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Efficacy of endophytic fungi isolated from Azadirachta indica roots against Alternaria causing early blight of tomato

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ABSTRACT

Many medicinal plants are reported to host a myriad of beneficial endophytic microbes. Among the well-known medicinal plants is Azadirachta indica (Neem; Family Meliaceae), which has gained worldwide importance due to its extensive array of therapeutic and insecticidal qualities. The use of A. indica extracts in the treatment of plant pathogens has been the subject of extensive investigation, but its endophytic microbes as potential biocontrol agents have received very little attention. In this study, the efficacy of endophytic fungi isolated from A. indica roots against Alternaria, which causes tomato early blight, was examined. Isolation and characterization of Alternaria species and endophytic fungi were done in the laboratory using standard procedures. An in-vitro assay of the endophytic fungi isolates against Alternaria was conducted in a complete randomized design in order to determine the percentage zone of inhibition. The colonies of Alternaria isolates were fast-growing, black to grayish-brown, and suede-like. The conidial length from different isolates was statistically significant ($p < 0.05$) and ranged from 15 µm to 46 µm. The conidial widths were not statistically significant ($p > 0.05$) and ranged from 8 µm to 15 µm, while the conidial area ranged from 120 µm to 690 µm. A total of seven species of endophytes were isolated from the root of Azadirachta indica: Phoma, Actinomycetes, Chaetomium, Trichoderma, Verticillium, Penicillium, and Fusarium. There was a significant difference in the zones of inhibition ($p < 0.05$), which ranged from 0.0 mm (Actinomycetes) to 3.44 mm (Trichoderma). These isolates could be used to create brand- new organic antifungal substances that are efficient against a variety of plant fungal pathogens.

Keywords: tomato early blight; Alternaria; Endophytes; Azadirachta indica

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1. Introduction

Important microorganisms called endophytes live inside plant tissues without causing illness. (Varma et al., 2017). They coexist harmoniously with the plant they are living on, and frequently the microorganisms serve as the plant's defense mechanism against outside phytopathogenic fungi (Alvin et al., 2014). They can improve plant growth under all conditions (Qureshi et al., 2019) and have the ability to facilitate crops' ability to withstand environmental stress as well as protect them from other pathogenic microorganisms (Taware and Rajurkar, 2015). Current attention has been focused on the use of antagonistic endophytic fungi as biocontrol

agents as a desirable option for managing various plant diseases with little environmental impact (De Silva et al., 2019). They suppress pathogen growth by involving various mechanisms such as direct antagonism through production of antimicrobial metabolites, induction of systemic resistance, and increasing resistance in plants against pathogens (Kumar and Dara, 2021).

It is believed that nearly every plant species has one or more endophytes, but only a small percentage of existing microbes are well known (Al-Daghari et al., 2020). While receiving little attention for their potential utility in the management of phytopathogens, endophytes have the capacity to serve as a source of candidate strains for prospective biocontrol applications (White et al., 2019). Hence, managing plant diseases through an understanding of endophytic fungi's interactions are essential to sustainable agricultural output (Kumar and Dara, 2021).

Many medicinal plants are known to host a myriad of beneficial endophytic fungi (Alvin et al., 2014). Among the well- known medicinal plants is Azadirachta indica A. Juss. (Neem), belonging to the family Meliaceae, it has grown in significance due to its therapeutic and insecticidal qualities (Dubey and Kashyap, 2016). The plant has an extremely large bio-control potential against a number of pathogens causing various plant diseases (Dohroo et al., 2016). Neem products can be blended with the bio-products of some species of Trichoderma, Gliocladium, and Bacillus thuringiensis to have effective control of various plant diseases (Dubey and Kashyap, 2016).

Endophytic Streptomyces have been isolated from the stem and root tissues of A. indica and were found to have plant growth-promoting activities (Varma et al., 2017). However, much research has been focused on the use of neem extracts in the management of plant diseases (Dheeba et al., 2015; Raza et al., 2016), with very little attention on endophytic microbes as potential biocontrol agents (Jabaen et al., 2013). Dheeba et al., (2015). Together with other commercially significant crops like pepper, eggplant, and potato, the tomato (Lycopersicon esculentum (L.) Karsten) is a member of the Solanaceae family (Dez & Nuez, 2008). It is crucial for research on the basic ideas of plant growth and development as well as for consumption as a fresh crop and in prepared dishes (Hobson & Grierson, 1993). It is the fourth-ranked vegetable in the world (Peralta & Spooner, 2007), with the largest producers being China, the United States, India, Turkey and Egypt (FAO, 2002). The crop is also one of the most extensively grown vegetable crops in sub-Saharan Africa, significantly contributing to both food security and income (Sibomana et al., 2016). The crop can be cultivated in a variety of agro- climatic environments, but it is best grown in well-drained, light loam soil with a pH of 5-7 (Prativa & Bhattarai, 2011).

In sub-Saharan Africa, Kenya is a leader in the production of tomatoes. In terms of value and output, tomatoes are the second-highest vegetable, with production making up 14% of all vegetable produce in the nation (Sigei et al. 2014). It is among the key crops in the horticultural industry in the country and one of the major income-generating vegetable crops (Masinde et al., 2011). In Kenya, the product accounts about 7% of all agricultural output (Ochilo et al., 2019). The leading county in tomato production is Kirinyaga County (14%), followed by Kajiado (9%) and Taita Taveta (7%) (Mwangi et al., 2015). Cultivation of tomatoes may be carried out in different environments with altitudes of 1150–1800 m above sea level (HCDA, 2013). Kenya has increased its tomato production throughout time. Nonetheless, yields are still low because of biotic and abiotic factors, such as disease and insect pests (Ojiewo et al., 2010). Fungal infections have significant economic importance in Kenyan tomato farming (Anastacia et al., 2011).

Tomato early blight, which is caused by a number of Alternaria species, including Alternaria alternata and Alternaria linariae (which includes Alternaria solani and Alternaria tomatophila), is one of the terrible diseases threatening Kenya's tomato output (Adhikari and Panthee, 2017). On a variety of hosts, Alternaria is an opportunistic pathogen that causes leaf spots, rots, and blights on various plant sections. The disease causes significant harm to plants at all phases of growth, and its severity is greatest in areas with high rainfall, temperatures and humidity (Chaerani et al., 2006). Unfortunately, despite the fact that early blight resistance has been bred out of tomato plants, the quantitative expression and polygenic inheritance of the resistance have made it difficult to create cultivars with high levels of protection (Chaerani and Voorrips, 2006). As a result, many smallholder tomato growers employ some cultural control techniques, although synthetic pesticides are mostly used to manage the disease (Ochilo et al., 2019).

Cultural methods, however, are not successful when used alone and require integration with other management methods (Kimani, 2014). On the other hand, fungicides are not economically feasible and have harmful effects on the environment (Waqas et al., 2016). Continuous use of chemicals over a long period of time can lead to some level of fungal resistance (Damalas, 2009). The presence of traces of chemicals and

higher levels of chemical residues are some of the reasons for bans against Kenyan produce on international markets (Rodino et al., 2014). Lack of knowledge of other, more cost- effective, and environmentally friendly methods of pest control may be the cause of the significant reliance on synthetic pesticides (Adhikari and Panthee, 2017). An alternative to the current management practices is therefore necessary to reduce early blight incidence and prevalence.

Natural plant products have been used as important sources of new biocontrols (Naziha and Dalia, 2010). Some natural plant products have the ability to minimize the populations of foliar pathogens and control disease development (Raza et al., 2016). They are environmentally friendly alternatives and potential components of integrated disease management. Biocontrols used in agriculture include microorganisms such as bacteria, fungi, viruses, and protozoa, as well as botanicals such as neem, garlic, pyrethrum, and turmeric extracts, among others (Alexandrov, 2011). Some microorganisms have been used in the management of plant pests and diseases (Amerasan and Jayakumar, 2019; Hounmalon et al., 2014). The most typical strategy involves choosing opposing microorganisms, researching their modes of action, and creating a biological control product. Indirect action through induced resistance of the host plant or microbial interactions directed against the pathogen, which primarily occur during its saprophytic phase, is the antagonistic effects responsible for disease suppression (Alabouvette et al., 2006). In this work, the efficacy of endophytic fungi isolated from A. indica roots against Alternaria, which causes tomato early blight, was examined.

2. Materials And Methods

2.1. Isolation and characterization of Alternaria species

Infected plant samples showing the symptoms of Alternaria black spots, rots, and blights were collected from tomato fields in Tharaka-Nithi County, Kenya. Afterwards, they were cleaned three times with sterile distilled water after being chopped into little pieces and submerged in 1.3% sodium hypochlorite for five minutes. For seven days, they were incubated at 25
°C after being plated on potato dextrose agar (PDA). According to Yu et al., (2016) the pure cultures were made by plucking a single spore and plating it on new PDA under a dissecting microscope. For identification, the cultural and morphological traits of the isolates were noted.

2.2. Isolation and characterization of endophytic microbes of Azadirachta indica

Plant samples of fresh roots of Azadirachta indica were collected from the field as described by Verma et al. (2009). Bark samples (about 5 x 5 cm) were cut from the roots at a depth of one meter and processed within 24 hours of collection. They were surface-sterilized using 70% ethanol for three minutes, 2% sodium hypochlorite containing 0.1% for 20 seconds, distilled water for two minutes, and then sterile distilled water for five minutes (Ujam et al., 2020).

The roots' outer bark was peeled off, and the cortex was thoroughly sliced. The tissues were then cut into 2 mm pieces inside the laminar air flow hood using sterile blades. On a water agar plate with streptomycin added, about 100 segments were plated. They were then cultured at 22
°C for 14 days. The growth of endophytic fungus on the plates was consistently observed. In PDA plates, the hyphal tips that developed on pieces of surface- sterilized bark were isolated and cultured for 2-4 days at 27 °C. After incubation, a number was given to each fungus. The isolates were sub cultured in PDA for seven days to produce pure isolates. The isolates' morphological and cultural traits were noted for identification.

2.3. In-vitro assay of Azadirachta Indica endophytes against Alternaria isolates

A laboratory experiment was conducted in the Biological Sciences Laboratory, Chuka University, Kenya. The experiment was arranged in a complete randomized design (CRD) using one representative isolate of Alternaria sp. and seven endophytic isolates, and replicated five times. Using a sterile cork borer (0.6 cm in diameter), the agar disc of the Alternaria isolate was cut 1.5 cm from the edge of the petri dish. A second identical- sized agar disc with the endophyte isolates was positioned in the same position but on the opposite end of the same petri dish containing the pathogen. The control plate was not inoculated with any endophyte. After inoculation, observations were conducted at two (2), four (4), six (6), and seven (7) days on all plates that were incubated at room temperature. The pathogen's colony diameter was measured, and the percent (%) inhibition was calculated using the formula below:

$$\%\ \text{inhibition} = \frac{(\textit{colony diameter without extract microbes} - \textit{colony diameter with extract microbes})}{\textit{colony diameter without extract isolates}}$$

2.4. Data analysis

The acquired data variables were subjected to an analysis of variance using SAS statistical software version 9.4, and significance means were separated using the least significant difference (LSD) test at 5% levels of significance.

3. Results And Discussion

3.1 Morphological characteristics of Alternaria isolates

Various symptoms were observed in the field during sampling (Fig. 1). These include spots of light-brown to dark brown, roundish-oval to irregular spots of 2 mm diameter in the early stage on foliar parts. This expanded when the infection reached an advanced stage. Black spots on the fruits with concentric rings were also observed.

Figure 1: Symptoms of tomato early blight disease caused by Alternaria spp.

The colonies of the Alternaria isolates were fast-growing, black to grayish-brown, and suede-like (Fig. 2). Conidiophores arose singly or in clusters of up to 10, which showed varied shapes from obclavate to ovoid to ellipsoidal. Spores formed in chains; club shaped with beaks of varying lengths

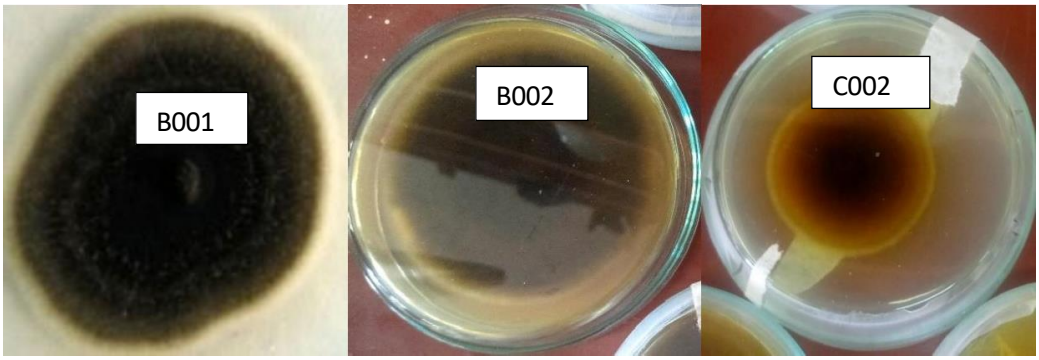

Figure 2: Cultural characteristics of Alternaria isolates on Potato Dextrose Agar (PDA) isolated from diffferent farms: B001 (Kathwana), B002 (Kairini) and C002 (Maara).

The conidia length of the isolates was statistically significant ($p < 0.05$) and ranged from 15 µm to 46 µm (Table 4). The conidial widths were not statistically significant ($p > 0.05$) and ranged from 8 µm to 15 µm,

while the conidial area ranged from 120 µm to 690 µm. Microscopic examination showed a septate mycelium that was profuse and grayish in color. The mycelia were long, irregularly branched and multicelled.

Table 1: Conidia Length, Width and Area of Alternaria Isolates

Isolate ID	Conidia length (µm)	Conidia width (µm)	Area of conidia (µm)
A001	46[a]	15[a]	690[a]
A002	44[a]	12[ab]	528[ab]
B001	42[b]	13[b]	546[ab]
B002	30[b]	8[ab]	240[b]
C001	38[b]	10[a]	380[b]
C002	15[b]	8[ab]	120[b]
Mean	35.8333	13.333	417.3333
LSD (α = 0.05	17.9167	11	1110.3443
CV (%)	8.73	10.58	18.73

aMeans followed by the same letters are not significantly different at 5% probability level.

3.2. Characterization of Endophytic Microbes isolated from the roots of Azadirachta indica

From the roots of A. indica, seven different endophyte species were discovered. These were: Phoma, Actinomycetes, Chaetomium, Trichoderma, Verticillium, Penicillium,and Fusarium (Table 5).

Table 5: Morphological characterization of endophyte isolates from Azadirachta indica root

Isolat	Colony texture	Surface color	Zonation	Spores	Hyphae	Endophyte
P001	Powdery to velvety	White-cottony	Smooth margin	Chlamydospores	1.55 x 1.08 µm	*Phoma*
T005	Powdery	Dark green to yellow	Irregular margin	Present	3-7 phialides	*Trichoderma*
A002	Velvety	Grey	Regular margin	Spore chains	Hyphae with septum	*Actinomycetes*
P003	Rough and tuberculate	Greyish-green	Regular	Penicilli spores	Hyphae(100-200µm)	*Penicillium*
C004	Powdery	Brown-whitish	Irregular	Ascospores present	Brownish hyphae(30-40µm)	*Chaetomium*
V005	Fluffy myecilium	Creamy white	Irregular	Present	Microsclerotia with cylindrical hyphae	*Verticillium*
F001	Chamydospores are smooth	Paleto peach-violet	No zonation	Present	Hyaline-septated	*Fusarium*

3.2. Antifungal activity of Azadirachta indica root endophytic isolates against Alternaria

The zones of inhibition of endophytic isolates varied significantly ($p < 0.05$), which ranged from 0.0 mm to 3.44 mm (Table 6). The highest zone of inhibition was shown by Trichoderma (3.44 mm), followed by Verticillium (3.24 mm) and Chaetomium (3.18 mm). Actinomycetes showed no zone of inhibition.

The variation in morphological characteristics among Alternaria isolates observed in this study is similar to those reported in other studies. Hubballi et al. (2010) reported a variation in cultural characters among 15 Alternaria isolates. The mean mycelial growth in different media ranged from 80.23 to 89.80 mm. As observed in a polyhouse condition, Alternaria generated abundant mycelium with an average width of 4.42 m, conidiophores of 42.26 x 4.29 m, and conidia measuring 47.16 × 13.49 m, respectively (Nagrale et al., 2013). The mycelium was hyaline at first, but as it developed, it became gray-brown, multicelled, septate, and irregularly branching. Sein et al. (2020) observed no significant variation in Alternaria colony color, pigmentation, margin, or growth pattern.

Table 6: Effects of endophytic isolates of Azadirachta indica root against Alternaria

Endophytic isolates	**Zone of inhibition**
Trichoderma	3.44[a]
Verticillium	3.24[a]
Chaetomium	3.18[a]
Fusarium	2.64[b]
Penicillium	2.52[bc]
Phoma	2.18[c]
Actinomycetes	0.00[d]
Mean	2.4571
LSD	0.3462
CV (%)	10.88

aMeans followed by the same letters are not significantly different at 5% probability level

The isolates had grayish, compact mycelium with a dark center and small, dark concentric rings and circular margins. A study by Kirareia et al. (2019) also reported the same morphological characteristics, where conidiophores arose singly or in clusters of up to 10. Spores were large and appeared dark with short beaks and a fine, long septum, as reported by Saleem and Amany (2022). Cluster findings show two classes of small-spored Alternaria species in molecular identification of Alternaria species: The Alternaria infectoria group and the Alternaria alternata group, each of which could be further classified into three groups (Andersen and Thrane, 1996). Additionally, an examination of chemical and physiological data revealed that 39 Alternaria isolates clustered into three separate groupings that may be distinguished morphologically as Alternaria alternata, Alternaria longipes, and Alternaria gaisen (Andersen et al., 2001), similar to the report by Roberts et al. (2000).

The seven fungal isolates from the roots of A. indica are similar to those isolated from the same plant by Al-Daghari et al. (2020). Chaetomium, an endophytic fungus, demonstrated a broad spectrum of antifungal action against the pathogenic microorganisms under test (Kaur and Singh, 2020). Moreover, according to Zhang (2011), Chaetomium has compounds with potent antifungal action against fungi that cause plant diseases. The fungus has been utilized to combat Candida albicans, Escherichia coli, Bacillus subtilis, and Pseudomonas fluorescens (Awada et al., 2014). Verticillium, on the other hand, is a fungal pathogen that causes wilt in many plant species but also acts as an endophyte in others (Wheeler et al., 2019). The physiological status of the host, fungus, and/or environment controls the timing of transitions between the two trophic stages. Due to the crude extract's potent antifungal activity against Pyricularia oryzae P-2b, the endophytes were isolated from the roots of wild Rehmannia glutinosa and chosen for chemical and biological research (You et al., 2009).

According to reports, endophytic Penicillium may colonize ecological niches and defend its host plant against a variety of pressures. This is done by displaying a wide range of biological functions that can be used for a wide range of applications, including agricultural, biotechnological, and pharmaceutical ones (Toghueo and Boyom, 2020). Penicillium endophytes may provide gibberellins to plant hosts, which is crucial when the plant is experiencing biotic or abiotic stress (Leito & Enguita, 2016). Antibiotics have also been made using some Penicillium species (Kharwa et al., 2010). The search for new biologically active metabolites from endophytes have been studied extensively, and more than
280 substances with antimicrobial, anticancer, antiviral, antioxidant, anti- inflammatory, antiparasitic, immunosuppressant, anti-obesity, anti- fibrotic, neuroprotective, insecticidal, and biocontrol activities have been identified (Toghueo and Boyom, 2020; Sullivan and White, 2000). In addition, the fungus Phoma has been shown to be an efficient biocontrol agent for powdery mildew (Zhang et al., 2012).

Additional substances from the same genus demonstrated considerable growth inhibition against the pathogens in bioactivity experiments against two plant pathogenic bacteria and four plant pathogenic fungi (Wang et al., 2012). To combat Verticillium, Ceratocystis, Cercospora, and Sclerotinia, the fungus secretes a special concoction of volatile organic chemicals (Strobel, 2011). Hence, endophytic Phoma is a viable source of new and beneficial natural metabolites.

On the other side, Trichoderma is a bio-fungicide that has been applied to soil and seeds to prevent problems brought on by fungi (Ahuja and Bhatt, 2018). Due to the species' well-known biological control mechanisms for plant diseases, plant development, and the synthesis of secondary metabolites in agroecosystems, it has been widely used in agricultural applications (Nur and Badaluddin, 2020). When Trichoderma hamatum isolate (DIS 219b) colonized cacao, drought-induced alterations in stomatal conductance, net photosynthesis, and green fluorescence emissions were postponed (Bae et al., 2009) while another isolate prevented cacao's black pod rot, caused by Phytophthora palmivora (Hanada et al., 2008).

In a different investigation, the parasitic and antibacterial potential of six Trichoderma isolates against Phytophthora capsici in hot pepper was examined (Bae et al., 2011). All six isolates colonized roots, generated defense-related expressed sequence tags (ESTs), and postponed the onset of illness.
Fusarium encompasses genetically and phenotypically diverse strains, some of which are major soil-borne pathogens of economically important plants (Kang et al., 2014). It is the causative agent of Fusarium wilt, a disease affecting a wide range of commercially domesticated plants and crops (Joshi, 2018). However, Fusarium has been known to be a biological control agent against Cannabis sativa and striga weed (Tiourebaev et al., 2010). Native grasses with a variety of phylogenetically distinct tissue types were also demonstrated to have mycotoxin-producing Fusarium species, albeit the grasses appeared to accommodate these toxigenic fungi differently than farmed crops (Lofgren et al., 2018). These endophytic bacteria may offer defense mechanisms and survival strategies in their host plants due to the synthesis of a variety of secondary metabolites that are both chemically unique and structurally unheard of (Toghueo, 2020).

Actinomycetes on the other hand are unicellular, gram-positive bacteria and important soil organisms that produce biologically active compounds that have been used as antibiotics and insecticides (Ala et al., 2018). Bioactivities such as cellulases, mannanases, xylanases, lipases, proteases, and antimicrobial substances are secreted by actinomycetes (Jeffrey et al., 2011). Actinomycetes have the potential to develop novel antibiotics that are effective against gram-positive bacteria and dermatophytes, according to research by Rotich et al, (2017).

The various endophytic fungi isolated from A. indica have also been tested for antimicrobial activity in vitro (Rajagopal and Suryanarayanan, 2000; Verma et al., 2011), and have been effective in controlling various microorganisms (Jabaen et al., 2013). This effect could be attributed to antagonism, possibly through the production of fungicidal compounds or extracellular components (Chatterjee et al., 2019; Girish, 2020). Endophytic fungi from different tissues of A. indica have been reported to play a major role as antimicrobial, antioxidant, and pathogenicity target compounds (Chutulo and Chalannavar, 2018). The differences in antifungal activity observed among isolates may be attributed to the production of different biologically active volatile organic compounds (Choińska et al., 2020). Some endophytes such as Trichoderma, Penicillium, and Chaetomium have been effective against Alternaria that causes leaf necrotic spots of Syzygium cumini (Shafique et al., 2019), fungal diseases of banana (Win et al., 2021), and leaf spots of tomato (Fayyadh, 2019), respectively. Another instance is where antimicrobial activity of endophytic Actinomycetes from
A. indica was tested against a variety of pathogenic fungi and bacteria and 60 percent of the isolates exhibited inhibitory action toward one or more harmful fungus and bacteria (Verma et al., 2009). Endophytic bacteria are also present in the leaves of A. indica and are reported to control some gram-positive and gram- negative bacteria (Singh et al., 2017). These isolates may be used to create novel natural antimicrobial compounds as well as possible bio-control agents against a variety of harmful fungus and bacteria (Verma et al., 2009).

4. Conclusion

Alternaria, the causative agent of tomato early blight, is a plant pathogen that causes severe losses in tomatoes. This study revealed that fungal endophytes isolated from A. indica roots are capable of managing the disease and, therefore, can be used in integrated disease management. Identification of bio-compounds produced by the isolated endophytes is necessary, as is molecular characterization of Alternaria isolated from tomatoes. The fungal isolates inhibited the pathogen hence field experiment is required to determine the efficacy of microbial isolates of A. indica as a biocontrol.

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