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Isolation and morphological characterization of novel bacterial Endophytes from Citrus and evaluation for antifungal potential against *Alternaria solani*

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ABSTRACT

Endophytes have always been a topic of interest for researchers due to their wide variety of benefits to their hosts and their diversity of geographical distribution. In this study, bacterial endophytes were isolated from the leaf midrib of different varieties of citrus cultivated in the Sargodha region of Punjab Pakistan. The endophytic bacterial community associated with citrus was characterized and screened for antifungal activity against *Alternaria solani* which causes losses to crops. A total of twelve strains were identified based on morphological and biochemical tests following Bergey's manual of systematic bacteriology. The antagonistic potential of bacterial endophytes to *A. solani* was explored using the agar absorption method. This study showed the antifungal potential of *Pantoea sp*. (35.66%), *Ensifer adhaerens* (35.33%), *Citrobacter diversus* (33.03%), and *Azotobacter nigricans* (31.56%) to check the growth of pathogenic fungi compared to controls. *Aureobacterium liquifaciens* (27.66%), *Acinetobacter sp.* (25.66%), *Bordetella pertussis* (26.63%) also showed equal potential for inhibition. In contrast remaining isolates *Enterobacter cloacae* (19.33%), *Azomonas agilis* (17%) and *Kurthia sp*. (19%) were less efficient as compared to the others. Bacterial endophytes are colonized inside plants and have antagonistic potential for fungal pathogens. These endophytes should be further explored for disease control. Ongoing study in this area will help to the innovate biological control of plant pathogenic fungi.

Key words: Phenotypic characterization, antifungal potential, bacteria, biological control

INTRODUCTION

Citrus is an economically most important fruit in Pakistan. Many plants are in constant contact with different types of bacteria (Prober *et al*., 2015). These bacteria that live inside host plant tissues are commonly known as endophytes. Singly or collectively, endophytes show different kinds of associations: mutualism, commensalism and latent pathogenicity (Boyle *et al*., 2006). Endophytes have attained high density in microbial niches (Brader *et al*., 2014). About 30,000 plant species inhabiting the earth contain one or more endophytes (Lacava *et al*., 2014). Endophytes have received great attention in last 20 years due to their ability to control pathogenic bacteria and fungi (Rodríguez *et al*., 2017). Endophytic bacteria do not cause a visible harm to the host plants and can be gram positive or negative (Ibanez et al., 2017).Chemical fungicides are widely used to prevent the infection of pathogens. Theses chemical agents are very costly and cause serious environmental pollution and remain in soils for a long time. These chemicals also induce resistance of the pathogens to the fungicide so as to reduce the efficacy against certain pathogens (Bardin et al., 2017). Many of the of these studies have been focused on biological control of plant diseases which are ecosystem friendly and an alternative to chemical fungicides (Thakur, 2017).These endophytes can be isolated from external (Phyllosphere) or internal (Endophytes) plant tissues. Maximum number of the bacterial endophytes have been cultured from different tissues of several plant species, such as potato, maize, sorghum, wheat, cotton and rice (Sharma et al., 2009). Basically bacteria enter in plant tissue through roots, flowers, stems and cotyledons (Bacon *et al*., 2004). The colonization of endophytes inside plants depends on the extent of the localization either at the point of entry or may spread throughout plant tissues (Kandel *et al*., 2017).

The isolation of microorganisms from citrus leaf and their proper identification provides the information among the variability in isolated strains. The 16 s ribosomal DNA analysis and other

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sequence analysis have been widely used for studying phylogenetic diversity among various microbial communities (Hauben *et al*., 1997; Vero *et al*., 2002; Almeida *et al*., 2001). Endophytic bacteria both have gram positive and gram negative nature isolated from a variety of plants (Sturz *et al*., 2000). Some endophytes are agronomically very important for enhancing and promoting the growth of plants as well as controlling the various diseases of plants and for immune system stability for increasing resistance against various pathogens (Azevedo *et al*., 2000; Scherwinski *et al*., 2007). Edophytic bacterial species diversity based on culture independent techniques analysis with reference to citrus plants also suggest that bacterial endophytic populations having more diversity than previously reported (Arau´jo *et al*., 2002; Lacava *et al*., 2006).

Although these endophytes can reside within cells, in the intercellular spaces or in the vascular system of a host (Zinniel *et al*., 2002). They have many biological functions due to their colonization and alteration of properties inside host tissues. They can prevent infection of the plants from pathogens, improve anti-disease capability by competing with different pathogens or production of antifungal substances and induction of host systemic resistance against pathogens. Subsequently, endophytic bacteria have the potential to work as bio control agents to control plant pathogens (Kloeppe *et al*., 1999). The use of these bacterial endophytes as biocontrol agents has already been reported in various studies on potato, tomato, cotton, maize, cabbage, rice, cucumber (Chris & Chanway, 2002). The molecular basis of the mechanisms involved in the biological control are still not known but comprises of ecological niche competition for the production of compounds that could obstruct the pathogen effects on plants, induce systemic resistance (ISR) for a wide range of plant pathogens, or reduce Stresses caused by abiotic factors (Vallad & Goodman, 2004).

The effect of endophytes on phyto-pathogens were reported by Upreti & Thomas, (2015). Most of endophytes isolated from citrus are resistant against root pathogens(Siala *et al.,* 2006), Black rot of cabbage was controlled by colonization of endophytic bacteria *Xanthomonas campestris* (Jetiyanon,1994), hence endophytes enhance the growth and physiological functioning of host plants (Hallmann *et al*., 1997). Endophytic bacteria provide protection against the oak wilt pathogen. By the colonization of endophytes in plants the activity or growth of pathogens decreases (Sturz *et al*., 2005). It has been reported that *Serratia marcescens and Pseudomonas putida* can control cucumber mosaic virus, *Fusarium* wilt and anthracnose in cucumber (Roberts, 2005). However sixty one bacterial endophytes cultured from potato stem functioned as a biocontrol agent counter to *Clavibacter michiganensis* (Bargabus *et al*., 2002), *Bacillus pumilis* has been used to control disease of *Cercospora* leaf spot in sugar beet crop. These bacteria also have ability to produce antibiotics (Sessitsch, 2004). They can control the fungal pathogen in wheat and the *Phytophthora capsici* infection of black pepper. It has been reported that these bacteria also produce hyperparasitic activity when they attack pathogens, they secrete cell wall hydrolase enzymes (Chernin & Chet, 2002). Endophytes are also important for biocontrol of soil borne diseases and their antifungal potential (Yuliar, 2013). The purpose of the present work was to isolate bacterial endophytes from the leaves of different varieties of citrus, characterize them based on morphological and biochemical tests and to determine the antifungal potential against *A. solani*.

MATERIALS AND METHODS

Sample Collection

A comprehensive survey was conducted to take the leaf samples of different varieties of citrus from Citrus research institute (CRI) in Sargodha Punjab Pakistan. Bacterial endophytes were isolated from the leaf midrib portions of citrus varieties such as CRI-78, CRI-7, Natal (*Citrus sinensis*), Dancy (*Citrus reticulata*), and Trifoliate (*Citrus trifoliata*) displaying visible signs of citrus greening. The leaves were washed with running tap water, dried and stored at -80 ^{o}C.

Isolation and Identification of Bacteria

The majority of the strains were isolated by normal isolating method and leaves were immersed in sterile water 5-6 times after surface disinfection with 70% alcohol. The xylem of the leaves were removed and implanted in bacterial culture medium and incubated at 37 °C for 24 to 48 hours. After this pure culture were obtained for further studying (De Oliveira *et al*., 2012; Bahig *et al*., 2012). Isolation of pure culture is vital for characterizing a single species, otherwise presence of contamination can lead inaccuracy in results (Jing *et al*., 2011). After this the bacterial population density was estimated and bacterial strains were grouped on the basis of various phenotypic traits i.e. morphology, colony colour, texture, shape and mobility etc. (De Oliveira *et al*., 2012; Bahig *et al*., 2012). On the other hand gram staining was performed to differentiate the bacteria in to two groups.

Identification of Bacterial Isolates

Morphological characterization of isolated bacterial cultures was based on colony morphology. Biochemical tests were used including catalase test, methyl red test, citrate utilization test, hydrogen sulfide test and indole test, were done following standard protocols (Garrity,2005).

Evaluation of Antifungal Activity of Endophytic Bacteria

The antagonistic effect of bacterial endophytes against *A.solani* (FCBP Accession No. 1403) was test by the agar absorption method (Kalemba, 2003) compared to test bacterial strains. Bacterial isolates were incubated at 37 ^{o} C on BSMA for 24-48 h before use. Potato dextrose agar media (20ml) were poured into each sterile petri dish (90mm diameter). 200 µl suspensions of each bacterial endophyte was applied directly on the plate and spread. Well of 5 mm diameter were punched in the agar plate (5mm). The *A. solani* plugs were inserted into the wells of agar plates directly. Inoculated plates were incubated at 37 ^{o}C. The diameter of fungal growth/ inhibition in Treated (*T*) and control (*C*) Petri dishes were measured in three different directions for seven days. Percentage of growth inhibition (*I*) was calculated using the formula *I* (%) = [(*C* -*T*)/*C*] x 100 (Sztejnberg *et al*.,1989).

Statistical Analysis

All data was statistically analyzed using analysis of variance (ANOVA) at probability level 0.05 by using software package DSAASAT.

RESULTS

In this study twelve different bacteria were isolated from different cultivars of citrus and identified them on the basis of morphological and biochemical test of following Bergey's manual of systematics. A detail for all the isolated bacterial strains is described in (Table 1).

Table 1: List of different citrus varieties identifications and FCBP accession no

Sr. No	Sample	Identified bacteria	FCBP Accession #
1	Natal 1	Pantoea *sp.*	606
2	Natal 2	*Enterobacter cloacae*	607
3	Natal 3	*Azomonas sp.*	567
4	Natal 4	*Citrobacter diversus*	568
5	Dancy	*Bordetella pertussis*	610
6	Dancy 5	*Azomonas agilis*	611
7	Dancy 6	*Aureobacterium liquefacians*	613
8	CRI-7	*Ensifer adhaerens*	566
9	CRI-78	*Acinetobacter sp.*	570
10	Trifoliate	*Kurthia sp.*	612
11	Trifoliate 1	*Azobacter nigrieans*	609
12	Trifoliate 2	*Ensifer adhaerens*	608

Morphological Characterization

Morphological characters of all the isolated bacterial strains are described in (Table 2).

Biochemical Characterization

Biochemical characterization of bacteria using catalase test, indole test, methyl red test, and hydrogen sulphide test are also helpful in identification of bacterial isolates. Most of the tested endophytes are gram negative and they have concave elevation. *Azotobacter nigricans* has flat elevation. Most have spore forming ability and are motile. But *Azomonas agilis, Aureobacterium liquefacians* cannot form spores. *Azomonas agilis,* and *Bordetella pertussis* are catalase negative (Table 3).

Evaluation of Anti-fungal Activity

The zone of inhibition on agar plate for antifungal activity of bacteria against *A. solan*i for different test bacterial strains is shown in (Fig. 2). The 12 isolated strains *(Pantoea sp, Enterobacter cloacae, Azomonas agilis, Aureobacterium liquefaciens, Ensifer adhaerens, Kurthia sp, Acinetobacter sp., Bordetella pertussis, Azotobacter nigrieans, and Citrobacter diversus)* were used against the pathogenic fungus. These endophytes have ability to control the fungus. Some actinomycets have also ability to control the pathogenic fungus. *Acinetobacter sp.* also controlled the fungus by producing metabolites.

Table 2: Morphological characterization of isolated bacterial strains from different varieties of citrus

Bacteria	Margins	Colony color	Shape	Texture	Gram type	Elevation	Mobility	Spore forming
Pantoea sp.	Irregular	Lemon color	Oval	Slightly rough	-ve	Concave	+ve	-ve
Enterobacter cloacea	Wavy	Off white creamy	Round	Smooth	-ve	Concave	+ve	-ve
Azomonas agilis	Wavy	Yellow	Lemon shape	Smooth slightly rough	-ve	Concave	+ve	+ve
Aureobacterium liquefaciens	Wavy	Creamy off white	Oval	Smooth slimy	+ve	Concave	+ve	+ve
Ensifer adhaerens	Irregular	Lemon yellow	Lemon shape	Smooth	-ve	Flask	+ve	-ve
Kurthia sp.	Round wavy	Dirty white	Round	Slimy smooth	-ve	Concave	+ve	-ve
Acinetobacter sp.	Wavy	White	Round	Smooth slimy	+ve	Concave	+ve	-ve
Bordetella pertussis	Wavy	Dirty off white	Round	Smooth slimy	-ve	Concave	-ve	-ve
Azomonas sp.	Wavy	Creamy off white	Round	Smooth slimy	-ve	Concave	-ve	-ve
Citrobacter diversus	Wavy	Yellow	Round	Slimy crust	-ve	Concave	-ve	-ve
Azobacter nigricans	Irregular	Yellow	Large oval	Slimy shiny	-ve	Concave	-ve	-ve
Ensifer adhaerens	Irregular	Lemon yellow	Lemon shape	Smooth	-ve	Flask	+ve	-ve

Table 3: Biochemical characterization of isolated bacterial strain from different varieties of citrus

Bacteria	Indole test	Catalase test	Methyl red test	Hydrogen sulphide	Citrate utilization test
Pantoea sp.	-ve	+ve	-ve	-ve	+ve
Enterobacter cloacae	-ve	+ve	-ve	-ve	+ve
Azomonas agilis	-ve	-ve	-ve	-ve	+ve
Aureobacterium liquefacians	-ve	+ve	-ve	-ve	+ve
Ensifer adhaerens	-ve	+ve	-ve	-ve	+ve
Kurthia sp.	-ve	+ve	-ve	-ve	+ve
Acinetobacter sp.	-ve	+ve	-ve	-ve	+ve
Bordetella pertussis	-ve	-ve	-ve	-ve	+ve
Azomonas sp.	-ve	+ve	-ve	-ve	+ve
Citrobacter diversus	-ve	+ve	-ve	-ve	+ve
Azobacter nigricans	-ve	+ve	-ve	-ve	+ve
Ensifer adhaerens	-ve	+ve	-ve	-ve	+ve

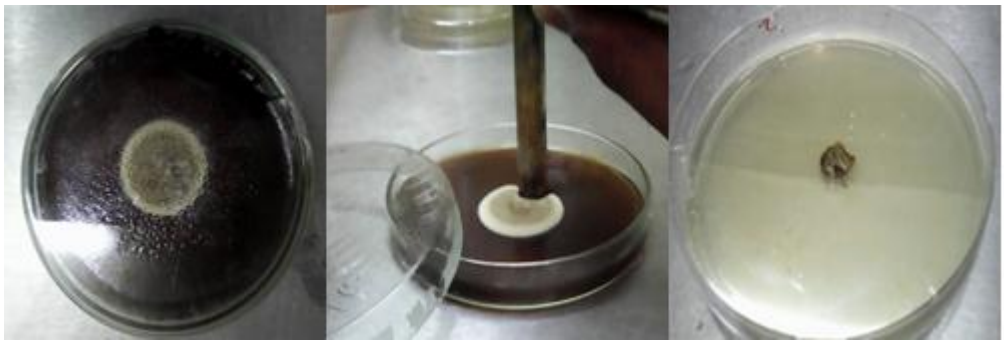

Fig. 1: Potato dextrose agar plates of *A. solani* used for the antibacterial activity of endophytes bacteria

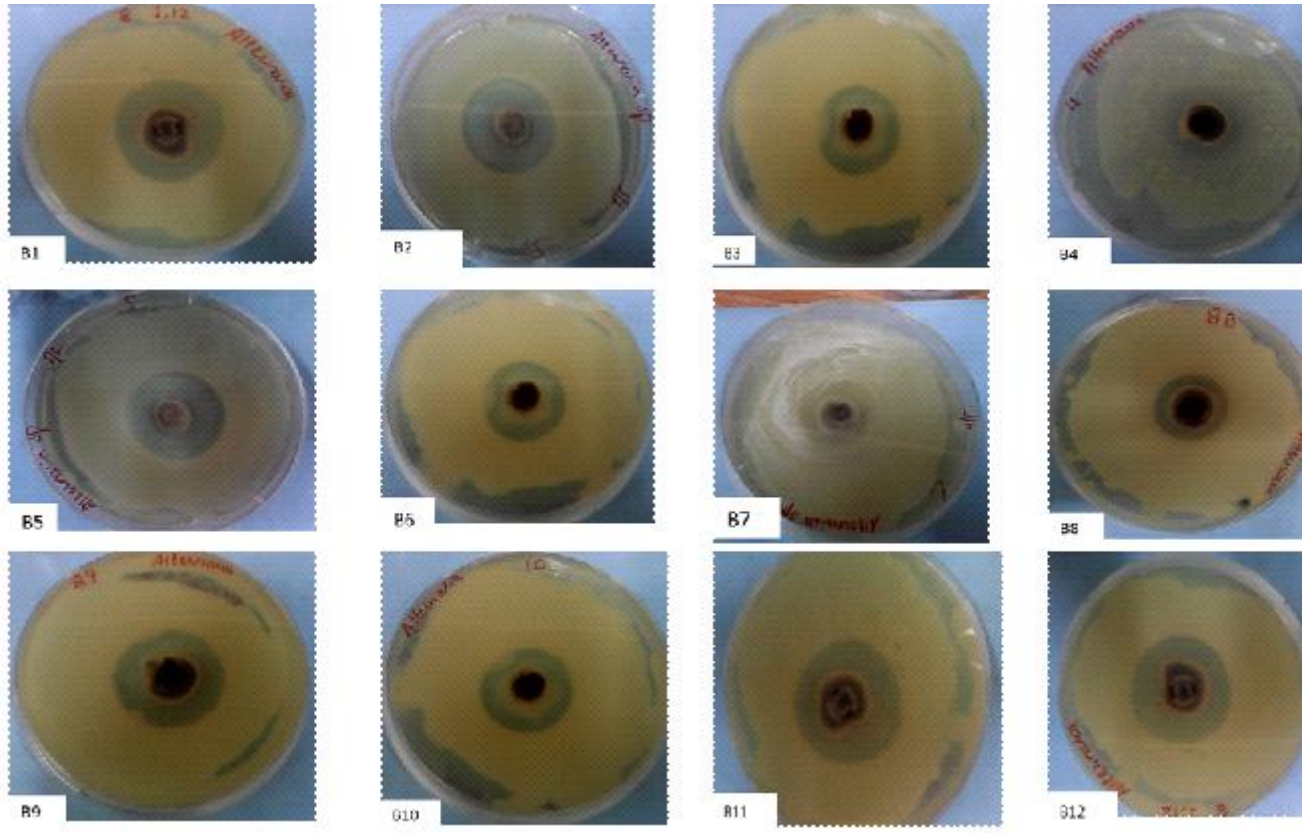

Fig. 2: The zones of inhibition on agar plate for antifungal activity of bacteria against *Alternaria solan*i against different test bacterial strains. **B1)** *Pantoea sp*. **B2)** *Enterobacter cloacae* **B3)** *Azomonas agilis* **B4)** *Aureobacterium liquefacians* **B5)** *Ensifer adhaerens* **B6)** *Kurthia sp.***B7)** *Acinetobacter sp.* **B8)** *Bordetella pertussis* **B9)** *Azomonas sp.* **B10)** *Citrobacter diversus* **B11)** *Azotobacter nigricans* **B12)** *Ensifer adhaerens.*

Pantoea sp. (35.66mm), *Ensifer adhaerens (35.33*mm*)*, *Citrobacter diversus (33.03mm), Azotobacter nigricans* (31.56mm) had the same ability to control the growth of *A. solani*. While *Aureobacterium liquifaciens (27.66*mm*)*, *Acinetobacter sp. (25.66*mm*), Bordetella pertussis (26.63*mm*)* showed equal potential for inhibition. *Enterobacter cloacae (19.33*mm*)*, *Azomonas agilis (17*mm*)* and *Kurthia sp*. (19mm) has nearly similar potential and were less efficient compared to others (Fig. 3; Table 4).

Table 4: Antagonistic activity of selected endophytic bacteria strains against *Alternaria solani* on PDA plates

Treatments	Diameter of inhibition zone in (mm)
Control	64.66±1.65 a
Pantoea sp.	35.66±0.981 b
Enterobacter cloacae	19.33±0.72 f
Azomonas agilis	17±0.942 f
Aureobacterium liquefaciens	27.66±1.186 de
Ensifer adhaerens	35.33±0.232 b
Kurthia sp.	19±0.471 f
Acinetobacter sp.	25.66±0.72 e
Bordetella pertusis	26.63±0.745 de
Citrobacter diversus	33.03±0.47 bc
Azobacter nigricans	31.56±0.65 c

Notes: The data presented in this table represent the mean value of three replicates; ± SE of the mean; Duncan's Multiple range test showed that same letters are not significantly different at (P=0.05).

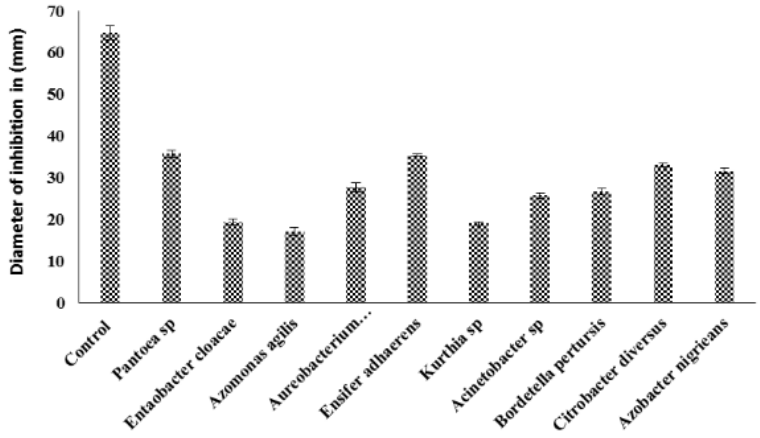

Fig. 3: Antifungal potential of selected bacterial strains against *Alternaria solani*

Pantoea sp. showed more potential to inhibit the growth of *A.solani* as compared to other isolated test bacterial strains. The exploitation of further bacterial strains for the control of these fungi has not been reported yet. In this study, evidence on the culturing and characterization of bacterial isolates having antagonistic potential for *Alternaria solani* is represented with the potential to be used as biological control agents.

DISCUSSION

Production of tremendously diverse bioactive compounds by endophytic bacteria and their potential use as biological control agents has been reported to be dependent on many constraints including taxonomical position, physiological characters and geological conditions (Sharma, 2009). Endophytic bacteria might either become localized at the entry point or spread throughout the plant tissues. They can also antagonize phytopathogens via secretion of various bioactive molecules since they exist in the same system (Ongena, 2008). Endophytic bacteria can release a wide array of extracellular bioactive metabolites with high capability to inhibit the growth of various bacterial and fungal species thus they can be used to manage different plant diseases (Liu *et al*., 2015). Many bacterial endophytes have been isolated from rice such as *Pseudomonas sp., Burkholderia sp., Herbaspirillium seropedicae, Rhizobium leguminosarum, Klebsiella sp.* (Olivares

et al., 1996). There are also many report of *Enterobacter sp.* from *Citrus sinensis* and other crops (Kuklinsky-Sobral *et al*., 2005), while *Pantoea sp.* species has been reported from sugarcane and soybean (Loiret *et al*., 2004; Magnani *et al*.,2010).Nitrogen fixing *Enterobacter sp.* also isolated from citrus mid rib is a phosphate solubilizing bacteria.

Morphological characterization of endophytic bacteria is essential to understanding the taxonomy (Araujo *et al*., 2002), they can be gram positive and gram negative (Mardaneh & Dallal, 2013), *Aureobacterium liquefaciens* is a gram positive bacteria with a colony color yellow and a rod shape bacteria (Yokota, 1993). A wide variety of endophytes have been isolated from Citrus plants including *Bacillus specie* from Rough lemon (Araujo *et al*., 2002).

Endophytic streptomyces, have been found by observing zones of inhibition in Petri plates (Larkin *et al*., 1998). Bacterial strains isolates produced a zone of inhibition on agar plates browning the pathogens and growing rapidly to stop the growth of pathogen (Franicevic, 1993). Yazici *et al* (2011) reported that several bacterial species have highest antifungal activity and very effective against *alternaria solani* in vitro tests. Bacterial isolated strains have more diversity and having more ability to control pathogens spreading various diseases in agriculture crops (Sardi *et al*., 1992; Coombs & Franco 2003; Taechowisan *et al*., 2003; Franco *et al*., 2007).

Endophytic bacteria provide a better environment for the plant against the fungal pathogen (Whipps, 1997). Mostly antagonistic endophytic bacteria use antibiosis and competition lysis against the fungal pathogen. Work on antagonistic effect of bacterial endophytes on different species of fungi has been reported, while endophytic bacteria can be antagonistic against a fungus (Berg & Hallmann, 2006). Use of biological ways to control the agricultural pests and diseases is an effective substitute compared to pesticides, which may accumulates in soil and become lethal to the soil borne microbial communities (Nagórska *et al*., 2007).Since the use of environmental friendly procedures for the improvements are often based on the search for new genetic, chemical, and biological sources that are effective for Plant health related problems (Gillican, 2008), there are several reports on the use of different microorganisms as biological control agents to reduce the incidence of phytopathogens by the production of antibiotics (Raaijmakers *et al*.,2002).

Various Gram-negative bacteria have antifungal activity through the production of extracellular lytic enzymes, siderophores, salicylic acid, antibiotics, and volatile metabolites, such as hydrogen cyanide (Manwar *et al*.,2004) to control fungal plant pathogens. Hence there is possibility that Gram-negative bacterial isolates may produce antifungal compounds (volatile compounds or certain enzymes) that could be lost during the processing of these bacterial compounds. However the presence of bacteria is necessary for production of the substances responsible for inhibition, and could be associated with the bacterial communities (Afsharmanesh *et al*., 2006). However *Pantoea sp.* are used is in post-harvest biological control of fungi in fruits and especially citrus, where it can inhibit the development of *Rhizopus stolonifer*, *Penicillium digitatum, Penicillium expansum*, *Monilinia laxa*, *Botritis cinereae* and could be useful an alternative to chemical fungicides (Morales *et al.,* 2008). Many natural products produced by endophytes have proven to be antifungal, antibacterial, antidiabetic, antioxidants and immune suppressive and great source of bioactive natural products. The majority of endophytic bacteria produce novel antibiotics like Munumbicins, Ecomycins, Pseudomycins, and Kakadumycins. These compounds inhibit the growth of pathogenic bacteria and fungi (Christina, 2013).

CONCLUSION

From the study it can be concluded that, some of bacterial strains isolated from citrus may be very useful for controlling the various plant pathogens having a very strong antifungal activity. *Pantoea sp.* is most effective one that can be used as a biological control agent for controlling of various fungal diseases of other crop plants along with other species i.e. *Ensifer adhaerens* and *Citrobacter diversus*. The information obtained from the study is equally beneficial for microbiologists, researchers, students, scientists and farmers community for adopting and utilization of such types of endophytes to control pathogens for increasing the yield potential of the crop.

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