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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF RHIZOBACTERIA ASSOCIATED WITH PEACH PLANT AND EVALUATION OF THESE RHIZOBACTERIA AGAINST ITS FUNGAL PATHOGENS

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Abstract

Rhizospheric bacteria directly or indirectly affect plant growth. Management of plant diseases through biocontrol agents is one of the best approach that mayreduce the use of synthetic chemical based formulations. In this study, antagonistic activities of *Bacillus subtilis*was evaluated *In vitro* against: *Monilinia fructicola, Taphrina deformans* and *Alternaria alternate* which cause Brown rot, Peach leaf curl and Alternaria rot. In dual culture technique, microbial antagonists inhibited the spore germination and mycelial growth of *Moniliniafructicola* and *Alternaria alternata.* Pathogenicity test for *Taphrina deformance* was also performed on leavesof Peach seedlings while pathogenicity of *Monilinia fructicola* and *Alternaria alternata* were done on Peach fruit being fruit rotting fungi. Out of 16 isolates,3 isolates *B.subtilis* viz., Rh3, Rh7 and Rh12 showed strongest activity against *Monilinia fructicola*, having 12mm, 12mm and9mm zone of inhibition for *Taphrina deformans;* 9mm, 9mm and 7mm and for *Alternaria alternata* and 8mm, 7.5 and 6mm zone of inhibition against. *B. subtllis* showed better results against *Monilinia frecticola* as compared to other pathogens *Taphrinadeformans* and *Alternaria alternata*. Many researchers are working on the biological control of fungal plant pathogens using antagonistic rhizo bacterial strains belonging to the genus *Bacillus*. The results of this study identify *B. subtilis* as encouraging biological control agents for further testing against Brown rot, *Alternaria* rot in fruit and peach leaf curl disease in peach leaves.

Key words: *Prunus persica, Taphrina deformans, Pseudomonas, Bacillus subtilis,* Antagonistic.

Introduction

Peach is the most important among the stone fruits because of its taste, color and high nutritive value and also for its diversified uses. Peachconsists of 2% protein, 10-14% sugarand rich in Ascorbic acid, Vitamins A and B besides Calcium, Iron, and Phosphorus. Total production of peach in world is 21 million tones and Pakistan occupied an area of 6339 hectares with the production of 66847 tones. (Anon., 2015).Commonly, brown rot is the most critical disease risk for stone fruits in humid, warm climates. In Pakistan brown rot disease caused 20% losses of peach fruits, due toinitial application of fungicides on stone fruits due to brown rot disease. Soft decay, blight of blossoms and twigs of peaches spread because of thisdisease. Increase inthe susceptibility against brown rot in fruits was observedlast 2 to 3 week duration before harvest. Expanded disease is related with an expansion in sugar content as the fruits mature. At first, tan-dark colored and roundabout spots are obvious on the fruit surface. Under moist conditions, dim brown masses of conidia make on these injuries(Anon., 2018; Zehr, 1982).

The peach leaf curl diseases is caused by *Taphrina deformans* is wild spread in all peach growing area around the world (Sharma *et al*., 2007). In AJ&K district Bagh and Rawalakot almost 80% peach fruits are affected by peach leaf curl. Yellow to radish area appear on young developing leaves these areas progressively thicken and pucker and causing the leaf to curl. Infected leaves abbacies prematurely or sometime remain attached and gradually turning dark brown on severely infected trees (Broom & Ingels, 2012).

Alternaria rot is also an important disease of peach.In the primary stages of '*Alernaria rot'* and "moldy heart" affected fruits do not give appearance of any external symptoms but growing a gray or black mycelium into fruit pulp or stone, respectively. With the passage of time starting from the pedicel the fruit surface turns brown, and soon after, fall the fruit on the ground (Logrieco *et al*., 2003).

For the control of every single fungal pathogen chemicals use are great practice.Pesticides are utilized to enhance yield and visual nature of collected items (Wilson & Tisdell, 2001). Nonetheless, because of their destructive impacts on nature (Geiger *et al*., 2010)and perhaps on purchasers' and implements' wellbeing (Mostafalou & Abdollahi, 2013), the reasonability of regular editing frameworks is nowaday's broadly addressed. The European Union, as of late, settled a mandate to diminish pesticide utilization and advance the utilization of non-chemical strategies wherever conceivable. Postharvest biological control using microbial antagonists has emerged as one of the most promising alternatives to synthetic pesticide applications (Janisiewicz & Korsten, 2002).

So, it was longing to need to present non chemicals based choices that are the reason in this research we presented rhizobacteria against fungal pathogen of peach rhizosphere. The significance of biological control considered as the most recent ecologically benevolent measure to control the diseases of various fruits. Biological control implies methods for controlling disease or decreasing the sum or the impact of pathogens that depends on biological mechanisms or life forms other than man. One reason for the developing enthusiasm for biological control is that it is by all accounts the main conceivable other option of pesticides utilization (Butt *et al*., 2001).

Materials and Methods

Survey and sample collection: A comprehensive survey was conducted in peach growing areas of Rawalpindi and two districts of AJK (Bagh & Rawalakot). Peach fields were randomly selected for diseased sample collection. 10-15 samples were taken from each orchard. Soil samples from the rhizosphere of healthy peach trees were also collected for the isolation of beneficial rhizobacteria. All the samples were collected in polythene bags and labelled properly. Samples were brought to laboratory and were kept at -4^{o}C in a refrigerator till the further use in experimentation.

Isolation and purification of rhizobacteria: Nutrient agar was used for isolation and culturing of rhizobacteria by using serial dilution technique. One gram soil homogenized in 10 ml sterile distilled water and isolation was performed by serial dilutions method. Several dilutions of homogenate were prepared and the dilutions 10^{-1}, 10^{-3}, 10^{-5}, $10^{-7}$and 10^{-8} was streaked on nutrient agar (NA) media for bacterial growth. The petri plates were kept in incubator at 28 ±2°C. After 24 hours, the bacterial growth was observed on the petri plates and colony color, shape and size was noted carefully. Streak plate method was used to obtain single pure colony. Single bacterial colony was picked with sterile toothpick and was transferred in 5ml nutrient broth glass tubes. The tubes were placed on shaking incubator and pure bacterial culture was multiplied on Yeast extract-dextrose-calcium carbonate (YDC) and Crystal violet pectate (CVP) for growth and colony characterization.

Isolation and purification of fungal pathogens: Disease specimens of peach trees infected by fungal pathogens were collected and taken to the laboratory. Disease portion along with healthy portion were surface sterilized in 1% sodium hypochlorite solution and diseased pieces of specimens were dipped in distilled water for five times and placed on PDA media plates and these plates were incubated at 25±2°C for 5-7 days. Then identification of fungi was done on the basis of morphological characteristics after consulting Doctor.Fungus.com.org and different fungal identification keys (Ellis, 1971; Nelson *et al*., 1981; Sivanesan, 1987; Jeffries *et al*., 1990; Simmons, 2003). Hyphal tips growing on PDA medium were transferred on new plates for getting pure culture of pathogen. Fungus was purified by using hyphal tip technique (Whipps, 2001).

Pathogenicity test of fungi: For inoculation of *T. deformance* pathogenic fungi of leaves, fungal suspension was made by mixing mycelium containing media in distilled water. Fungi suspension inoculated in leaf of the plants in the form of suspension. Peach seedlings were sown in sterilized soil. One year old seedlings of peach were inoculated with *Taphrina deformans* inoculum of concentration 10^{8}cfu/ml by leaf inoculation technique. For control treatment a set of two seedlings were inoculated with sterile distilled water. The plants were then observed after 30-50 days for the symptoms.

Whereas for *M. fructicola* and *A. alternate* which are fruit rotting fungi so fruits are inoculated by using pin prick method. For this healthy fruits after washing in sterilized distilled water were inoculated by dipping the tooth pick in inoculum of *M. fructicola*and *A. alernata* and inserted in the fruits. For control treatment one fruit is inoculate with distilled water. The fruits were then incubated for 3 to 5days for symptoms development at 25±2°C. By utilizing the isolation method re-isolation of the fungus *Taphrina deformans* from the leafs was done after 8-10 weeks. Whereas for *M. fructicola* and *Alternata* were reisolated the pathogens from fruits after 5-7 days.

Fungal isolates which showed positive test were considered aggressive and selected for further characterization. Fungal suspension of all the aggressive isolates were injected separately after setting their OD_{600}=1 in healthy peach plants. Symptoms of peach leaf curl were observed the progressively thicken and pucker yellow to radish area appear on young developing leaves and causing the leaf to curl. On severity infected trees infected leaves abbacies prematurely or sometime remain attached and gradually turning dark brown. Out of 8 plants, almost 4 plants severely affected and 2 infected gradually with progressive curling. Remaining 2 plants was not as healthy as untreated were. This shows that the pathogen has reduced the growth of plant and plant has not performed according to its genetic potential.

Through symptoms of *Alternaria* rot, it was confirmed that the isolates were pathogenic. Out of 10 fruits 4 were severely affected 4 get disease gradually and remaining 2 were not as healthy as untreated were. This shows that pathogen has reduced the growth of fruits and plant has not performed according to its genetic potential.

***In vitro* evaluation of rhizobacteria against fungi:** Antagonistic activity of isolated rhizobacteria against pathogenic fungi of peach were determined by dual culture technique (Malek & Ishac, 1968). The fungal isolate was assayed using PDA media plate and in a same plate having PDA media, rhizobacterial isolates were also streaked opposite portion of pathogenic fungi of peach and incubated for 5-7 days at 25±2°C. The inhibition of pathogenic fungi caused by rhizobacterial isolate is also known as zone of inhibition technique and measured in mm.

Morphological identification and Biochemical test of rhizobacteria: Morphological identification of the rhizobacteria was done based on colony colour, cell morphology and colony size. Similarly different biochemical test were performed for identification of bacteria such as:

Gram staining test was done to differentiate between Gram Positive and Gram negative bacteria by following the procedure described by (Simak *et al*., 2001).

Potassium hydroxide KOH (Loop) test was performed in this test the rhizobacterial isolates which were reported to be negative will behave positive against potassium hydroxide (loop) test. Bacterial culture will look like of slimy threads when blended with 3 % potassium hydroxide, these bacteria will shows arrangement of separate layers over the glass slide by following the method (Suslow *et al*., 1982).

During Catalase Oxidase Test, the rhizobacterial isolates shows the aerobic nature and produce gas bubbles when on glass slide mixed with the drop of hydrogen per oxide. Occurrence of aerobic bacteria indicated by production of gas bubbles (Anwar *et al*., 2013).

Indole Acetic Acid (IAA) Test, resulted to generate IAA by rhizobacterial isolates. In the tube when Salkowski reagent was added at that point, after 20-25 minutes all the tube change their color into red that's shows the positive results. (Asghar *et al*., 2000).

Phosphorus Solubilizing TestAll the isolates respond positive to this test when they were produced halo zones thus this conformed the phosphorus solubilizing performance of the isolates (Yoav*et al*., 2013).

Siderophore Production Test will be performed to check the rhizobacterial isolates to chelate iron which is of prime significance to the pathogens by following the procedure (Sayyed & Patel, 2011).

Results

Isolation and culturing of rhizobacteria: Sixteen isolate of rhizobacteria were isolated from the different samples on morphological basis which have different color of the colony, elevation, shape and size of the colony. On NA media colonies appeared as circular, raised, shiny and creamy white after 5-7 days of post incubation at 28˚C. Then, by using streak plate method single bacterial colony was picked after 24 hours shown in Table 1.

In vitro* evaluation of rhizobacteria against fungal pathogen of peach:In vitro* evaluation of rhizobacteria against fungal pathogen of peach showed that, a large number of isolates don't have antagonistic activity against the fungal pathogen of peach plant and did not exhibit any zone of inhibition. Among 16 isolates Rh-3. Rh-5 and Rh-12 showed the maximum antagonistic activities against the pathogenic fungi of peach plant. Among them Rh-12 showed maximum zone of inhibition than Rh-5 which inhibited the pathogenic fungi at 11mm, 9 mm and 8 mm of the inhibition zone. In case of *A. alternate* Rh-7 and Rh-9 showed maximum inhibition zone than Rh-5 as they inhibited at 9mm, 9mm and 8mm of zone. Among all 16 isolates the three isolates showed maximum antagonistic activity. Among 16 isolates 7 isolates Rh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16 were identified as *B. subtilis* by following the biochemical part of the procedure (Zeng *et al*., 2008) and they inhibit the pathogen 7mm, 8mm, 9mm, 6mm, 9mm, 5mm and 4mm respectively Fig. 1. While in case of *T. deformans* isolates Rh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16 inhibited the growth of pathogen 6mm, 7mm, 8mm, 5mm, 7.5mm, 4mm and 3.5mm respectively (Fig. 2).Among all these 7 isolates Rh-7 gave comparatively better results as compare to Rh-12 and Rh-5 as they inhibited the pathogen as 8mm, 7.5mm and 7mm Table 3. Selected isolates of Rhizoacteria Rh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16 were also identified as *Bacillus subtilis*. *In vitro* experiments these isolate were found to be most antagonistic to fungal pathogen of peach plant as shown in Table 2.

Biochemical test of rhizobacteria

Gram staining test: Results demonstrated that 5 of the bacteria that displayed the antagonistic activities against the pathogen were gram negative where other 2 isolates revealed gram positive to the test. Every isolates were rod shape uncovered Investigations of cell, under high amplification.

Potassium hydroxide loop test: Results demonstrated that 2 rhizobacteria showed KOH positive while other while 5 showed KOH negative.

Catalase oxidase test: Results showed that all tested rhizobacteriaRh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16 produced gas bubbles when on glass slide mixed with the drop of hydrogen per oxide.This test indicating aerobic nature of these bacteria.

Table 1. Colony characteristics of rhizobacteria.

Sr. No.	Isolates	Colony color	Colony Morphology	Colony size(mm)
1.	Rh-1	Off white	Round large wavy	2.5-3
2.	Rh-2	White	Large round slightly raised	2-4
3.	Rh-3	Creamy white	Round medium sized	1-2
4.	Rh-4	White small sticky	Circular entire margin	1-3
5.	Rh-5	Creamy white	Round medium sized slightly raised	1-2
6.	Rh-6	Light yellow	Circular, conver, small	1
7.	Rh-7	Pale yellow	Round small centrally raised	0.5-o.7
8.	Rh-8	Light green	Flate, large,circular and margin undulated	2-4
9.	Rh-9	Creamy white	Round medium size slightly raised	3-4
10.	Rh-10	Pale orange	Wavy, medium size, flate	2
11.	Rh-11	Small grayish white	Round, large, flate, wavy margin	3
12.	Rh-12	Off white	Round, large, wavy	1
13.	Rh-13	White small	Circular, convex with an entire margin	3-5
14.	Rh-14	Whitish creamy	Circular, convex small	1
15.	Rh-15	Creamy white small	Flate, large and undulated margin	1-2
16.	Rh-16	Whitish colony	Round ,medium,small raised	2-3

Table 2. Diameter of zone of inhibition calculated in (mm) *In-vitro* by rhizobacterial isolates.

Sr No.	Isolates	*M. fructicola*	*A. alternata*	*T. deformans*
1.	Rh-1	4mm	2mm	2mm
2.	Rh-2	0mm	0mm	0mm
3.	Rh-3	9mm	7mm	6mm
4.	Rh-4	5mm	4mm	2.5mm
5.	Rh-5	10mm	8mm	7mm
6.	Rh-6	2mm	1.5mm	1mm
7.	Rh-7	12mm	9mm	8mm
8.	Rh-8	3mm	2.5mm	2mm
9.	Rh-9	8mm	6mm	5mm
10.	Rh-10	0mm	0mm	0mm
11.	Rh-11	1.5mm	1.5mm	1mm
12.	Rh-12	12mm	9mm	7.5mm
13.	Rh-13	3mm	1.5mm	1mm
14.	Rh-14	7mm	5mm	4mm
15.	Rh-15	5mm	3mm	3mm
16.	Rh-16	5mm	4mm	3.5mm

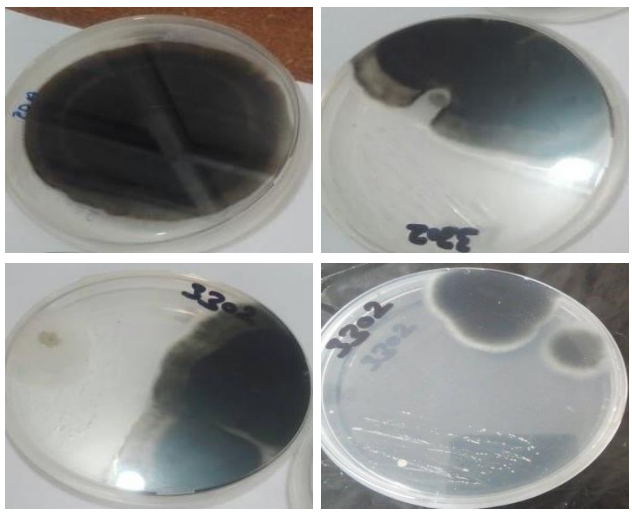

Fig. 1. *In vitro* antifungal activity of *Bacilussubtlis*againt fungal pathogen of peach *Taphrinadeformans and Alternaria alternata*.

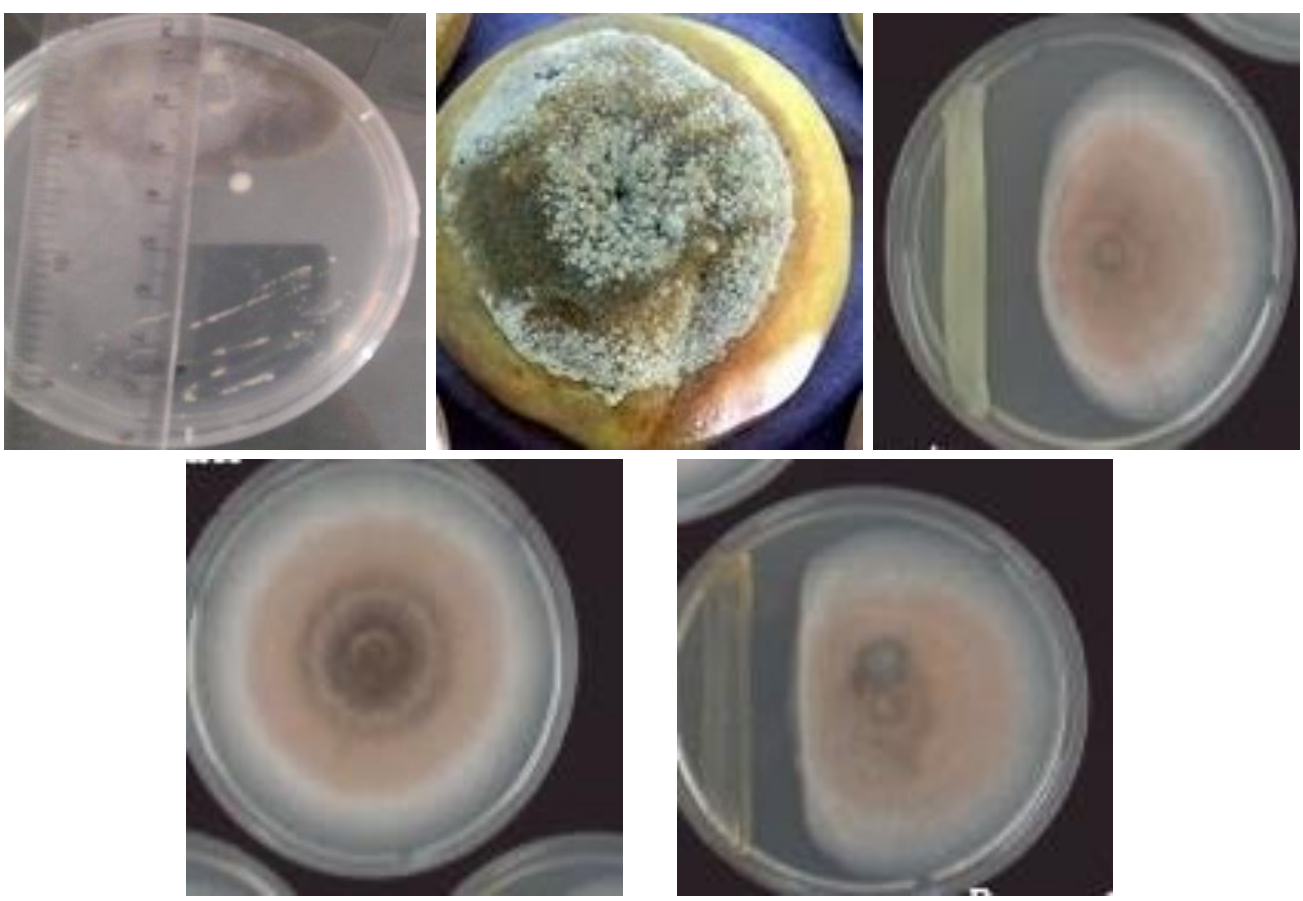

Fig. 2. *In vitro* antifungal activity of *Bacilus subtilis* againt fungal pathogen of peach *Moniliniafructicola*.

Indole acetic acid (IAA) test: As far as IAA production is concerned, in the tube when Salkowski reagent was added at that point, after 20-25 minutes all the tube change their color into red that's showed the positive results.More IAA was delivering in isolate Rh-3 than Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16. This was apparent from Salkowski reagent that the red color got fade later in Rh-12 as the time passed while in Rh-3, Rh-5, Rh-9, Rh-14 and Rh-16 fadedness was faster. Thus it could be says that Rh-12 may be more synthesizer of IAA than others.

Phosphorus solubilization test: All these isolates responded positively to this test when they were produced halo zones thus this conformed the phosphorus solubilizing performance of the isolates.Rh -3 results were more reliable than Rh-7 and Rh-12 because the diameter of halo zone was larger. Rh-3 halo zone was 14mm in diameter so describing its ability to solublize more Ca-p than Rh-5, Rh-7, Rh-9, Rh-12, Rh-14 and Rh-16 and isolate which produce 5mm, 8mm, 11mm, 9mm, 6mm and 12mm of halo zone respectively.

Siderophore production test: For siderophore production it was observed all the strains were to be positive Rh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16. In the medium around the colony the color was diffused.Rh-5, Rh-7, Rh-9, Rh-12 and Rh-14 produce yellow to greenish color while Rh-3 and Rh-16 produced orange to brown color. Results of all tests shown in Table 3.

Discussion

The results of this study demonstrated that *Bacillus subtili* streatments have potential to control brown rot on peach fruit, caused by *M. fructicola*, *Alterneria rot* caused by *A. alternata*. These rhizobacteria were evaluated for their antagonistic ability to decrease in the percentage of disease of pathogenic fungi under *In vitro* conditions. It has previously been reported that in peach *Bacillus subtilis* strain CPA demonstrated its potential to control *Monilinia* spp. (Kotlyar *et al*., 2011).

Similarly the efficacy of the antagonist in the suppression of postharvest decay of Chinese winter jujube caused by *Alternaria alternate* was studied and it was found that antagonist significantly controlled the decay and there was natural development of the decay (Wang *et al*., 2009).

Significant levels of decay control have also been reported with other microbial antagonists (Qin and Tian 2004; Tian *et al*., 2004) and specifically by antagonistic rhizobacteria (Ghosh *et al*., 2015).

However, not all the PGPR strains exert their positive effect on plant growth via increasing nutrient status of host plants. PGPR seem to promote growth through suppression of plant disease (Yazdankhah *et al.,* 2001; Zehnder *et al*., 2001), or through production of phytohormones and peptides acting as biostimulants (Glick *et al*., 1998; Jimenez *et al.,* 2004).

Our results were according to the prior findings of different researchers (Wang *et al*., 2008; Vessey, 2003) for the suppression of disease by the rhizobacterial isolates. The rhizobacterial isolates are root colonizer that serve and proliferate along with plants roots, resulting plant defense and plant growth against pathogens (Whipps, 2001).

There is no doubt about the necessity of developing alternate strategy to control pests of crop plants by using microorganisms which are enemies to the pests. Such microorganisms, popularly known as biocontrol agents, kill the pathogens by direct destruction of their cellular structures, by competing for nutrients (Pal and Gardener, 2006) or by producing some antagonistic metabolites (Cazorla *et al*., 2007, Naseby *et al*., 2001).

Rhizobacteria and their zone of inhibition were apparently because of materials (cell wall degrading enzymes or antifungal substance discharged by the bacteria into the culture medium. Rh-3, Rh-5, Rh-7, Rh-9, Rh-12, Rh-14, and Rh-16 inhibited the zone of fungal growth in better way as compare to other isolates. It is also reported that Bacillus subtilis CPA-8, a strain with demonstrated ability to control Monilinia spp. in peaches, was studied to elucidate its mechanisms of antifungal activity. Growth inhibition assays using cell-free supernatants and butanolic extracts showed strong antifungal activities against Monilinialaxa and Moniliniafructicola. By comparison with the reference B. subtilis strains UMAF6614 and UMAF6639, fengycin, it urin and surfactin lipopeptides were identified by thin layer chromatography in butanolic extracts from cell-free supernatants, indicating that antibiosiscould be a major factor involved in the biologicalcontrol ability of CPA-8 (Yanez *et al*., 2012).

Rhizobacteria that exert beneficial effects on plant development are normally termed "Plant Growth-Promoting Rhizobacteria" (Kloepper and Schroth, 1978). PGPR was found to be mainly involved in enhancing plant nutrition, stress tolerance or health (Vacheron*et al*., 2013). This is mainly due to their effect associated with enhanced availability of nutrients (Bashan *et al.,* 2013; Lugtenberg & Kamilova, 2009; Drogue *et al*., 2012), phytohormones-mediated stimulation of root system (Somers *et al*., 2005) and induced systemic resistance (Zamioudis *et al*., 2013).

As in our *In vitro* experiment ability of rhizobacteria to antagonize the fungi Purified isolates were evaluated. It was found that out of (16) isolate (7) isolate were found better and had showed antagonistic effect on the pathogen. But 3 isolates has stronger antagonistic effect against *M. fructicola* and *A. alternata*. For more biochemical and other tests these bacteria were chosen. Siderophore production and antifungal compounds by the rhizobacteria can inhibit the development of pathogen.

To control postharvest diseases of fruits utilizing of *B. subtilis* strains is a good alternative to reduce the chemical fungicides open the way in fruit management Biological formulation development based on *B. subtilis* strains for postharvest is considered a new and ethentic field in Pakistan. To control the brown rot of stone fruits *B. subtilis* strains were previously used due to its antagonistic activities.

After the completion of the in-vitro experiments for the possible use of rhizobacteria to antagonize the pathogenic fungi through using zone of inhibition method. And the zone of inhibition shows that these rhizobacteria can control the fungal pathogens on the fruits to reduce post-harvest loss.

Hence it can be concluded that rhizobacteria can reduce the diseases and also can improve the health of the peach fruits, by reducing post-harvest losses.

Table 3. Isolates response to gram staining, morphology, loop test, H_2O_2 test reaction, Catalase oxidase test and Indoleactic acid test.

Sr. No.	Isolate	Gram stain reaction	Cell morphology	KOH test reaction	H_2O_2 test reaction	Catalase oxidase test	Indole acetic acid test
1.	Rh-3	+	Rod shaped	–	–	+	+
2.	Rh-5	+	Rod shaped	+	–	+	+
3.	Rh-7	+	Rod shaped	–	–	+	+
4.	Rh-9	+	Rod shaped	–	–	+	+
5.	Rh-12	+	Rod shaped	+	–	+	+
6.	Rh-14	+	Rod shaped	–	–	+	+
7.	Rh-16	+	Rod shaped	–	–	+	–

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