



ISOLATION AND IDENTIFICATION OF CHITINASE PRODUCING ANTIFUNGAL RHIZOBACTERIA.

Microbiology

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ABSTRACT

The public concern over the harmful effects of chemical pesticides on the environment and human health has enhanced the search for safer, environment friendly control alternatives. Control of the plant pests the application of biological agents holds great promise as an alternative to the use of chemicals. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volume of pesticides. It is widely distributed in nature as an integument of insect and crustaceans and as a cell wall component of fungi and algae. Chitinases poly (1,4- (N; acetyl ; B; glucosaminide) glycanohydrolase are a group of enzymes that able to degrade chitin directly into low molecular weight products. In this study 18 soil samples were randomly collected from the rhizosphere of wheat from different villages of district, Faridabad. These soil samples were used for the isolation of antifungal chitinase producing bacterial strains. A total of 40 chitinase producing bacterial isolates were obtained. Antagonistic activity of all the isolates was tested against fungal pathogen *Fusarium oxysporium* using standard dual culture technique. Out of the forty isolates eight isolates proved to be positive antagonists of this fungal pathogen i.e *Fusarium oxysporium*. The inhibition of tested fungal pathogen by bacterial isolates varied from 32.00%- 65.00% and average percentage inhibition was calculated as 56.00% R6 has been found as the best isolate showed their terms of percentage inhibition of *Fusarium oxysporium* i.e 65.00%. Due to the importance of chitinolytic enzymes in insects, Nematode, and fungal growth and development, they are receiving attention in regard to their development as biopesticides or chemical defence proteins in transgenic plants and microbial biocontrol agents. In this sense, biological control of some soil-borne fungal disease has been correlated with chitinase production. This study will lead to isolating antagonistic Rhizobacterial strains having chitinolytic activity against the prevalent fungal pathogens affecting crops, such isolates may be developed into successful soil inoculants strains.

KEYWORDS

Chitinase, rhizobacteria, *Fusarium oxysporium* and chemical pesticides.

Introduction

Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as Plant Growth Promoting Rhizobacteria (PGPR). Application of PGPR may become a promising bio control agent for plant diseases. Large scale application of PGPR to crop as inoculants will substantially reduce the use of chemical environment (Bloembergen and Lugtenberg) (2001). The whitefly, *Bemisia Tabaccae* Genn, (Hemiptera: Aleyrodidae) is an important pest of many crops. It attacks more than 500 plant species belong to 63 plant families. The insect is a vector of plant viruses member of Gemini virus group (Damayanti et al, (2007). The beneficial plant-microbes interaction in the rhizosphere are determinants of plant health and soil fertility Jeffries et al., (2003). For agriculture production, these interaction play a pivotal role in transformation, mobilization solubilization etc from a limited nutrient pool in the soil and subsequently uptake of essential plant nutrients by the plants to realize full genetic potential of the crops. In the biogeochemical cycles of both inorganic and organic nutrients in the soil and in the maintenance of soil health and quality, soil microorganisms are very important Jeffries et al., (2003). It is necessary to improve the efficiency of the meager amount of external inputs by employing the best combination of beneficial microbes. Soil bacteria isolates from rhizosphere which have been shown to improve plant health or increase yield, are usually referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth) (1978), Suslow and Schroth (1982). The beneficial effects of PGPR have been observed in many crops including horticultural, oilseed crops etc. However in wheat, reports are scanty especially in biocontrol aspects Hoisington et al., (1999). Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance (Wisniewski and Wilson, 1992). The use of environment friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture.

Fungal plant pathogen is among the most important factors that cause serious losses to agriculture products annually (Ekundayo et al., 2011).

Management of fungal disease using antagonistic microorganisms, known as control, has been the focus of intense research worldwide (Killani et al., 2011). Biological control of plant pathogens is considered as a viable alternative method to chemical control. Non-pathogenic soil bacteria, the plant growth promoting rhizobacteria (PGPR) living in association with roots of higher plants enhance the adaptive potential of the hosts and increase their growth through a number of mechanisms. *Bacillus* spp. have also been known to produce compounds which promote plant growth directly or indirectly viz., hydrogen cyanide (HCN), siderophores, indole acetic acid (IAA), solubilized phosphorus and antifungal activity.

They produce toxins, fumonisins and trichothecenes. *F. Oxysporum* has a variety of hosts that include sugarcane, garden beans, cowpeas, spinach, potatoes, banana, watermelon, prickly pear, tomato, cucumber, pepper, muskmelon, tobacco, cucurbits, Sweet potatoes, asparagus, vanilla, strawberry, and cotton Naik et al., (2010).

Evidence shows the intervention of rhizosphere microorganism as protective agents against soil-borne plant pathogen. In many instances a correlation has been established between antagonist in vitro and protection in the field (Paland Gardner 2006). The present study has been taken up with the objective to isolate and characterize the isolated *Bacillus* spp. for their plant growth promoting by various mechanisms and their antagonistic effect on *F. Oxysporum* (Javed et al 2013). The plant growth promoting fungus *Fusarium equiseti* GF183 effectively controlled *Fusarium wilt* of spinach caused by *Fusarium oxysporum* *F. spp. spinciae* in transplanting systems using paper pots. Reduction in disease severity ranged from 43.5 to 91.8%. Double application of *F. equiseti* GF183 increased the protective effects (Van Loon et al., 1998). The antifungal properties of chitinolytic soil bacteria may enable them to compete successfully for space and nutrient with fungi. The production of chitinase may be a part of the lytic system that enables the bacteria to use living hyphae rather than chitin as the actual growth substrate, since chitin is an important constituent of most fungal cell wall. Lysis of living fungal hyphae by chitinolytic bacteria has been reported; however these reports mostly

deal with bacteria that had been selected because of their mycolytic properties (Chet et al., (1990).

Spinach varieties are classified by leaf types, and there are three types grown in Nova Scotia; Savoy (wrinkled), semi – Savoy and flat. Savoy and semi-Savoy are used for fresh markets, while smooth(flat) types are used for baby spinach. Spinach prefers a cool climate. The minimum temperature for seed germination is 2°C with a maximum germination temperature of 30°C and an optimum range of 7 to 20°C. Young plants can withstand temperatures as low as -9°C. Best crop growth occurs at 15 to 20°C with a minimum temperature of 5°C and a maximum of 30°C. Spinach bolts rapidly when days are both long and hot (Hoffland et al., (1996) Baranwal A et al., 2013). Induced Resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated Van Loon et al., (1998). In 1991 two research groups independently described ISR as the mode of action of disease suppression by non pathogenic rhizosphere bacteria Van Peer et al., (1991). Fusarium wilt starts out looking like vein clearing on the younger leaves and drooping of the older lower leaves, followed by stunting of the plant, yellowing of the lower leaves, defoliation, marginal necrosis and death of the plant. *Fusarium oxysporum* is split into divisions called *formae speciales*. There are over 100 *formae speciales* divisions, each with one or two different races. Each *forma specialis* within the species are host-specific (i.e. specific to a certain plant) and produce different symptoms. *F. oxysporum* causes vascular wilt in tomato. *Fusarium oxysporum* is the most widely dispersed of the *Fusarium* species and is found worldwide. *F. oxysporum* has no known sexual stage, but produces three types of asexual spores: microconidia, macroconidia, and chlamydospores. *F. oxysporum* is a common soil saprophyte that infects a wide host range of plant species around the world. It has the ability to survive in most soil—arctic, tropical, desert, cultivated and non-cultivated. Though *Fusarium oxysporum* may be found in many places and environments, development of the disease is favoured by high temperatures and warm moist soils. The optimum temperature for growth on artificial media is between 25-30°C, and the optimum soil temperature for root infection is 30°C or above. However, infection through the seed can occur at temperatures as low as 14°C. *F. oxysporum* is a major wilt pathogen of many economically important crop plants. There are no exact figures published on the damage caused by the Fusarium wilt. However, planting susceptible varieties were noted to produce more than 50% losses. Total losses in US, of Barley & wheat crops between 1991 & 1996 have been estimated at \$ 3 billion.

Objectives:

As there are many harmful effects of using chemicals or other types of inorganic pesticides for crop prevention so we are focusing on eco friendly method of using antifungal chitinase producing rhizobacteria as potential bio control agents. Thus, the present study has been designed to isolate and identify the chitinase producing antifungal rhizobacteria associated with vegetable crops. This overall aim will be achieved through following objectives.

- 1 Isolation of rhizobacteria associated with vegetable crops.
- 2 Assessment of antifungal activity of the isolate bacterial strains.
- 3 Identification of potential chitinase producing antifungal rhizobacteria.

Methodology

Collection of soil samples:

The soil sample was randomly collected from the spinach cultivation villages of district Faridabad, Haryana India. Sampling was carried out in such fields which have a long history for usage of capton for last many years. young plant were chosen and completely uprooted to get the roots and the adhering soil particles .the loosely bound soil was removed with vigorous shaking , the foliage part was removed and the remaining root and soil samples were transferred to sterile polythene bags, and stored at 4°C till further use .The sample was carried out from 15th February 2013 to 20th february 2013. Total 20 soil sample were collected from twenty spinach cultivation villages of district Faridabad.

Fungal sample collected from Agriculture Research Center Faridabad, Haryana, India. Four fungal strains were *Fusarium Oxysporum*..We were stored in refrigrated at 4°C Temperature and further used as sample.

S. No.	Date	No. of soil sample
1-	16/01/2014-17/01/2014	Spinach soil sample
Area		
Bajirpur ,Pipali gaav, Jasona tigaw, Pipali gaav Chandpur, Khedi, Kuaapur, Tigaun, Jhajharwala,		
Mithapur, Neemka, Korali, Bhudena, Bhudena, Sharjapur, Mithapur and Jharjawala		

Table no 2: Soil samples collected from different villages of district Faridabad, Isolation of rhizobacteria associate with vegetable crop:

Isolation of rhizobacteria:

54 rhizobacterial strains were isolated from the rhizosphere of spinach using dilute plate methods. Different selective media like, Mineral salt media Agar for *Pseudomonas* spp. was used along with the general media i.e. Nutrient Agar. chitin adding in the mineral salt medium it is source of carbon and energy. The loosely bound soil was removed by vigorous shaking. The tightly bound soil particles were removed aseptically with the help of brush. Also 1gm soil weighing and soil were dispensed in 10 ml water and shaken for mix well for few 10-15 minuts. The resulting suspension was diluted 10 fold and used for isolation of rhizobacteria.

The prepared dilution was spread on the media plate and incubated for 24 – 48 hours at 30°C Temperature. After incubation the plates were observed for the discrete isolated colonies. The colonies from the plates were transferred by streaking on the agar slants (Fig:1). These cultures are further processed by transferring them in glycerol stocks for long term preservation.



Figure No 1: Isolates fom soil sample

Preparation of chitin :

Colloidal chitin was prepared from practical grade crab shell chitin (Loba chemie) described by Shu Loackwood 1975. 10Gms of chitin take from crab shell sigma and chitin crab shells crush in motar and pestal .And then 150 ml Hcl (Hydrochloric acid) added in the chitin . chitin incubate for 1hour in shaker incubator . after incubation time added child water 500ml in the chitin . mix well after few minuts filter the chitin with wattman filter paper . filtered chitin collected in biker and then added 500ml chiled water again . again filtered the chitin collected in biker this step repeat atleast four time . after filtered chitin four time collected in biker and stored at 4°C temperature for further use.

Preservation and Maintainance

Subculturing of selective isolates on agar slants was carried out to get the pure cultures and were preserved at 30°C to use them further in during dual culture technique.

Assessment of antifungal activity of the isolate bacterial strains.

Dual culture technique:

A 5 mm plug from activated fungal cultures was placed at the centre of PDA media plates. Activated bacterial cultures were parallel streaked at a distance of 2 cm from the fungal plug. Control was designed so as to contain only the fungal plug without rhizobacterial streaks. The plates were incubated at 25° C for 5-7 days and observed for radial growth of fungus. The diameter of radial growth of fungus was measured for all the test plates as well as control. All the experiments were performed in duplicates.

Percentage inhibition of fungus was calculated using the formula:

$$I\% = \frac{(C-T/C) \times 100}{I}$$

Inhibition C = Conrot T= Test

1. Strong antagonist: Growth of biocontrol agent was very fast, it covered the entire medium surface and completely overgrew the

- pathogen.
- ii. Antagonistic: Growth of biocontrol agent was very fast, it covered at least 2/3rd medium surface but without showing mycoparasitic action.
- iii. Moderate antagonist: Growth of biocontrol agent was very slow but rapidly it overgrew the pathogenic one in contact.
- iv. Slow antagonist: Growth of biocontrol agent and pathogen was similar in magnitude, none appeared to be dominant to the other.
- v. Poor antagonist: Growth of the pathogen was fast, it colonized at least 2/3rd of the surface and appeared to withstand encroachment by the biocontrol agent
- vi. Non antagonist: Growth of the pathogen was very fast, it completely overgrew the biocontrol agent and covered the entire medium surface.

Isolation microbial strains capable of producing chitinase

Mineral salts medium on agar plate supplemented with pesticide as sole carbon and energy source was prepared for isolation of chitin

Identification of potential chitinase producing antifungal rhizobacteria:

The most efficient chitinase producing isolates were characterized based on their morphological and physiological characteristics according to Bergey's manual of determinative bacteriology (Holt et al., 1994).

Morphological Identification and Gram staining procedure

The isolated isolates were examined for their colony and cell morphology. Different morphology features including, size, shape, margins, colour and consistency of colony, cell shape, cell arrangement, gram reaction, were examined as per the standard produces described by Aneja (2003) and Cappuccino and Sherman (2010). The activated culture was taken on the clean glass slide with the help of the loop and One drope of normal sline mix with loop making a thin smear and dry in air . It was stain with crystal violet for 1 minute and washed with water followed by staining with iodine and washed with acetone after 1 minute. The smear was then stained with safrinin o for 1 minute and then washed with tap water observation was made under compound microscope .The purple coloured isolates were recorded as Gram positive bacteria and pink coloured isolates recorded as Gram negative bacteria.

Statistical analysis:

The data obtaine for various experiments carried out in present investigation was subjected to one way analysis of varience (ANOVA) for completely randomized design where applicable with P < 0.05 being accepted as significant with the help of Microsoft excel.

Results :

Here we present the results of the study aimed at isolation and identification of 8 bacterial isolates obtained from the rhizosphere of spinach for their antagonistic potential against diseases caused by *Fussarium oysporium*. All the 8 isolates from the healthy rhizosphere of wheat, from different regions of District Faridabad, Haryana were examined for their antagonistic potential against *Fussarium oysporium*.

Isolation:

54 bacterial isolates were obtained from the 16 soil samples enriched with chitin given the no R1. R2.

R3.R4. R5. R6. R7.R8.....R54 differend no kind of isolates were obtained from 16 soil sample. It was interesting to note that 100% (R1 to R54) of the bacterial isolates showed growth on mineral salt medium and nutrient media . The details of isolates obtained from various sample are given in table.

S.N.	Sample name	Isolates
1	7,13,&14	2
3	03,6,9,11, 15 &16	3
4	01,2,5,8,12 &13	4
4	04	5

Total No 5. of isolates obtained from each soil sample with their name

Antifungal activity:

A total of 54 bacterial strains were isolated from the 16 soil samples

collected from wheat fields of Faridabad district ,Haryana. The antagonistic behavior of isolates against was tested against *Fussarium oxysporium* using Dual culture technique (Egamberdieva, 2007). Out of the tested 40 strains only 8 isolates i.e showed significant antagonistic potential against the test R1, R2, R3, R4, R5, R6,R54 ted fungal pathogen i.e. *Fussarium oxysporium* .However, variation in inhibition was observed although inoculums load was same for all isolates (P <0.05). The results obtained were as follows .

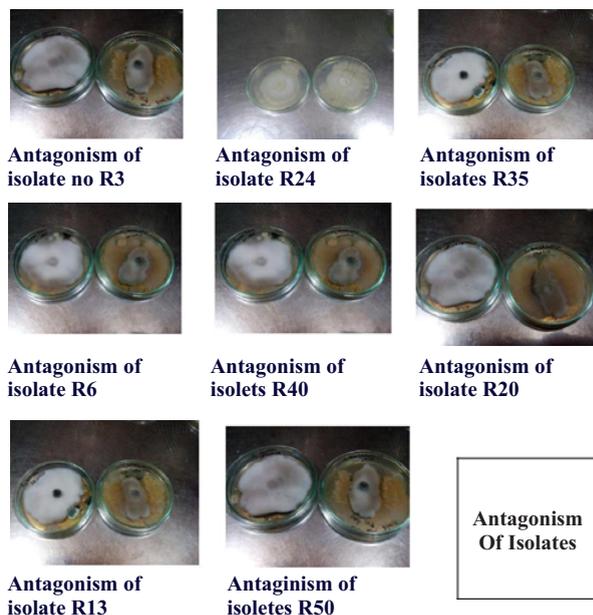
All the isolates obtained were analysed for their antifungal activity against *Fussarium oxysporium*.

Sample no.	Isolates name	Antagonistic behavior
1-2, 4-5, 7-12, 14-19,	R1-2, R4-5, R7-12, R14-19, R36-	-
36-39, 41-49, 51-52	39, R41-49, R51-52	
3, 20	R3, R20	+
6, 50	R6, R50	++
13, 24-34, 35, 40, 53	R13, R24-34, R35, R40, R53	A+
- = no inhibition, += positive isolate, +	+ = fairly positive isolate, A+ = Strongly positive isolate	

Table no 6: Isolates analysed for antifungal activity

Plates in which in ability of isolates to inhibit the growth of fungus was tested:

Below are the plates representing the positive antagonists which showed inhibition against *Fussarium oxysporium*. The growth of fungus in the test sample is lower as compared to control sample.



In these pictures we can clearly see that growth of fungus in control plate is less than the corresponding growth in the test plate. Further, we checked the growth inhibition and percentage inhibition of the positive isolates (Table7)

S. No.	Isolate name	Radial growth of fungus in (mm)	Percentage inhibition
1	R3	28 ± 5.45	64.5 ± 6.06
2	R6	32.5 ± 0.90	67.5 ± 0.70
3	R13	31 ± 2.41	63 ± 2.41
4	R20	35.5 ± 3.53	63.5 ± 5.94
5	R24	28.5 ± .80	45.5 ± 2.12
6	R35	33 ± 1.41	42.56 ± 2.82
7	R40	32.5 ± 3.53	37.5 ± 3.53
8	R50	32 ± 2.28	30 ± 1.82

Table 7 : Percentage nhibtion of tested fungus by isolated bacterial strains.

Isolate varied significantly in terms of percentage inhibition of the

tested fungus ($P < 0.05$) and the average percentage inhibition against *Fusarium oxysporium* was observed as 53% while its maximum inhibition was 65% by the isolate R3, R6, R13, R20. These results show that out of the positive antagonists; R3, R6, R12, R20, and R6 showed maximum percentage inhibition of test fungus and isolate R50 showed minimum inhibition. As compared to the control the zone of inhibition was clearly visible at 5th day after incubation. The percentage growth inhibition was found to be between 30%-65%. Growth inhibition of *Fusarium oxysporium* may be attributed to production of chitinase or other inhibitory substance antagonists.

Identification of chitinase producing Antagonistic isolates:

All the eight strongly positive antagonistic isolates which showed maximum inhibition against prevalent fungal pathogen i.e. *Fusarium oxysporium* were further identified for their morphological characteristics on the basis of colony characteristics and Gram staining. During the identification some non-motile and motile rod shaped bacteria were seen which were pink in color i.e. gram negative. On the basis of their colony characteristics, gram staining and motility test they have been tentatively identified as belonging to the genus *Flavobacterium* (R6, R24, R35, R50). On the other hand some isolates which showed violet color i.e. gram positive and were circular in form of a chain and pairs have been tentatively identified as belonging to the genus *Streptococcus* (R3, R13, R20, R40).

S.No	Name of the isolate	Presumptive
		identification
1	R3, R13, R20 and R40	<i>Streptococcus</i>
2	R6, R24, R25 and R50	<i>Flavobacterium</i>

Table 7 : Identification of the antifungal bacterial isolates.

Discussion:

From the results, it can be inferred that 8 isolates have been confirmed to possess antifungal activity against the fungus *Fusarium oxysporium*. Out of those, 8 isolates are likely to be *Bacillus* species and two of them may be *Pseudomonas* species. They have been isolated on specific media. This result is in accordance with the previous researches on the related issues by the researchers. It has previously been reported that application of mixture of isolates inhibits pathogen growth more efficiently than single isolate (Marjan et al., 2003). The reason why application of single isolate does not control disease in better way might be related to insufficient root colonization. Therefore, these mechanisms by applying a mixture of the isolates lead to more effective biocontrol of fungal diseases in the plant. These results are also supported by Kumar and Narula (1999, 2006) who isolated PGPRs from wheat rhizosphere that had ability to produce IAA and to solubilize phosphate. Some other mechanism such as hydrocyanic acid, siderophores and induction of resistance may also play a role in the action of PGPR. So that rhizobacterial agents will probably be one of the most significant strategies for disease management (Javed A et al., 2015; Luz, 1996). Therefore, the PGPR used in our study were promising as plant growth stimulator and biocontrol against fungal disease in barley.

Agriculture in modern times is getting more and more dependent upon the steady supply of artificial fertilizers and pesticides with the introduction of green revolution technologies. Fertilizers and pesticides may be introduced directly into the environment in a liquid phase, as a dispersion or solution or in a solid form as powder or granular form. Sprays are directed to the foliage. Solids are applied to soil surface or foliage. Some fertilizers are systemic that is they get absorbed in the plant tissues and end up in the consumers. Some of them are contact, which are applied on the surface to fight pests and diseases.

These biocides tend to remain active long after destroying the target i.e. pests, weeds, fungi and rodents. On continued application these agrochemicals causes contamination of food of food materials, disruption of natural balance of ecosystem by killing non target species and gradual increase in the immunity of target organisms to these chemicals. Further since most of these chemicals are not biodegradable they enter the food chain and persist in plant and animal bodies.

Continued use of huge amounts of different kinds of poisonous agricultural pesticides increase their concentration in the organism and multiplies through food chain and a phenomenon called biomagnification

is caused which moves up in the food chain and affects the apex species in the food pyramid. Man also situated at the higher trophic level of food accumulates these poisons and many cases of food poisoning and contamination are reported.

Conclusion:

Our findings establish the possible application of chitinase releasing antagonistic *Streptococcus* and *Flavobacterium* strains in the biocontrol of a prevalent fungal pathogen of Spinach i.e. *Fusarium oxysporium*. The study highlights the importance of lytic enzymes (chitinase) produced by isolates for biocontrol of fungal diseases. Further detailed investigations using efficiency test of the isolated bacterial strains under field and pot conditions are needed to clarify their potential in control of prevalent fungal pathogen i.e. *Fusarium oxysporium*. The world population is growing by 160 people per min and wheat is predicted to be the most important cereal crop to feed the ever increasing world population. The total area under crop is about 29.8 million hectares in the country. The production of wheat in country has increased from 75.81 million MT in 2012-2014. The productivity of wheat which was 2602kg/hectare in 2004-2005 has increased to 3140kg/hectare in 2012-2014. The major increase in productivity of wheat has been observed in states of Punjab, Haryana, and Uttar Pradesh. Higher coverage area is reported from MP in recent years (Fatima et al., 2009). Fungus are the most important

pest group in wheat production worldwide.

The incidence and impact of pathogens especially

Fusarium oxysporum, *Alternaria solani*, *Septoria* spp. and rust fungi, increase with intensity of crop productivity (i.e. with attainable yield). Annual losses of wheat due to *Alternaria solani* is in range of 32-57% (Fatim et al., 2009). Farmers have been using various types of fungicides, insecticides, herbicides for protection of the crops over the centuries. Insecticides like DDT, organophosphates, carbamates, phenoxy-acetic acid derivatives as herbicides and thiram as fungicide are the major chemical agents for crop protection. Over the last 60 years progress in chemical crop protection is extraordinary (Muller, 2002). But there are various types of harmful effects of these chemical pesticides on the human health and the environment, so biocontrol of these fungal pathogens is of utmost importance. Biological control is environmentally safe and is only option available to protect plants from pathogens. Biological control is defined broadly as the "use of natural or modified organisms, genes, or gene products" to reduce the effects of pests and diseases (Javed et al., 2013; Heydari et al., 2010).

PGPR or plant growth promoting rhizobacteria are considered to be the most suitable biocontrol agents for control of fungal diseases. Nonpathogenic soil bacteria, the plant growth promoting rhizobacteria (PGPR) living in association with roots of higher plants enhance the adaptive potential of the hosts and increase their growth through a number of mechanisms (Shobha et al., 2012). PGPR can be figuratively divided into three groups. The first group is free living microorganisms that specifically interact with plants under favorable conditions. The second group is rhizospheric and phylospheric species.

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