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EFFECT OF BACTERIAL ENDOPHYTES ISOLATED FROM THE CITRUS ON THE PHYSICAL PARAMETER OF BITTER GOURD (*Momordica charantia* L.)

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ABSTRACT

Bacterial endophytes have a symbiotic relationship between different parts of the plant and could play an important role to improve plant health. The effects of endophytic bacterial communities in bitter gourd have not been studied yet in Pakistan. In the present study, ten different bacteria that belong to class Alpha, beta and gamma Proteobacteria as well as bacilli were injected into bitter gourd seedlings under glass house conditions and five weeks after physical parameters (SL, RL, leaf area, leaf number, wet and dry weight of plant, root and shoot ratio) were studied. According to results *Azomonas agilis* and *Ensifer adhaerens* give good results as compared to control for all parameters studied. These inoculants could be used as bio fertilizer for sustainable crop production system.

Key word: Host pathogen interaction, inoculation, physical parameters and bitter gourd.

INTRODUCTION

Any organism that is lived inside plants and do not cause any visible harm to plants is termed as endophyte. Endophytes developed different type of associations with other hosts such as mutualism, commensalism and latent pathogenicity (Barbara and Christine, 2006). Endophytes have achieved higher numbers in microbial niche. About 30,000 plant species residing the earth contains one or more endophytes (Araújo *et al.*, 2001; Lacava and Azevedo, 2014). Endophytes received attention in the last 20 years (Azevedo *et al.*, 2000). Endophytes and epiphytes are two growing models of leaf bacteria (Hallmann *et al.*, 1997). Santoyo *et al.* (2016) described that endophytes are the bacteria that do not cause visible harm to the host plants. They reside inside the host plant tissues and thus are protected from the outer biotic and abiotic environmental stresses. Hung and Annapurna (2004) said that it has been observed that endophytes are present in almost every part of the plant, i.e. flowers, root, leaves, shoots, stem etc. According to Zinniel *et al.* (2002), they can be gram positive and gram negative. They may remain concentrated at the point where they enter. They may also spread throughout the plant system. Endophytes can also be finding by hybridization (Hallmann *et al.*, 1997). Endophytes present in broad range of plants, citrus plants (Araújo *et al.*, 2001), potatoes (Garbeva *et al.*, 2001), poplar (Ulrich *et al.*, 2008), red clover (Sturz *et al.*, 1998), rough lemon (Lodewyckx *et al.*, 2002), rice (Stoltzfus *et al.*, 1997), soybeans (Okubo *et al.*, 2009), sugar beet (Magnani *et al.*, 2010), strawberry (Lodewyckx *et al.*, 2002), sugarcane (Dong *et al.*, 1994) and wheat (Conn and Franco, 2004). *Enterobacter* genus frequently found in stems of plants like soya bean and *citrus sinensis* (Magnani *et al.*, 2010). *Pantoea* species identified in sugar cane and soya bean (Loiret *et al.*, 2004). The study of endophytes just not for understanding their ecological role, but it is important for their application and biotechnology. Gouda *et al.* (2016) said that endophytes have great significance in the field of medicine agriculture and industry. Endophytes identification and molecular characterization lead toward the invention of antibiotics, immune-suppressants, and anticancer compounds. Endophytes bacteria high in roots and this concentration decrease in stem than in leaves. Endophytes bacteria are advantageous in contributing the growth of plants. They enhance biological nitrogen fixation and increase rate of mineral uptake phosphate solubilization (Yuliar *et al.*, 2013). Plant growth promoting endophytes decrease the metal toxic effect in host plants. Endophytes concentration and ability of toxin production is affected by mesic character, soil pH insect pest resistance and temperature change (Azevedo *et al.*, 2000). Endophytes bacteria has the ability to minimize the disease symptoms in host plants (Pleban *et al.*, 1997). Mostly endophytes are obtained from the surface sterilization by chemicals and culture on media LBA (Rosenblueth and Martínez-Romero, 2004). Beneficial endophytes are extracted from the roots of citrus plants for increase in sustainability and agro ecosystem. Uses of citrus and citrus greening (HLB) model change the bacterial community against the phytopathogens. Now a days a lot of research trend shift toward the study of hot pathogen interaction and some interactions leads to the beneficial effects on the physiology of their host plants. Therefore this type of interactions is gaining more importance with respect to use of their bio active compounds in biotechnology and that are mostly produced by bacteria. These interactions has ability to create immunity or resistance to combat diseases and also reduced symptoms by inducing systemic induced resistance in plants. Specific growth hormones produce by this interaction improve health of plant. Endophytic bacteria improve nutrient uptake and

nitrogen fixing ability of plants and also important for use as bio fertilizers. One of the most important roles of these endophytic bacteria is degradation of heavy metals from soils and makes it more fertile for plants growth and development. In other words they play beneficial role in agriculture, medicine, biotechnology and in the field of food science.

OBJECTIVES

The basic aim to conduct this study was to check either these bacterial endophytes are imposing beneficial effects on plants with respect to their physical growth or reduce the plant health.

MATERIAL AND METHODS

Sample collection: Leaf samples of different varieties of citrus were collected from Citrus Research Institute Sargodha, Punjab Pakistan in September, 2015. The leaf samples showing symptoms of citrus greening were collected.

Isolation and identification of bacteria: Isolation of bacterial endophytes were performed from midrib portion of citrus leaf by method describes Mushtaq *et al.* (2018) Morphological characterizations of isolated bacterial cultures were based on colony morphology (colony color, colony shape, texture, elevation, margins, gram type, spore type and motility). Biochemical tests (catalase test, methyl red test, citrate utilization test, hydrogen sulfide test and indole test) were done following standard protocols (Garrity *et al.*, 2005).

Catalse test: About 15% H_2O_2 was used for the detection of catalase in anaerobic bacteria. In this test 2-3 mL of hydrogen sulfide were poured in the test tube. Sterilized bacterial loop with bacterial culture were dipped in tube containing hydrogen sulfide and formation of bubbles were observed. Formation of bubbles indicates the positive or negative result and this test were used to determine the presence of catalase enzyme in test bacterial strain.

Methyl red test: Endophytes bacterial species have ability to detect the ability of formation to produce stable acids end products form available glucose. Methyl red test were performed by inoculating the LB broth media with bacterial culture and incubated at 37°C for 24 hours and 0.5 mL of methyl red indicator were added to each tube. The appearance of yellow/red color was indication of the positive reaction with methyl red indicator.

Citrate utilization test: Citrate utilization test was used to determine the ability of bacteria to utilize sodium citrate. Simmons citrate agar slant were prepared by following recipe (ammonium dihydrogen phosphate 0.25 g, dipotassium phosphate 0.25g, sodium chloride 1.25 g, sodium citrate 0.5 g, magnesium sulfate 0.05 g, agar 3.75 g, bromthymol blue 0.02 g for 250 mL media) in test tube. Then these tubes were inoculated with bacterial culture and incubated these tubes at 37°C for 24 hours. Appearance of blue or green color was the clear indication for the positive reaction.

Hydrogen sulfide test: This test was used to detect the hydrogen sulfide production by the micro-organism (Cappuccino and Sherman, 2002). For this test SIM agar (peptone 1.5 g, beef extract 2.5 g, ferrous ammonium sulfate 0.2 g, sodium thiosulfate 0.025 g, agar 3 g for 500 mL) media was used. Tubes were filled with media, allow solidifying and then inoculated with bacterial culture with the help of sterile loop and incubated at 37°C for 24 hours. Black precipitation indicates positive results.

Indole test: Indole test was used to determine the ability of an organism to break amino acid tryptophan to form the compound indole. Indole production was detected by Kovac's reagent which contains (4 p-dimethylamino benzaldehyde)**,** in this reaction red color produce (Cappuccino and Sherman, 2002). SIM agar (peptone 1.5 g, beef extract 2.5 g, Ferrous ammonium sulfate 0.2 g, sodium thiosulfate 0.025 g, agar 3 g for 500 mL). Media was poured into sterile test tubes and inoculated with bacterial culture. Then incubate it at 37°C for 24 hours and 1 mL of Kovac's reagent was added into each tube. Yellow or red coloration was observed through this test. The identified cultures were submitted to the (first fungal culture bank of Pakistan) FCBP with accession numbers FCBP# 567, FCBP#606, FCBP# 607, FCBP#568, FCBP#610, FCBP#611, FCBP#613, FCBP#566, FCBP#570, FCBP#612, FCBP#609 and FCBP#608.

Plant growth promoting traits: Bitter gourd (Faisalabad long) variety seedlings were used to test the effect of characterized bacterial strains on the physical parameter of plants under controlled conditions in glass house. The bitter gourd seedlings were planted on sterile soils in pots having mixture of compost and sandy loam soil in equal proportion.

Inoculum preparation and inoculation: Single celled colony were picked from pure culture and inoculated o test tubes contusing 5 mL LB medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g and agar 2 g per liter) and were placed in the shaker having temperature 37°C for overnight as followed the method describe by Mushtaq *et al.* (2018). 0.1 mL of bacterial suspension (10^8 CFU mL^{-1}) was inoculated into bitter gourd seedlings by injecting 1 mL of bacterial suspension into the intercellular spaces of the leaves with syringe needle. Completely randomized block design was followed by three replications per treatment. The control treatment was non inoculated plants that were just watered normally.

Physical parameters of plant: Physical parameter of bitter gourd plant were recorded and parameter studied were included root length, shoot length, leaf area, leaf number, dry weight of plant, wet weight of plant, root and shoot ratio.

Statistical analysis: Data obtained were statistically analyzed by using Analysis of Variance (ANOVA) at probability level 0.05 by using software package Statistix 8.1.

RESULTS AND DISCUSSION

Bitter gourd plants were inoculated with ten different endophytic bacteria to check the effects of these inoculums on host plant physiology. All the identified strains were belonging to class Alpha, beta and gamma Proteobacteria as well as Bacilli. Diagrammatic representation of the test strains used in this study and symptoms (necrotic lesions and vein yellowing) appears after inoculation is shown in figure 1. Biochemical characterization of endophytic bacteria and

morphological characters were important. This is helpful to identify the bacteria. 13 bacteria are isolated from citrus named *Acinetobacter spp., Aureobacterium liquefacians, Azomonas agilis, Bordetella pertussis, Citrobacter diversus, Enterobacter cloacae, Ensifer adhaerens, Kurthia spp.* and *Pantoea spp.* Nitrogen fixing *Enterobacter* also isolated from citrus mid rib. *Enterobacter* is a phosphate solubilizing bacteria. Morphological characteristics are important for identification of bacteria (Araújo *et al.*, 2001). Data on morphological and biochemical characteristics has been published (Mushtaq *et al.*, 2018).

According to Zinniel *et al.* (2002) they can be gram positive and gram negative. My isolated bacteria mostly were gram negative and some of these were gram positive. *Aureobacterium liquefacians* and *Kurthia spp.* is *gram* positive. *Enterobacter cloacae* were anaerobic bacteria. *Pantoea* species as gram negative bacteria (Mardaneh and Dallal, 2013). These bacteria have ability to form spore and some of these isolated endophytes were not able to form spore. The color of colony also very important in identification of bacterial colony isolated bacteria was mostly yellow white and off white in color. Some of these were slimy. Mostly endophytes have wavy margins. *Enterobacter cloacae* are anaerobic bacteria.

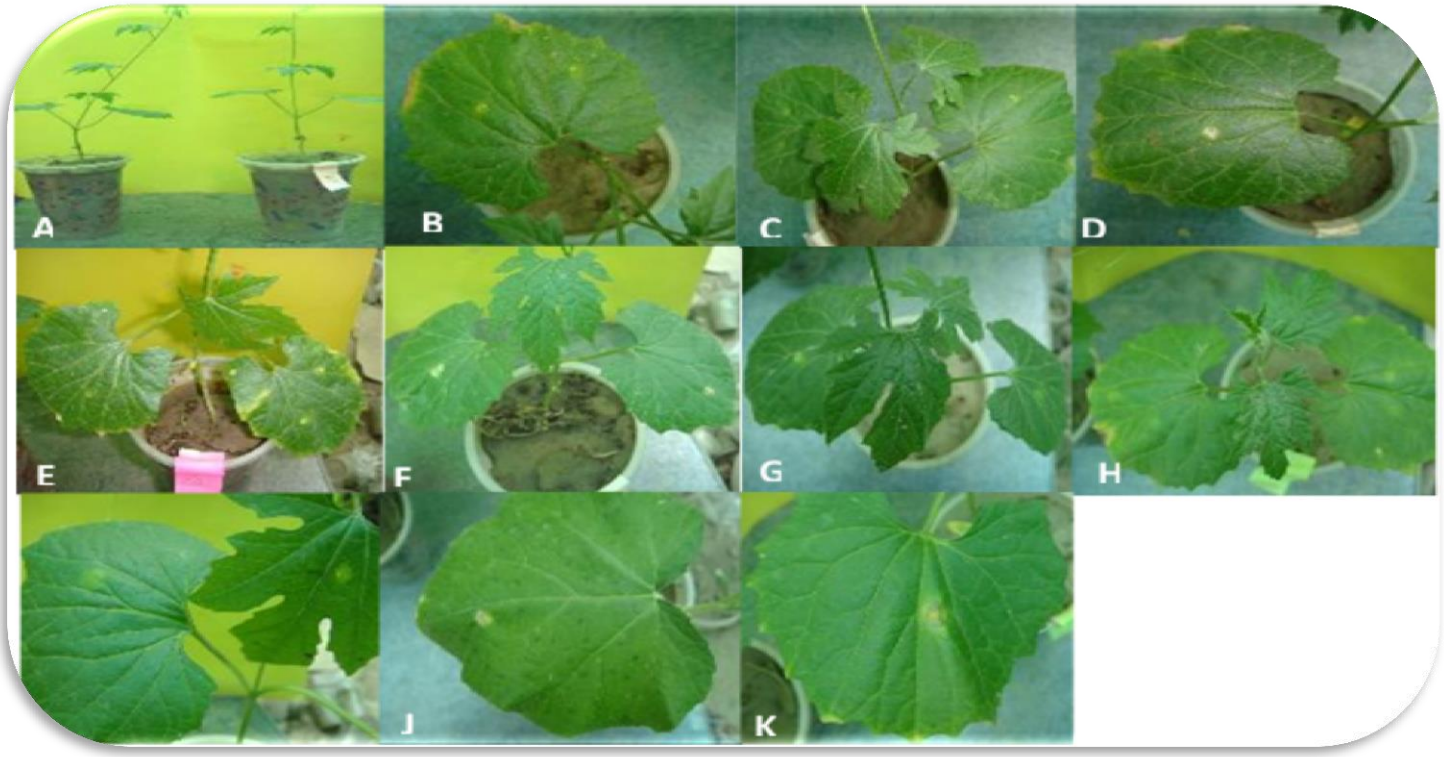

Figure 1: Symptoms of bitter gourd after inoculum with bacteria A) control (un inoculated) B) *Pantoea sp. c) Enterobacter cloacae D) Azomonas agilis E) Aureobacterium liquefacians F) Ensifer adhaerens G) Kurthia sp. H) Acinetobacter sp. I) Bordetella pertussis J) Citrobacter diversus K) Azotobacter nigrieans.*

Physical parameter shoot length show significant result after inoculum of bacteria changes occur after the treatment this thing indicate that endophytes influence on the growth of plants parts. Physical parameter root length also show significant result endophytes also influence on the root growth of all crops plants. Physical parameter leaf area and leaf number, wet weights were also showed significant result. It indicates that endophytes influence on the growth of plant. Endophytes promote the growth of plants. Maximum shoot length 12.67 cm were found in *Azomonas agilis* treated plants as compared to control 12.33 cm. while maximum 8.75 cm root length was found in plants treated with *Bordetella pertussis* as compared to control 867 cm as shown in table 1. In case of leaf area, leaf number, wet weight, Dry weight maximum values (4.57, 6, 2.03, 0.73) were found in seedling treated with *Azomonas agilis* as compared to control (un-inoculated) one. So the shoot root ratio was maximum 3 in case of *Ensifer adhaerens.* Endophytes bacteria have no such pathogenic effect on plants (Hardoim *et al.*, 2008). But after inoculum of citrus isolated endophytes these bacteria cause symptom on the bitter gourd. Endophytes are associated with different plants species. Endophytes 81 species were identified from this association Lodewyckx *et al.* (2002). There are two pathway of endophytes transmission in plants. Chi *et al.* (2005) suggested that endophytic bacteria enter into the host plants through roots from soil and become shield

around the intracellular spaces of plants and cause necrosis and some sort of symptoms. However in this experiment it was found that endophytes enter through the leaves and cover the intracellular spaces between the plant leaves and apparently leaves looks healthy without any sever type of symptoms.

Pantoea spp., Enterobacter cloacae, Azomonas agilis, Aureobacterium liquefacians, Ensifer adhaerens, Kurthia spp., Acinetobacter sp., Bordetella, Azotobacter nigrieans, Citrobacter diversus, Bordetella pertussis, and these bacteria cause symptoms in different hosts. Metabolites like flavonoids encourage the colonization of *Azorhizobium caulinodans* into wheat (Webster *et al.*, 1997). Mostly endophytes induce into plants increase the growth of plants at different growing stages. It differs from strain to strain (Rosenblueth and Martínez-Romero, 2004). Mid ribs of leaves conducting tissues and extra cellular spaces are more concentrated with endophytes as compare to other parts. Endophytic bacteria are motile in many cases (Reinhold-Hurek and Hurek, 1998). Inoculum between the veins of leaves helpful in penetrating the bacteria to the mid rib of plant leaves than endophytes colonized into other parts of plants through mid-rib. Their colonization promotes degrading the leaf structure like necrosis and leaf damaging. Germaine *et al.* (2009) has inoculated the roots of pea plants with bacteria and these bacteria increase the growth of pea plants. In my experiment these bacteria also influence on the growth of plants. Significant results were found on leaf area shoot and root ratio, root length shoot length, leaf number even wet and dry weight of the plants in response to bacterial inoculum. Endophytes attached with root bacteria and increase the plant growth and other beneficial mechanism in plants (Elbeltagy *et al.*, 2000).

Pantoea species ae gram negative bacteria and Mardaneh and Dallal (2013) described that *Aureobacterium liquefaciens* is gram positive bacteria and there colony color is yellow, rod shape bacteria. *Azobacter nigrieans* has flat elevation. Biochemical characterization of bacteria like catalase test, indole test, methyl red test, hydrogen sulphide test are helpful in understanding the isolated bacteria. Citrate utilization test of these bacteria were positive. While catalase test of some bacteria were negative. Yokota *et al.* (1993) said that *Aureobacterium liquefaciens* is gram positive bacteria and there colony color was yellow, rod shape bacteria.

De Weert *et al.* (2002) described that the mobility of bacterial endophytes through interactions with hosts plant happened by chemotaxis. Although carbohydrates and organic acids play vital role in below ground parts of plant during interaction (Bacilio-Jiménez *et al.*, 2003). Endophytes produce large variety of enzymes these enzymes production influence on the plant parts and cause symptoms of disease. Endophytes produce endoglucanases enzyme, this enzyme is also helpful in bacterial entrance (Reinhold-Hurek and Hurek, 1998). An additional enzyme named as endopoly galacturonidases provides the route for the entrance of endophytes bacteria (Elbeltagy *et al.*, 2000). Population dynamic of bacterial endophytes is also affected through the growing stage of plants and vary from host to host age wise.

CONCLUSION

This study suggested that these tested bacterial endophytes imposed beneficial effects on the plant growth and according to results infected plants were looks healthier as compared to un-inoculated ones. Different physical parameters studied were also showed significant difference with respect to control plants. Among test organisms *Azomonas agilis* and *Ensifer adhaerens* gives good results. These endophytes could be tested in field conditions to check their impacts in open environment. Then it could be suggested that either these beneficial microorganisms can be used as bio fertilizers or not. So the use of these endophytes would be important step towards sustainable agriculture. Recent use of endophytes such as a bio fertilizer biocontrol agent and phytoremediation has great scope in future.

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