ASSESSMENT OF ANTAGONISTIC POTENTIAL OF BACTERIA AS BIOCONTROL AGENT AGAINST ALTERNARIA LEAF SPOT OF TURNIP

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ABSTRACT

Alternaria leaf spot is a severe disease of turnip which produces necrotic spots on leaves. In the current study, six varieties of turnip were tested against this pathogen to check their susceptibility. Among these, purple gold variety was moderately susceptible on which three antagonistic bacterial isolates Bacillus subtilis, Streptomyces hydrogenans and Pseudomonas flourescene were evaluated against Alternaria brassicicola with three different concentrations 2, 3 and 5%. Among these B. subtilis gave best control with 23.852% disease incidence followed by Streptomyces hydrogenans (22.183%), Pseudomonas flourescene (21.742%) as compare to control. The pure sample was further used for dual culture technique and the inhibition zone technique against target pathogen. Results from the dual culture test exhibited that all the antagonistic bacteria repressed mycelial progress of pathogenic fungus but B. subtilis effectively reduced the development of fungi within 7 days with difference of 3.1Cm; Streptomyces hydrogenans inhibited the growth within 12 days of incubation with difference of 2.9Cm while Pseudomonas flourescene was also shown competitive effect but inhibit the growth after 15 days of incubation with difference of 1.12Cm. In case of inhibition zone technique three antagonistic bacteria with three different concentrations of 2, 3 and 5mL were effectively used against A. brassiccola. Among these three antagonistic bacterial isolates B. subtilis significantly exhibited the strongest antagonism against A. brassiccola with 2mL concentration followed by Streptomyces hydrogenans and Pseudomonas flourescene. A distinct inhibition zone of each treatment was measured. However, B. subtilis (2.8190Cm) gave a significantly greater diameter of inhibition zone than S. hydrogenans (2.8095) and P. flourescene (2.5048) after seven days of incubation respectively. Data were examined statistically to certify usefulness of bacterial antagonists against Alternaria brassicicola.

Keywords: A. brassiccola, B. subtilis, P. flourescene, S. hydrogenanas, Biological control, Turnip.

INTRODUCTION

Turnip (Brassica rapa) is a herbaceous annual or biennial root crop which belongs to Brassicaceae family. It’s starchy roots and leaves are edible. It is highly nutritious forage crop with a short growing season and grown in temperate climates worldwide (Barari et al., 2005). It is believed to have originated almost 4,000 years ago in Central and Southern Europe (Rakow, 2004). The total cultivated area of turnip in Pakistan is 15.766 thousand hectares with total seed production of 240 to 280kg/acre and 8 to 10 tonnes average yield per acre. Whereas in Khyber Pakhtunkhwa province of Pakistan the cultivated area for turnip constitutes 2.997 thousand hectares with production of 1.40 million tonnes having average yield of 0.042082million tonnes per hectare (Qasid et al., 2018). Turnip is a rich source of essential nutrients like vitamins,
minerals, water, protein, fats, carbohydrates and ash. Above-ground foliage parts normally contain crude protein (20 to 25%), consumable waterless substance (IVDDM) (65 to 80%), nonaligned cleansing agent (NDF) (nearby 20%) and harsh detergent fiber (ADF) (about 23%). The roots contain crude protein (10 to 14%) and IVDDM (80 to 85%) (Naglaa et al., 2017).

Turnip is prone to a number of pathogens including viruses, bacteria, nematodes and fungi. Among fungi Alternaria brassicicola is one of the important necrotrophic, Deuteromycetes fungal species which damages the plants of many families for example Cucurbitaceae, Brassicaceae and Solanaceae (Anuj et al., 2013). A. brassicicola is the causal agent of leaf spot of turnip, persist saprophytically outside their hosts. Usually its spores are produced during night and released in the day (Meah et al., 2017). Optimum temperature for spore production ranges between 23.8 to 27.7°C, which favors the development of new spores within short time. Prolonged dew period and frequent rainfall are favourable conditions for disease development (Su’udi et al., 2013). The main sources of transferring spores from one area to another are splashing water, tools, wind, human activity and animals (Manhas and Kaur, 2016).

Most of the progressive growers around the world often depend on chemical fungicides for disease management and over past hundreds of years these chemical fungicides have played a very important role in improving crop yields. However, the extensive use of toxic fungicides has created several environmental and economic complications. In addition, it is probably the main cause of development of resistant strains of plant pathogens against traditional synthetic pesticides. In this situation, biological control is the most sustainable approach for management of plant pathogens. It is the most accepted alternative option for plant disease management (Kumar et al., 2012). Among the microbial biocontrol agents, bacteria are able to produce wide spectrum of antifungal agents and effectively control phytopathogens both in-vivo and in-vitro conditions (Das et al., 2015). The establishment of any plant disease is due to the relationship between a vulnerable host, virulent pathogen and the conducive environmental factors (Gul and Tak, 2016). To reduce the frequency of disease outbreak, it is obligatory to build strong relationships between biological control mediators and the constituents of disease development triangle (Arun, 2008).

The biocontrol mechanisms contain prompted resistance, hypo virulence and inhibition of enzymes elaborate in plant pathogenicity (Xu et al., 2011). In the biological control number of antagonistic bacterial microbes such as Bacillus subtilis, pseudomonas fluorescens and Streptomyces hydrogenans have been tested with dual culture technique to control A. brassicicola (Abdalaa et al., 2014). These selected agents express powerful biocontrol abilities in-vitro than applied to field by directly introduced the suspension into plant tissues (Sakalauskas et al., 2014).

Research reports suggest that B. subtilis produce various types of antimicrobial compounds such as peptides (kim et al., 2010) secreted enzymes, proteins (Baysal et al., 2013) and unstable organic substances (Tan et al., 2013) which make them appropriate and effective biocontrol agents (Baffoni et al., 2015). Streptomyces hydrogenans is an actinomycetes and gram-positive bacteria which plays a vital role in controlling pathogen by manufacturing cyclic lipopeptide fengycin (Chandrarakar and Gupta, 2018). Due to production of enzymes, it hinders the expansion of possible fungal pathogens (Zaynab et al., 2018) which destroy the fungal cell wall (Errakhi et al., 2007; Getha et al., 2005). S. hydrogenans survive in the soil layers near the roots of the pharmaceutical plants and some belongs to water that are not considered as strong biocontrol agents (Thangapandian et al., 2007). Pseudomonas fluorescens is nonpathogenic saprophytes that inhabit soil, water and plant surfaces. It is a communal gram negative, rod-shaped bacterium. As the name indicates, it discharges a resolvable greenish fluorescent pigment called fluorescein, mainly when iron is less available (Naglaa et al., 2017). It include in the essential group of bacteria known as plant Growth Promoting Rhizobacteria (PGPR) which shows a significant role in the enhancement of plant, encouraged complete resistance, biological mechanism of pathogens etc (Babu et al., 2000). So keeping in view the problems and constrains of synthetic fungicides the and benefits of microbial bio control agents the present study is mainly designed to explore the antifungal potential B., S. hydrogenans and P. flourescene against A. brassicicola.

**MATERIALS AND METHODS**

**Establishment of disease screening trial:** Seed of six varieties of turnip were taken from Oilseed Research Institute, Ayub Agriculture Research Institute (AARI), Faisalabad. The experiment was set up as Randomized Complete Block Design (RCBD) with plant to plant 25 Cm and row to row 75 Cm distance having three replications
of each variety. All the cultural practices were done uniformly as required.

**Disease incidence:** The disease rating scale of Li et al. (2007), 0-6 disease rating scale for assessment of disease incidence was followed. 

\[
\text{Disease incidence} = \frac{\text{No. of diseased plants}}{\text{Total number of plants}} \times 100
\]

<table>
<thead>
<tr>
<th>Grade</th>
<th>Disease %</th>
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<tr>
<td>0</td>
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<td>Immune</td>
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<tr>
<td>1</td>
<td>1-10%</td>
<td>Highly Resistant</td>
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<td>2</td>
<td>11-20%</td>
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<td>3</td>
<td>21-30%</td>
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<td>4</td>
<td>31-50%</td>
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<td>6</td>
<td>&gt;75%</td>
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MR= Moderately Resistance, MS= Moderately Susceptible, S= Susceptible, HS= Highly Susceptible

**Isolation, purification and identification of A. brassicacola:** Turnip leaves having visible symptoms of Alternaria leaf spot were collected from research area, Institute of Horticultural Sciences (IHS), UAF and preserved at 4°C. A. brassicacola was isolated from infected leaves of turnip using a standard plating technique on PDA. The infected leaves were cut down into small pieces by using sterilized scissor along with some healthy portion. After cutting into small pieces, chopped parts were sterilized with 70% ethanol for 30 seconds. After three serials washing in sterilized distilled water these pieces were dried using blotting paper and inoculated on PDA plates. The inoculated plates were placed at 28±C in an incubator. Fungal growth was observed on plates which was further purified and examined under the microscope. The isolated fungus was identified on the basis of morphological characters including conidial shape and number of septa (Luna et al., 2002).

**Preparation of inoculum and Pathogenicity test:** Spores of fungus were separated from PDA culture with the help of glass separator in a container. Muslin cloth was used to stain the fungal suspension for removal of mycelial content (Zhang et al., 2021). The specifications of haemocytometer were divided into three dimensions; 0.0625 mm², 0.05 mm² and 0.04 mm². The number of spores were diluted to 1x10⁶ spores/mL as a standard spore mixture with 2, 3 and 5mL concentration (Garba et al., 2017).

For confirmation of Alternaria leaf spot of turnip on turnip plants, pathogenicity test was performed by following the Koch’s postulates. Pathogen was isolated from infected portions of turnip plants and was re-inoculated on healthy turnip plants. The plants sprayed with sterile distilled water were used as control, symptoms were observed at regular intervals. Later on, pathogen was re-isolated with the culture isolated from parental infected plant.

**Application of inoculum:** The turnip plants were sprayed with distilled water to increase humidity, after two hours of water application the spore suspension was sprayed twice in the evening. The inoculation process was repeated thrice at 4th, 8th and 12th days of initial inoculation. To make environment conducive for infection, the field was irrigated before and after the inoculation. Plants was observed daily for symptoms development. Initial symptoms appeared after 5 days and characteristic symptoms produced after 7 days of inoculation (Garba et al., 2017). Percentage of disease incidence was recorded.

**Collection and multiplication of antagonistic bacteria:** Three antagonistic bacteria Bacillus subtilis, Pseudomonas fluorescens and streptomyces hydrogenans were collected from the Phyto-bacterioly lab of the Department of Plant Pathology, University of Agriculture, Faisalabad. After collection, each of the antagonistic bacterium was further multiplied on Nutrient Agar (NA) plates.

**Antagonistic activity assay against A. brassicacola:** Three antagonistic bacteria (Bacillus subtilis, Pseudomonas fluorescens and Streptomyces hydrogenans) were collected from the Phyto-bacteriology lab of Department of Plant Pathology, University of Agriculture, Faisalabad. After collection, each of the antagonistic bacterium was further multiplied on Nutrient Agar (NA) plates. In-vitro antagonistic activity of the above bacterial strains was initially tested against A. brassicacola on PDA plates. The bacterial inoculum of the active isolates was picked aseptically and placed in the center of Petri dish containing fungal growth. The Petri dishes were
incubated at 28±2°C for three days. With the help of digital Vernier caliper inhibition zones were measured after 24 hours with regular intervals of time of 7 days.

**Evaluation of different antagonists against mycelium growth of A. brassiccola by Dual culture technique:** Antagonistic bacteria were evaluated for antagonism against *A. brassicicola* on PDA by dual culture technique. 20 mL of PDA was distributed in sterilized Petri plates and allowed to solidify. A mycelial plug from an actively growing *A. brassicicola* on PDA was taken and placed at one side of Petri plate. The antagonists bacteria was inoculated at just opposite side of the same plate by leaving 3-4 cm distance (Das et al., 2015). In case of control, only test fungus was grown in the centre of the plate. Petri dishes were incubated at 25±2°C. After incubation, the diameter of *A. brassicicola* was noted regularly until it covered the whole Petri plate in control treatment. The zones of inhibition were measured with digital vernier caliper in two perpendicular directions (Manoj et al., 2014).

**Preparation of Antagonistic bacterial suspension:** Fresh culture of three selected antagonistic bacteria (*B. subtilis, S. hydrogenans* and *P. flourescene*) were taken and prepared a suspension. For this fresh colony were picked and added to the nutrient broth medium. The inoculated broth was kept for overnight shaking in a shaking incubator (Robus Technologies, SI900R) at a rate of 200 rpm. The cfu level of the broth was measured using spectrophotometer (uv/vis spectrophotometer, uv-1100) and was adjusted to a final concentration of 1 x 10⁶ CFU/mL.

**Evaluation of different antagonistic bacteria against Alternaria leaf spot of turnip under field condition:** Prepared fungal spore suspension was applied to the turnip plants by spraying method to produce disease symptoms. After 15 days, symptoms were appeared, and the development of symptoms are noted with regular intervals of time.

The first spray of antagonistic bacteria was done as the first symptoms of disease were appeared and other subsequent sprays were done with 3 days interval. Each treatment was repeated three times. Disease incidence was recorded weekly and percentage of disease incidence was calculated by following formula.

\[
\text{Disease incidence (\%) = \frac{\text{No. of diseased plants}}{\text{Total number of plants}} \times 100}
\]

**STATISTICAL ANALYSIS**

Statistically data were analyzed by using SAS / STAT software (Filzonmer et al., 2018) which was work under Completely Randomized Design (CRD) for laboratory experiment and Randomized Complete Block Design (RCBD) for field experiment to test the significance of antagonistic bacteria for the management of *Alternaria* leaf spot disease.

**RESULTS**

**In-vitro assessment of antagonistic bacteria against Alternaria leaf spot of turnip by using inhibition zone technique:** Maximum diameter of inhibition zones were expressed by *Bacillus subtilis* (2.82 cm) followed by *Streptomyces hydrogenans* (2.81 cm) and *Pseudomonas flourescene* (2.5 cm) as compared to control (0). Interaction between treatment and their concentration (TxC) maximum inhibition zone (3.31 cm) are expressed by *Bacillus subtilis* at 2 mL concentration followed by 3 and 5 mL concentration at the same concentration remaining treatment (*Streptomyces hydrogenans* and *Pseudomonas flourescene*) expressed maximum inhibition zone as compared to control. Interaction between treatments and days (TxD) maximum inhibition zone (1.54, 2.36, 2.58, 2.98, 3.24, 3.41 and 3.62 cm) expressed by *Bacillus subtilis* among *Streptomyces hydrogenans* (1.71, 2.28, 2.58, 2.97, 3.13, 3.36 and 3.64 cm) and *Pseudomonas flourescene* (1.72, 2.13, 2.39, 2.61, 2.76, 2.88 and 3.04 cm) as compared to control (0) after 24 hours respectively. Interaction between Treatment, Concentration and Days maximum inhibition zone (2.20, 2.63, 3.07, 3.53, 3.70, 3.90 and 4.17 cm) expressed by *Bacillus subtilis* after 24 hours continue with 7 days at 2 mL concentration among 3 and 5 mL concentration, at the same concentration remaining treatments (*Streptomyces hydrogenans* and *Pseudomonas flourescene*) expressed maximum inhibition zone as compared to control (0.00) after 24 hours continue with 7 days respectively.

**In-vitro assessment of antagonistic bacteria against Alternaria leaf spot of turnip by using dual culture technique:** Maximum inhibition of mycelial growth was expressed by *Bacillus subtilis* with difference of 3.03 cm followed by *Streptomyces hydrogenans* (2.90 cm) and *Pseudomonas flourescene* (1.12 cm) as compared to control (5.48 cm). Interaction between Treatment and Days maximum growth of *Alternaria brassicicola* was inhibited by *Bacillus subtilis* with maximum difference of 3.03 cm among *Streptomyces hydrogenans* with 2.90 cm and *Pseudomonas flourescene* with 1.12 cm as compared to control 5.5 cm.
Figure 1. Evaluation of various antagonistic bacteria, concentrations and days against Alternaria leaf spot of turnip by using inhibition zone technique

Figure 2. Evaluation of various antagonistic bacteria and days against Alternaria Leaf spot of turnip by using dual culture technique
Management of Alternaria leaf spot of turnip through antagonistic bacteria under field condition:

Minimum disease incidence (21.74%) expressed in *Pseudomonas flourescene* after that *Streptomyces hydrogenans* (22.18%) and *Bacillus subtilis* (23.85%). Interaction among treatments and their concentrations (TxC) minimum diseased incidence (21.742%) expressed by *Pseudomonas flourescene* at same concentrations remaining treatments expressed less disease incidence as compared to control (Figure 1).

**DISCUSSION**

Various factors are involved for producing the symptoms of a fungal disease including genetic makeup of a host, infection time, strain of fungi, environmental conditions and plant age. Alternaria leaf spot is one of the most destructive fungal disease caused by *A. brassicicola*. It potentially reduces the production of turnip by attacking on all above ground parts of plant which results in complete drying of leaves. It’s estimated that leaf spot disease decrease yield up to 47% (Qasid et al., 2018). Development of disease in epidemic form depends on the virulence of pathogen, time of infection, favorable conditions of environment and susceptible host. To overcome these problems in future use of antagonists are effective and durable solution to save turnip crop from the infection of *A. brassicicola*. The effective solution includes the screening of available germplasm to check the relative susceptibility of plant material. Biological control of plant pathogens becomes an integral part of pest management in light of the environmental and health issues attributed to the use of fungicides in agriculture (Kaur and Manhas, 2014). It is environment friendly and appropriate alternative method with no adverse effect of plant. In this method different biocontrol agents such as bacteria and fungi are used to suppress the population of pathogen (Sanzani et al., 2014).

Antagonistic organisms were collected from Bacteriology laboratory, Department of plant pathology, University of Agriculture Faisalabad. The antagonistic activity against *Alternaria citri* was tested by Dual Culture technique. For this purpose 4 antagonistics (*Bacillus subtilis*, *Pseudomonas flourescens* and *Streptomyces hydrogenans*) were collected and applied through dual culture technique under lab conditions. Six turnip varieties were grown and treated with *Alternaria brassicaola*, out of this most susceptible variety (purple gold) was further used for research process against Alternaria leaf spot of turnip. Three antagonistic bacteria (*Bacillus subtilis*, *Pseudomonas flourescens* and *Streptomyces hydrogenans*) were evaluated against *Alternaria brassiccola* under both field and lab condition. Current study revealed that *B. subtilis* was proved most effective *in-vivo* condition by controlling 23.852 % disease incidence. However, under *in-vitro* condition *Bacillus subtilis* and *Pseudomonas flourescens* show maximum result.

In contemporary studies Tozlu et al., 2018 determined the antifungal activities of of *B. subtilis*, *B. megaterium*, *B. pumilus*, *P. polymyxa*, *P. flourescens*, *Bacillus thuringiensis subsp. kurstakii* and *Bacillus sphaericus* against *A. alternate*. *B. pumilus* controlled 87.6-65.89% disease among all antagonists. Sundaramoorthy, (2012) revealed that both *Pseudomonas flourescens* and *B. subtilis* indicated best results against phytopathogenic fungi. According to Gul and Tak, (2016) culture filtrates from two isolates (*Bacillus subtilis* and *Bacillus amyloboliquefaciens*) expressed strong efficacy against *Alternaria spp* under *vitro* condition. Abdalla et al.
(2014) isolated different species of Bacillus from the rhizosphere of tomato plants to be used as natural biocontrol agents against Alternaria alternata under in vitro condition. It was found that four (Bacillus B25, B35, B41, B45) exhibited strong antagonism against Alternaria spp.

Recent study showed similar results as reported by Ankur et a. (2018), Naglaa et al. (2017) who selected different species of Bacillus such as B. subtilis, B. megaterium, B. pumilus and B. cereus and evaluated against numerous species of Alternaria. Results indicated that B. subtilis and B. megaterium expressed significant effect by inhibiting maximum growth of fungus (Deleu et al., 2008).

It has been identified that B. subtilis is capable to enhance the growth of plant (Choudhary et al., 2009). By using various mechanisms, Bacillus subtilis improves plant growth, such as perfection of plant nourishment, generation of complete resistance, injuriousness to pests and as antagonist against pathogens (Ali et al., 2016). B. subtilis produces wide range of secondary metabolites mediating antibiosis recognized for decades. Various types of antimicrobial compounds such as peptides, enzymes, proteins, and unstable organic substances which makes them appropriate and proper biocontrol agents (Baffoni et al., 2015). At least 4-5% of its genome is specific for antimicrobial compounds (Sumi et al., 2015).

Bacillus species showed strong antimicrobial activity against genus Alternaria (Abbo et al., 2014). Upshots of the current study is supported by the findings of Matar et al. (2009), Trivedi and Pandey, (2008) and Velmurugan et al. (2009) who evaluated B. subtilis, B. megaterium, B. pumilus and B. cereus and reported that plates of genus Alternaria which were inoculated with B. subtilis and B. megaterium showed significantly less mycelial growth during the incubation period and indicated the ability of these bacteria to inhibit in vitro mycelial growth of many plant pathogenic fungi. However, no significant differences in the inhibition of genus Alternaria were observed between the species of B. pumilus, B. cereus and the control treatment. Kokalis at el. (2002) reported that in vitro inhibitory activity of these bacterial species translated well into the in vivo test; B. subtilis and B. megaterium showed the highest inhibition effect in vitro against mycelial growth of genus Alternaria. Their effects in vivo test on genus Alternaria disease incidence were also the best. The fourth species B. cereus showed low suppressive effects against disease incidence during both seasons. These results are in agreement with the laboratory findings published by (Kokalis at el., 2002).

Because of their wide diversity, bacteriocins display different modes of action such as protoplasm vesicularization, pore formation or cell disintegration. They are generally bactericidal and produce bacteriostatic activities due to their amphiphilic or hydrophobic properties (Gautam and Sharma, 2009). For example lantibiotic have dual mode of action, firstly they inhibit the cell wall synthesis of the pathogen through binding to lipid which is the major transporter of peptidoglycan subunits across the inner cell membrane (Chen et al., 2008). Secondly lipid are used as a docking molecule to insert the lantibiotic in the membrane leading to pore formation and ultimately to cell death (Chatterjee et al., 2005). This dual action has been reported for subtilin, a class of bacteriocin which is active against broad range of plant pathogens. It also produces Bacillomycin which helps in forming particular metabolic compounds to restrict growth of fungus. Bacillomycin interacts with the fungal cell wall and destroys leaf lamina which results in the leakage of contents eventually causes the death of cell and restricts the growth of fungus (Mardanova et al., 2016).

### CONCLUSION

It is concluded from the recent research study that;

- The most susceptible variety of turnip was purple gold which exhibit clear symptoms of Alternaria leaf spot.
- In both in vivo and in vitro experiments, amongst three antagonistic bacteria Bacillus subtilis control maximum disease incidence followed by Streptomyces hydrogenans and Pseudomonas flourescense.

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Contribution of Authors:

Insha Saeed : Designed and conduct experiments.
Sabir H. Khan : Supervised and layout the experiments.
Abdul Rasheed : Data analysis and critically reviewed the manuscript.
Muhammad M. Jahangir : Help in manuscript writeup.
Abdul Jabbar : Data collection and compilation.
Hafiz M. F. Shaheen : Help in manuscript write up.
Wasi-U-Din : Editing the manuscript.
Komal Mazhar : Reference correction and reviewed the manuscript.