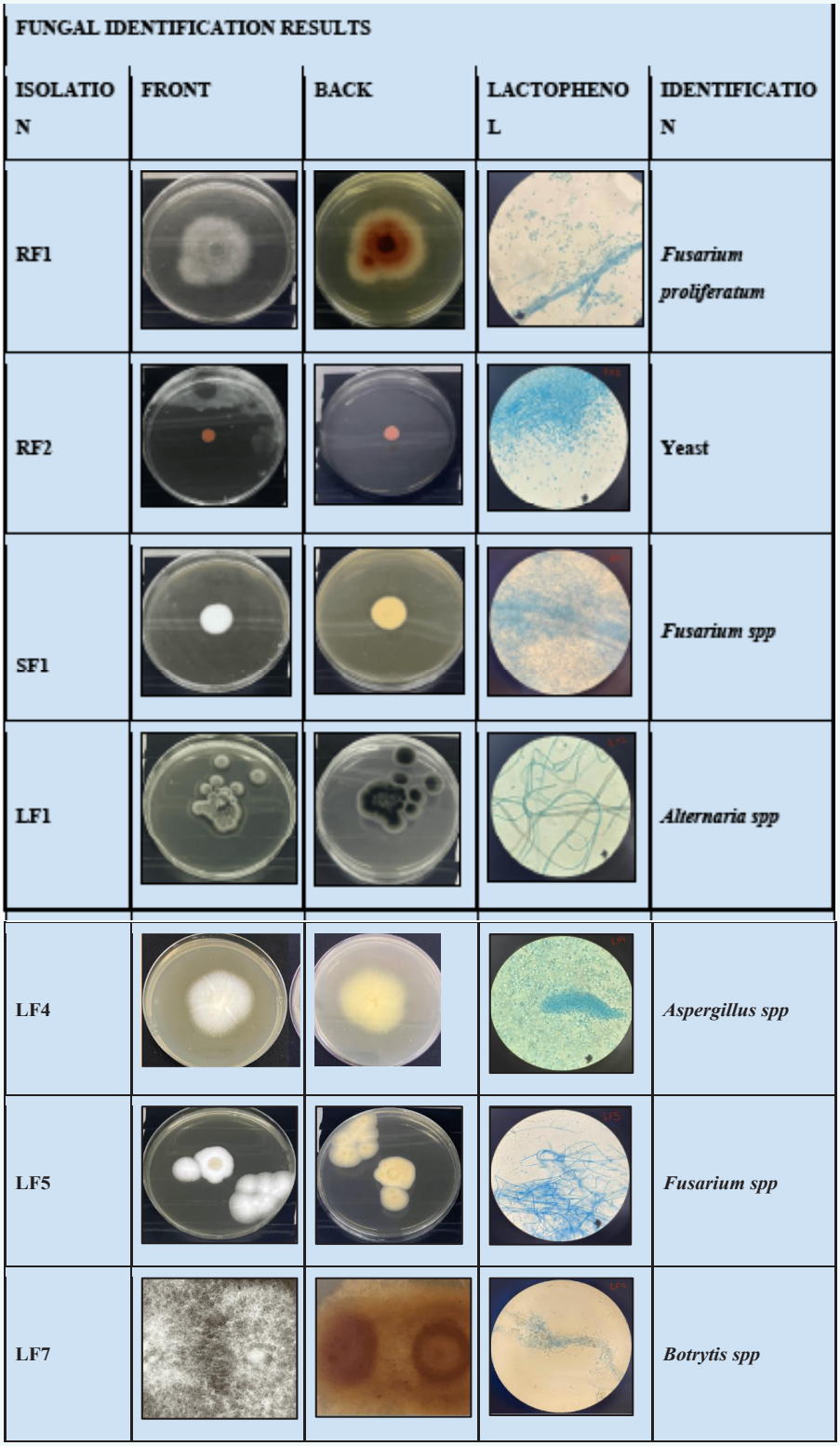


Figure 3: Morphological characteristics of some isolated fungi (Pycnidiophores were observed at x400).

Plate 2: Antifungal effect of fungal endophyte against *Colletotrichum higginsianum* in dual culture a) Normal growth of *Colletotrichum higginsianum* as control b) *Fusarium spp* antifungal effect against *Colletotrichum higginsianum*.

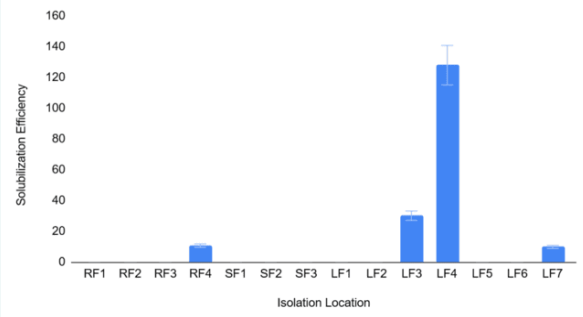


Figure 4: Phosphorous solubizationn efficiency (PSE) of the isolated fungal endophytes

and Ingle [14], found 91.6% root endophytes to be Gram positive. However, Bind and Nema [15], isolated endophytic bacteria from pigeon pea and noted that out of 40 endophytic bacterial isolates 25 of the isolates were Gram negative while 15 were Gram positive. The Gram's staining is based upon the biochemical characteristics of the cell wall because this reaction depends upon the presence of relative amount of peptidoglycan and lipids in the cell wall and on the presence of outer membrane.

Together, the experiments reported in this research support the overall hypothesis that plant growth and vigor are directly related to the composition of the endophytic community within the host plant and provide further evidence of biological nitrogen fixation in non-leguminous vegetable species. The nitrogen fixation observed in this study would support *B. carinata* ca colonization of nutrient-deficient sites. Plants are colonized by diverse bacteria that have the capacity to carry out PGP by producing ammonia gas [16]. The study further revealed that five bacterial and six fungal endophytes had the ability to solubilize phosphates on Pikovskayas media. These phosphate-solubilizing microorganisms transform insoluble phosphate into a soluble form through the production of organic acids, phosphatases, or other complex agents [17]. The predominant forms of organic phosphorus are phytates, which make up 60% of soil organic phosphorus [18]. For the phytates to be absorbed by plants, they must first be dephosphorylated with phosphatase enzymes [19]. Therefore, the application of phosphate solubilizing microorganisms to fields has been reported to increase crop yield and the extensively examined microbial mediated species has been the use of bacteria and filamentous fungi [20]. Endophytic yeast has been reported to play a role in P-solubilization by several mechanisms, such as lowering the pH by acid production, iron chelation, and exchange reactions in the growth environment [21]. Application of these important endophytes in agriculture could reduce inputs of water and inorganic fertilizers.

This article reported three bacterial and one fungal endophyte to have antifungal activity against *C. higginisianum in vitro.* The present finding proves that bacterial and fungal siderophores are potent agents that can be used against Brassica plant pathogens. The results corroborates with [22], who revealed that microorganisms produced siderophores during iron limiting conditions sequester iron (III), thus making it unavailable to the pathogen (Leong, 1986). Earlier findings have reported the use of siderophores in controlling a few pathogenic fungi such as P*ythium ultimum, Sclerotinia sclerotiorum,* and *Phytophthora parasitica,* causing diseases in plants [23].

CONCLUSION

This investigation revealed that Ethiopian kale accommodates wide range of bacteria and fungi that are symbiotic in the roots, stem and leaves. The nutrient-dense vegetable contained more bacteria in the microbiome as compared to the number of fungi. Most of the isolated bacteria were positive for Gram staining. Besides, a large percentage of bacteria from the roots had the ability to fix nitrogen and solubilize phosphorous on the respective media. Most of the fungi isolated belonged to the *Fusarium spp* Some of the fungi isolated have been found to be pathogenic while others are endophytic. One fungi was able to solubilize phosphates and this implies that this endophyte can be incorporated in the soil with the seed to improve phosphorous uptake in the soil. The presence of nitrogen fixing and phosphorous solubilizing endophytes gives a positive insight for future research to produce chia seed at low cost and limit the use of synthetic fertilizers that are pollute the soil and hazardous to man. Further research is however required.

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