Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Verticillium Wilt of Cotton (*Gossypium hirsutum* L.)

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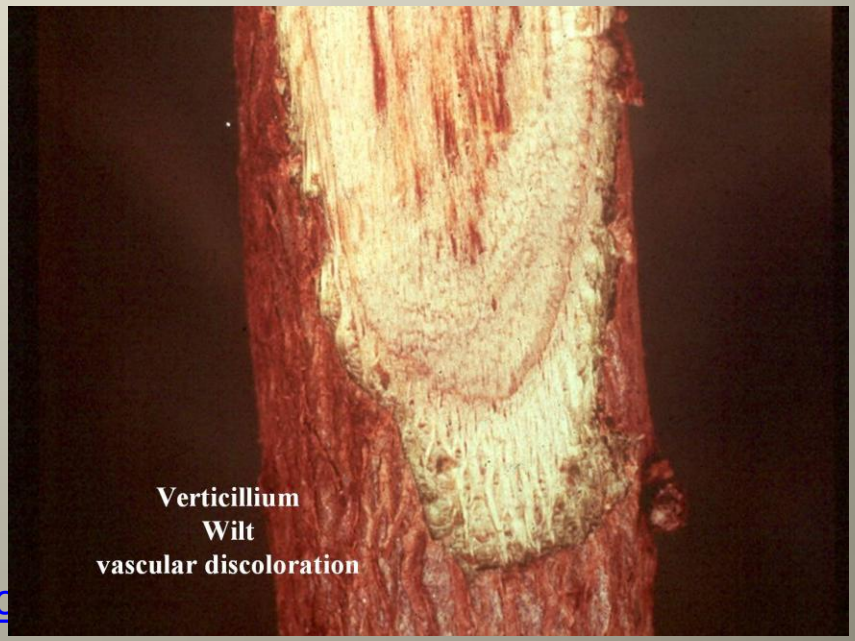
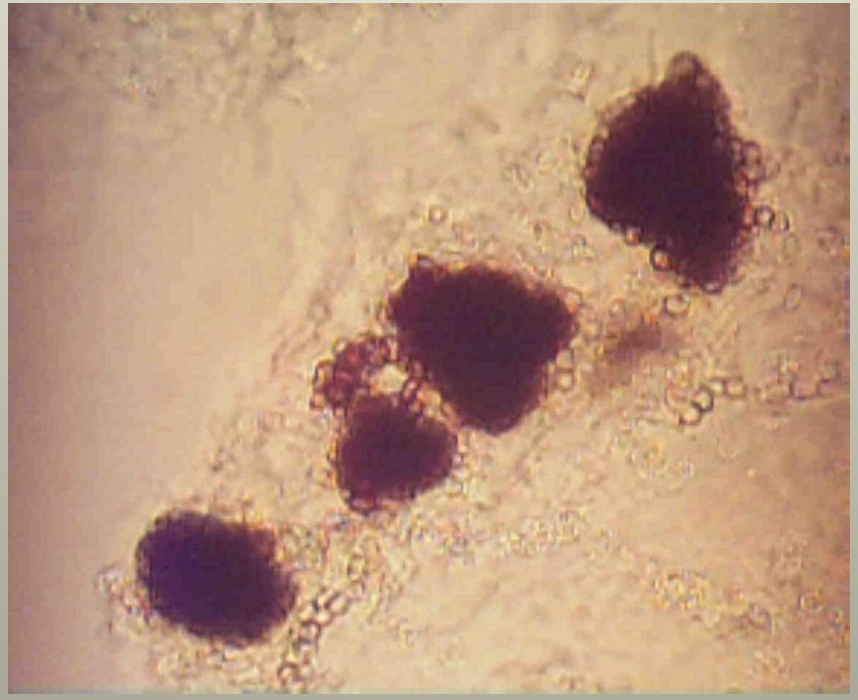
It was reported that Verticillium wilt on cotton is prevalent in all cotton growing areas of Turkey. Especially in Aegean and Mediterranean regions disease agent is *Verticillium dahliae* and it has caused seriously crop loses (Karaca et al., 1971; Esentepe, 1979).

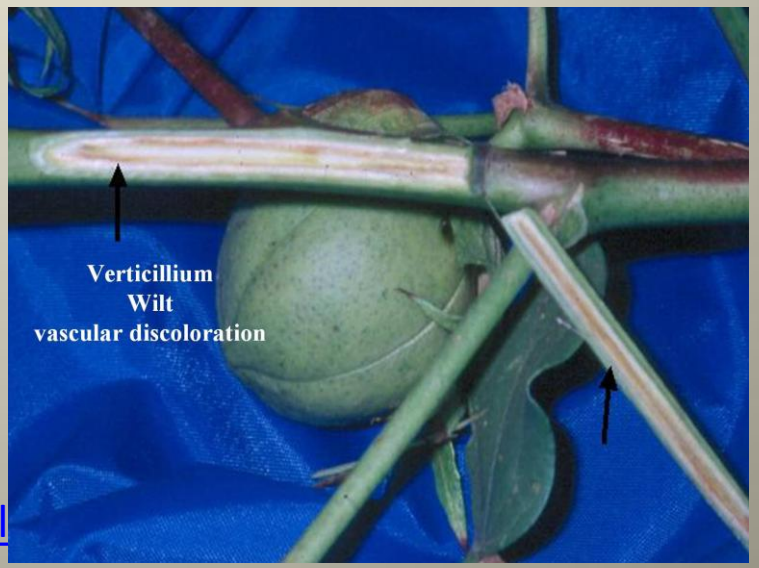


The fungus lives in the soil over 10 years due to microsclerots that is 10-120 µm length and resistant to negative conditions. Verticillium wilt dissease is caused by a soil-born fungus (*Verticillium dahliae* (Kleb.)) and it occures over 200 plant species.



http://treediseases.cfans.umn.edu/sites/treediseases.cfans.umn.edu/files/verticillium_microsclerotia._2.jpg

In case of existance of sensitive host plants microsclerotia germinate by root secretion and they penetrate to plant from the root and they move on xylem. Thus wilt semptoms occure on the plant (Agrios, 1997).



http://cotton.tamu.edu/Photos/diseasephotos/diseasephotos.html

In plant production phosphorus (P_2O_5) is one of the most important macro nutrient elements for biological growth and development.

Phosphorus cycle in biosphere occures a series of oxydation and reduction reactions and microorganisms have a great role in this cycle. Generally soluble phosphorus concentration in the soil is low and normally it is 1 ppm or less (Rodriguez and Fraga 1999).

Naturally phosphorus exist mineral forms such as phosphate rocks and apatite in the Earth and these minerals can not dissolve.

Plants can uptake phosphorus in the form of HPO_4^{2-}or $H_2PO_4^-$.

The second source of phosphorus for plants are fertilizers contain normally phosphorus in soluble form using particularly in agricultural fields.

A major part of inorganic phosphate given to soil in soluble forms clings to soil in a short time and it turns to insoluble form.

This case is known as phosphorus fixation and pH of the soil plays an important role.

Phosphorus attaches to soil and turns to insoluble form depending on soil structure by calcium in the limy soils having high pH and by iron and aluminium in acidic soils.

The third source of phosphorus in the soil is organic phosphate. In a lot of soils 30-50 % of total phosphorus originate from organic phosphates.

However they exist as compounds having higher molecular weight (nucleic acids, phospholipids, phosphodiesters).

Therefore it is necessary that they turn to soluble ionic phosphates (HPO_4^{2-}or $H_2PO_4^-$) or phosphate compounds having lower molecular weight because they can be taken by plants (Paul and Clark 1996).

A lot of microorganisms primarily root fungi living in plant roots (mycorrhizas) and numerious bacteria turn insoluble inorganic phophates to soluble form as a result of their metabolic function.

It is known that these bacteria depending on especially genus of Pseudomonas, Bacillus, Rhizobium and Burkholderia have ability of turning inorganic phosphates to soluble form with the asistance of organic asids and turning organic phophates to soluble form with the asistance of phosphotase enzymes (Rodriguez and Fraga, 1999).

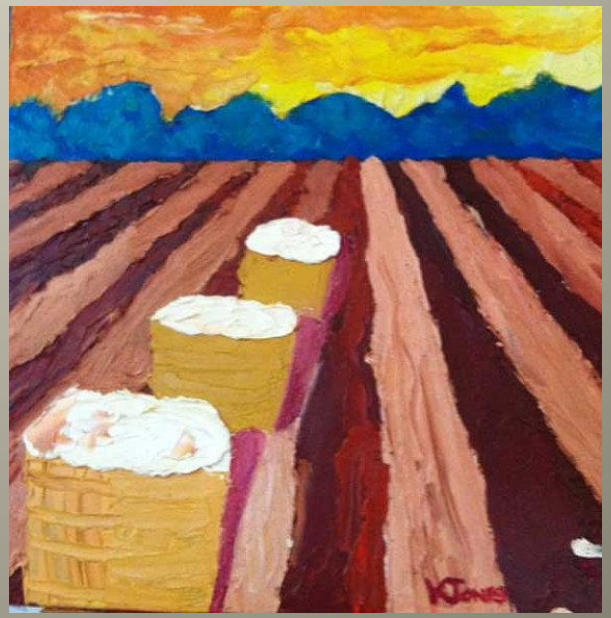
Studies in recent years have shown that phosphorus solubilizing bacteria are in root zone and near surroundings (rhizosphere) and at the same time they produce metabolites such as indole asetic acid and antibiotics.

Therefore researchers have reported that these bacteria have been used as biocontrol agent against plant diseases in adition to promotion of plant growth (Vassilev et al., 2006).

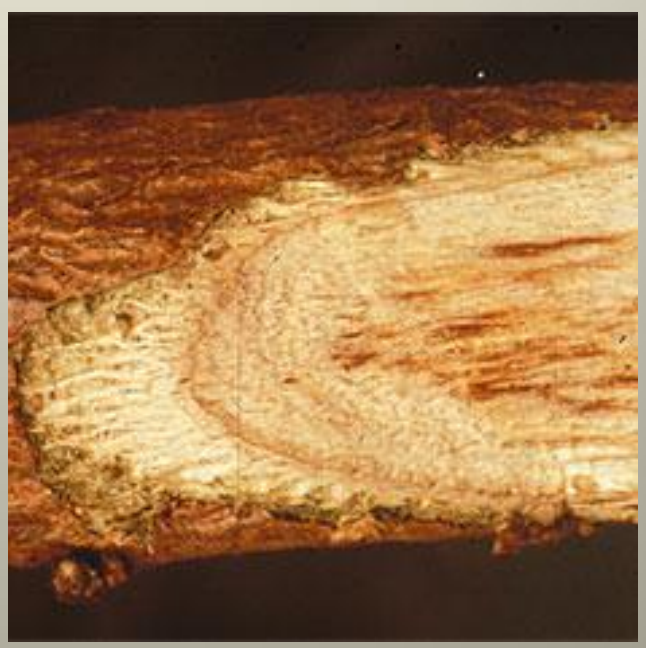
Among microorganisms Rhizobacteria promoting plant growth (Plant Growth Promoting Rhizobacteria; PGPR's) have vitally a role.

PGPR's are bacteria that can have useful efects to plants and they live free in the soil. PGPR's increase promotion of seedling emergency, colonization of roots and general plant growth.

At the same time PGPR's increase seed germination, root development, mineral nutrition and water uptake/use. Additionally these bacteria can also suppress plant diseases.



In this study, it was aimed at investigation of biological control possibilities using plant growth promoting Rhizobacteria (PGPRs) alternative to chemical control methods against Verticillium wilt. At the same time it was aimed at determining of effets of these bacteria on growth and development of cotton plant.

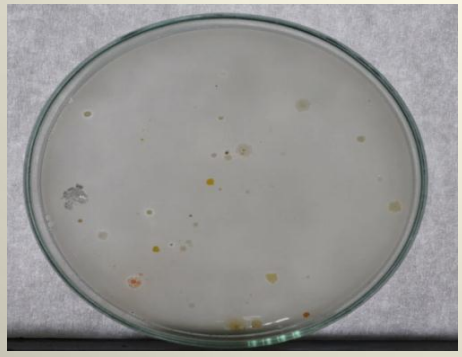


http://apps.cals.arizona.edu/cottondiseases/main.html#photos

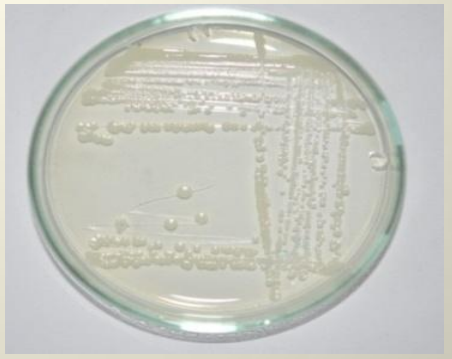
Materials and Methods

Materials

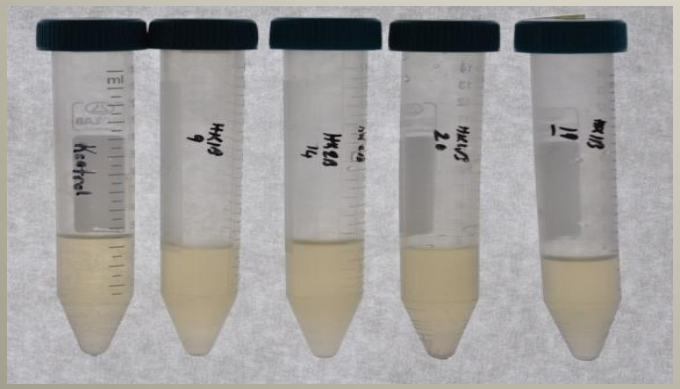
650 bacteria isolated from 25 soil samples taken from 25 cotton producing areas of Kahramanmaras province in different times (July and September 2013) and obtained bacteria from soil samples treated temperature at 80 ^{o}C, cotton (*G. hirsutum* L) seeds (the highest sensitive and tolerant cotton seeds supplied from Progen Seed Company) and seedlings, antagonist isolates, nutrient media, various chemicals and Biolog recognation kit were used as materials. In climate chamber and pot trials cv. Beren that is sensitive to disease (Verticillium dahliae Kleb.) and cv. Teks that is tolerant were used as material.



NBRIP nutrient medium



Solid King B nutrient medium



Liquid NB nurtient medium

As nutrient media were used solid NBRIP nutrient medium (National Botanical Research Institute Phosphate-Bromo Phenol Blue broth medium), solid King B (King's Medium B; KB), PDA (Potato Dextrose Agar), NA (Nutrient Agar), liquid NB (liquid Nutrient Broth) and Sucrose Nutrient Agar (SNA).

Methods

Taking of Soil-Root Samples

1-2 kg of Soil samples with root pieces from root zone of relatively healty plants were taken from 15-20 cm depth on 08.07.-03.09.2013. The samples were put in plastic bags and were labeled and they were brought to laboratory in ice bag and dried in plastic containers.

Isolation and Recognation of Resistantance Promoting PGPR's

Dried soils were screened 2 mm diameter and 10 g soil was shaken 2 hours at 150 rpm in phsiological water (0,85 % NaCl) of 90 ml. 0.5 ml suspention were aid to 15 ml falcon tube containing 4.5 ml phsiological water. After tube was santrifuged 0.5 ml suspention was taken from the tube and it was aid to tube containing 0.5 ml phsiological water. Similarly 10 times dilution series were prepared. 100 µl of second, third and fourth series of theese suspentions was taken and was sown to solid nutrient medium of NBRIP by surface sowing method and was incubated at 25±1°C 48 hours.

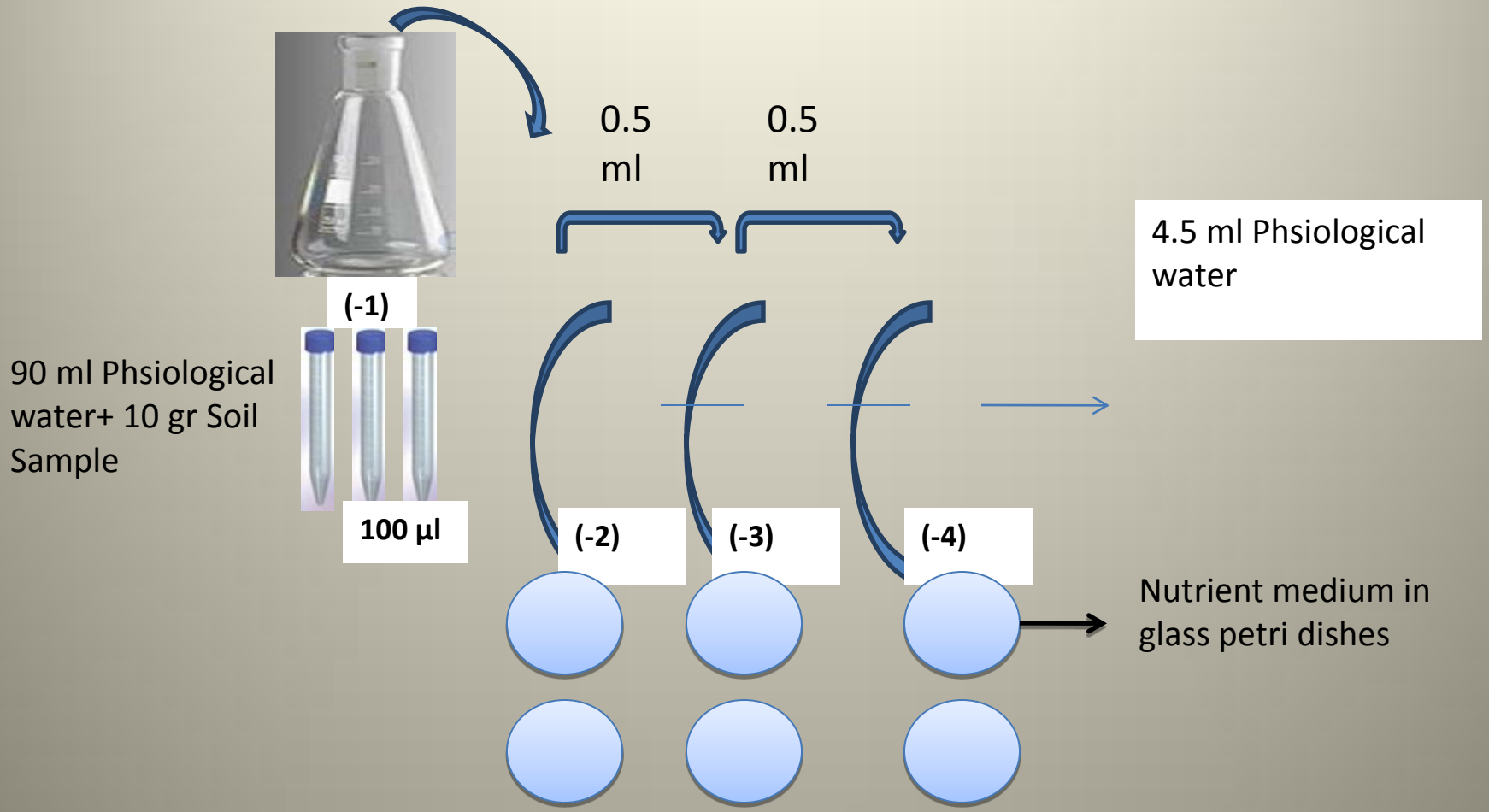
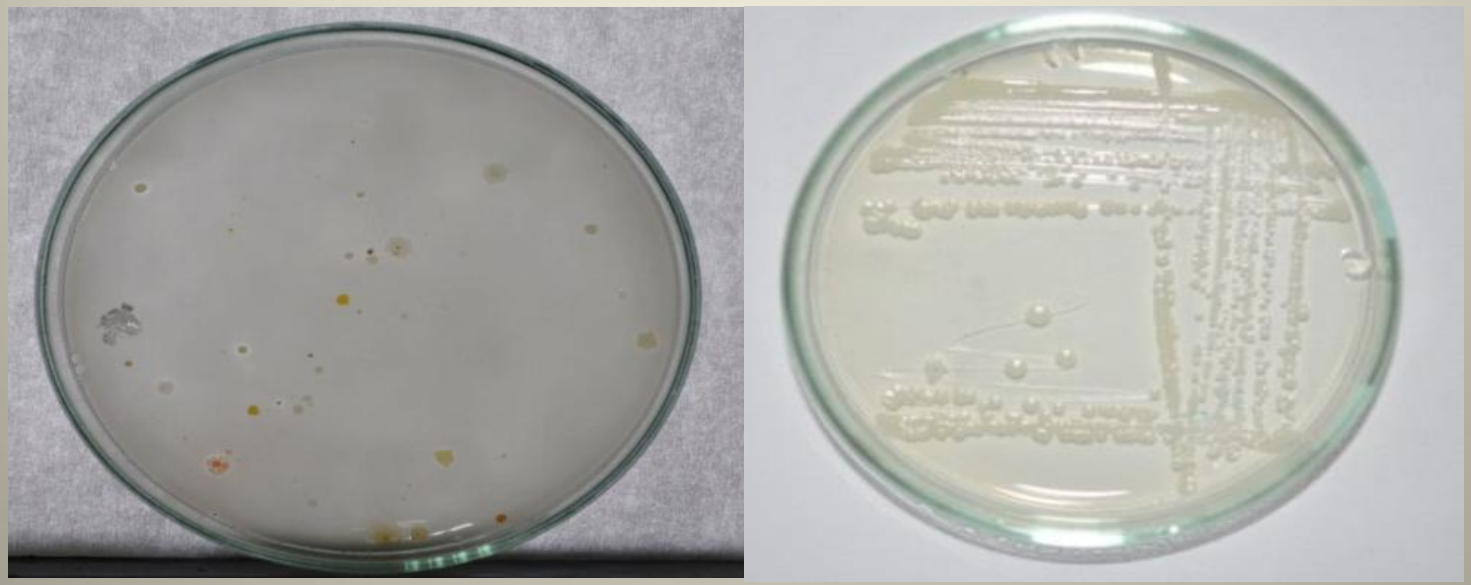


Fig. 1. Obtaining of candidate bacteria isolates

After incubation colonies solubiling phosphorus and formed zone were selected and were sown to solid King B (KB) nutrient medium until pure culture was obtained by line sowing method (Fig. 2.) .



(a) **(b)**

Fig. 2. a) Colonies having phosphorus dissolving zone on NBRIB nutrient medium **b)** Purification of colonies having phosphorus dissolving zone solid King B nutrient medium.

Also bacteria with fluoresans developing on solid King B nutrient medium and dissolved phosphorus were determined under UV light (Fig.3).

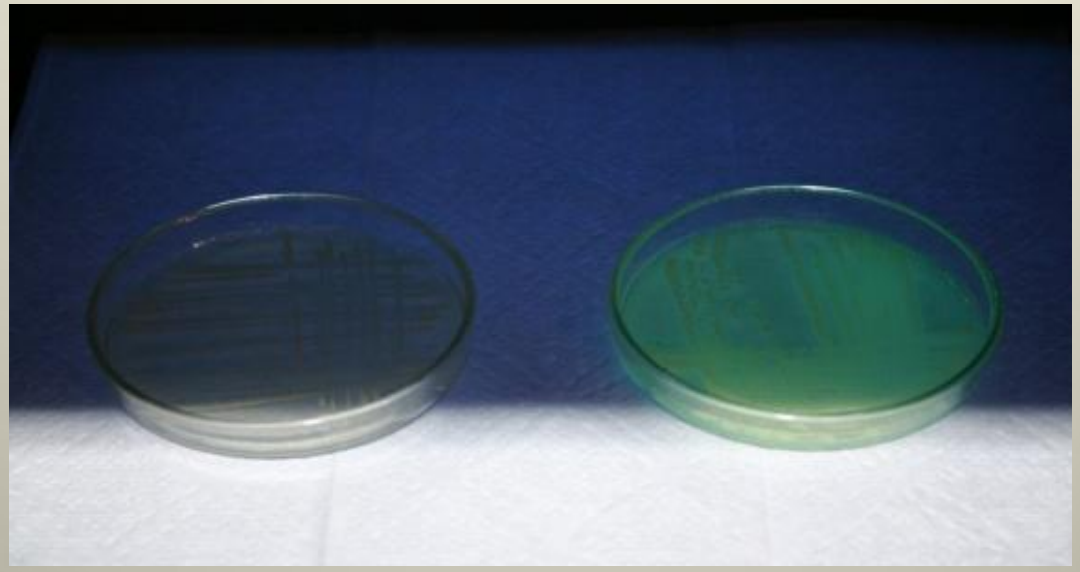


Fig. 3. Isolates with fluoresans under UV light

Obtained pure cultures were sown to 4.5 ml liquid Nutrient Broth (NB) in falcon tubes of 15 ml and they were incubated at shaker incubator at 25±1°C 48 hours. After incubation 200 µl of isolates which developed on liquid NB and 200 µl of 30 % Glyserine (70ml NB + 30ml Glyserine) were taken and mixed in cryo tubes of 2 ml and were labeled then were stocked at -20C^{o}.

In order to isolate bacillus bacteria from the soil samples a solution was prepered by using 10 g of soil sample and 90 ml of sterile physiological water (in 500 ml of flask with screw cap).

These solutions were kept in shaker water bath at 80^{o}C 30 min. at 150 rpm (Claus, 1964). Then, 10^{-1}, 10^{-2},10^{-3},10^{-4} dilution series were prepered from 4.5 ml of sterile physiological water at falcon tubes of 15 ml.

From these dilution series 100 µl was taken and was sown on solid Nutrient Agar (NA) surface. Then it was incubated at 28±1°C 24 hours.

After incubation colonies that formed on NA nutrient medium and have diffent morphology were selected and they were sown to solid NA nutrient medium using line sowing method until pure culture was obtained (Fig. 4).

(a) **(b)** **(c)**

Fig. 4. a) Colonies developing on 10^{-2} dilution series of solid NA nutrient medium**; b)** Colonies developing on 10^{-3} dilution series of solid NA nutrient medium**;**
c) Colonies developing on 10^{-4} dilution series of solid NA nutrient medium.

Obtained pure cultures were sown to 4.5 ml liquid Nutrient Broth (NB) at falcon tubes of 15 ml (Fig. 5.) and they were incubated at shaker incubator at 25±1°C 24 hours.

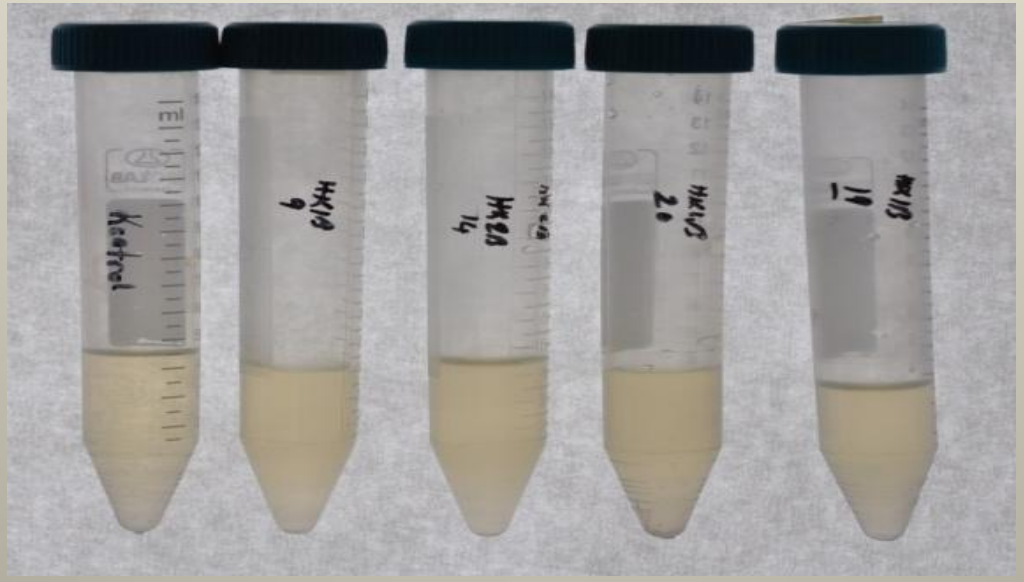


Fig. 5. Cultivating of pure colonies on liquid NB nurtient medium

After incubation 200 µl of isolates which developed on liquid NB and 200 µl of 30 % Glyserine (70ml NB + 30ml Glyserine) were taken and mixed in cryo tubes of 2 ml and were labeled then were stocked at -20C^{o}.

Biochemical Recognition Tests Treated Isolates

Biochemical recognition tests were applied to isolates from the soil such as Potassium Hydroxide, Oxidase, Catalase, Levan Formation, Pectolitic Activity and Hypersensitive Reaction on Tobacco.

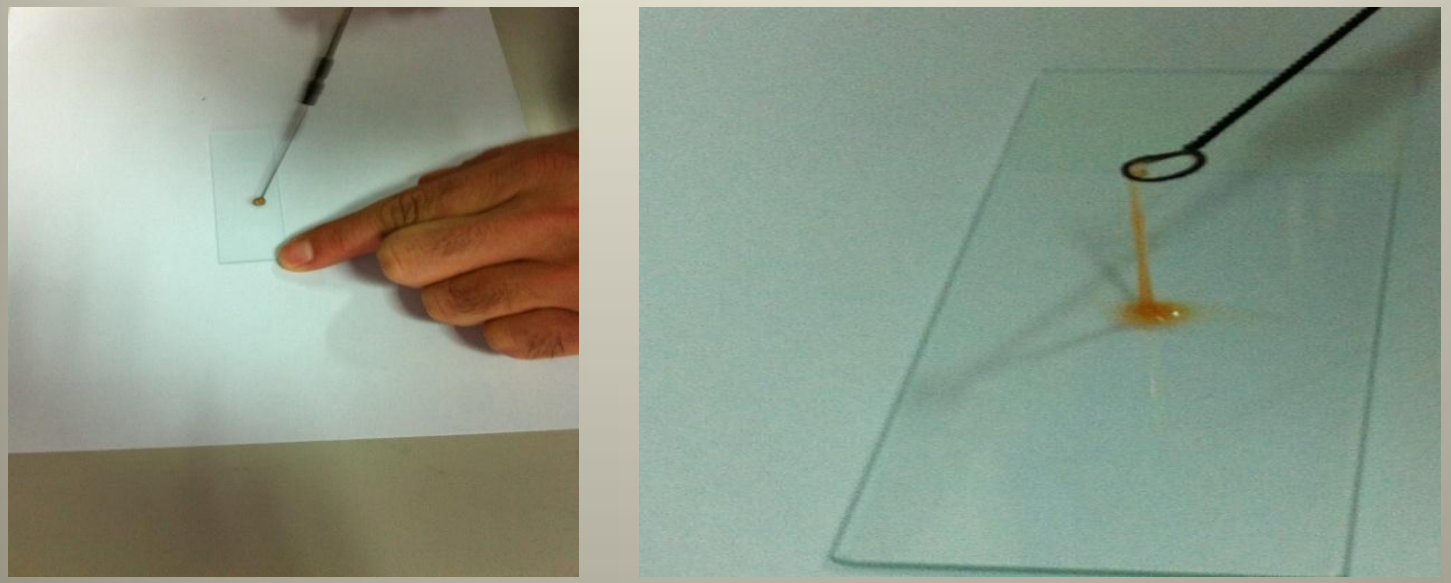


Fig. 6. Mucus formed by cultures that were gram negative at KOH test.

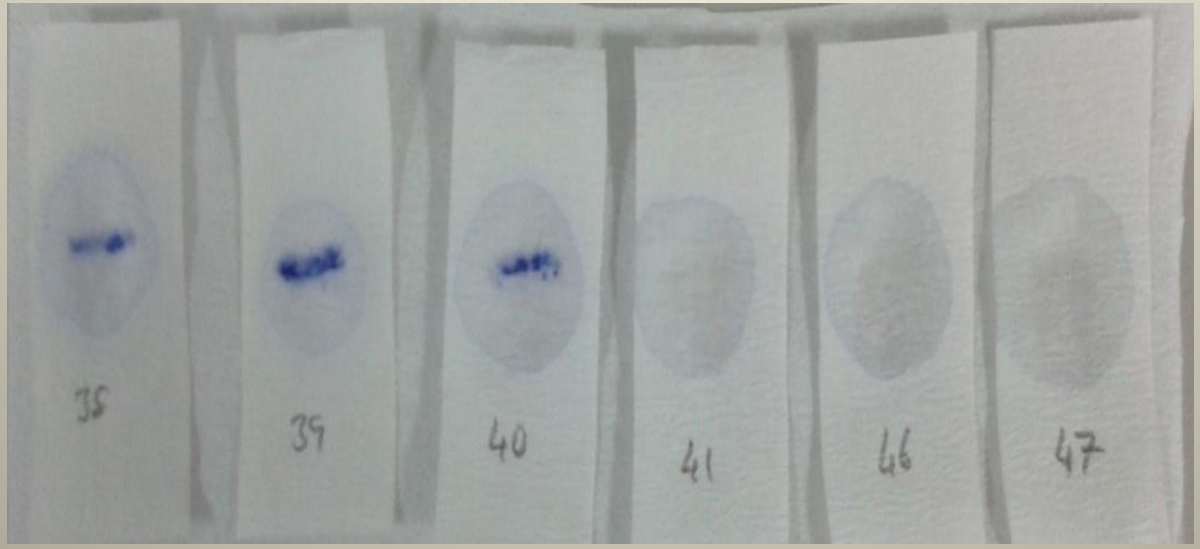


Fig. 7. Three isolates from left showed oxidase positive reaction, three isolates from right showed oxidase negative reaction at oxidase test.

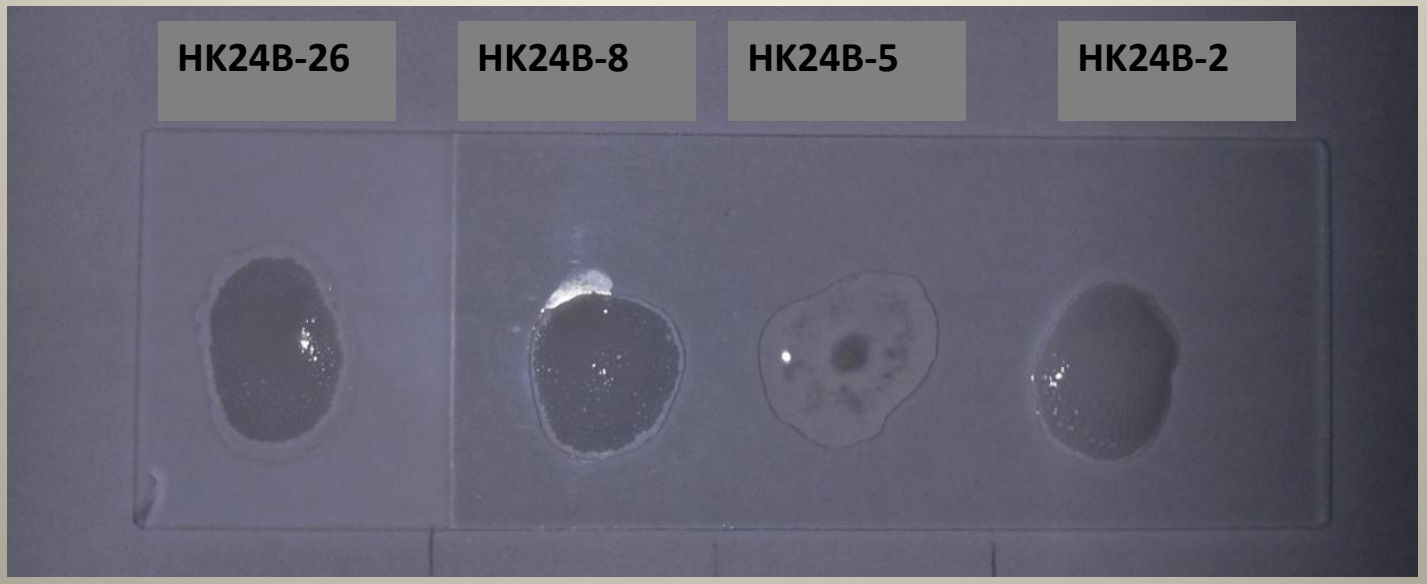


Fig. 8. Katalase positive reaction of HK24B-26,HK24B-8, HK24B-5 ve HK24B-2 isolates.

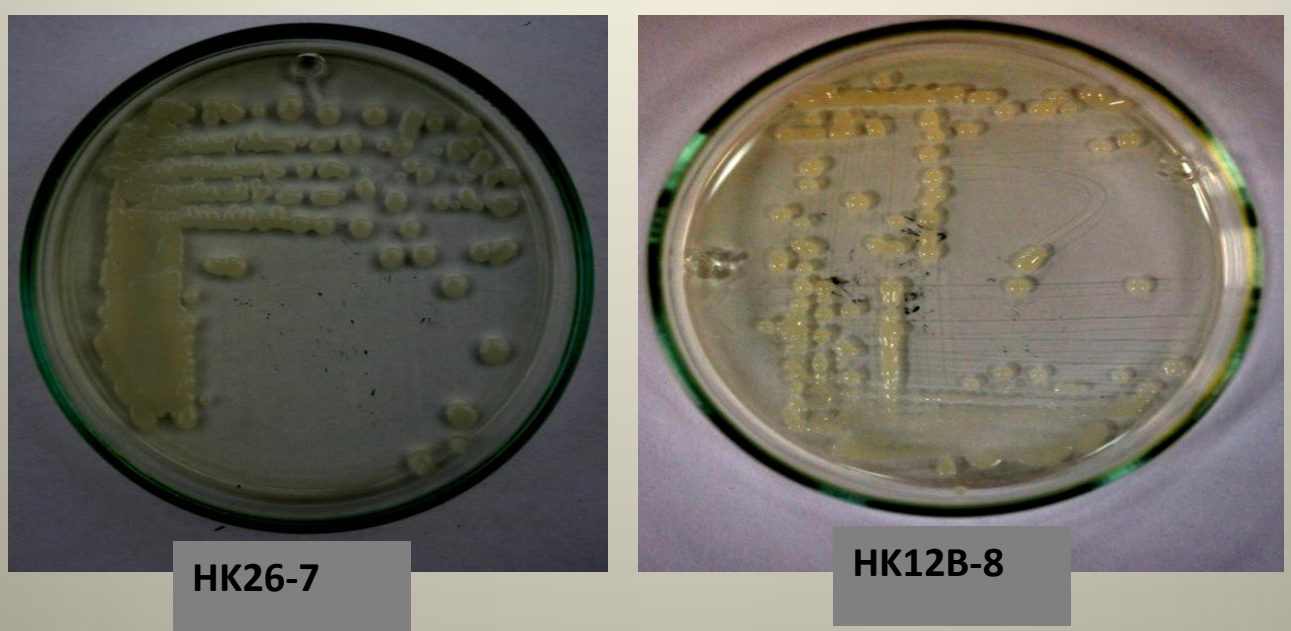


Fig. 9. Levan formation on HK12B-8 ve HK24B-3 isolates.

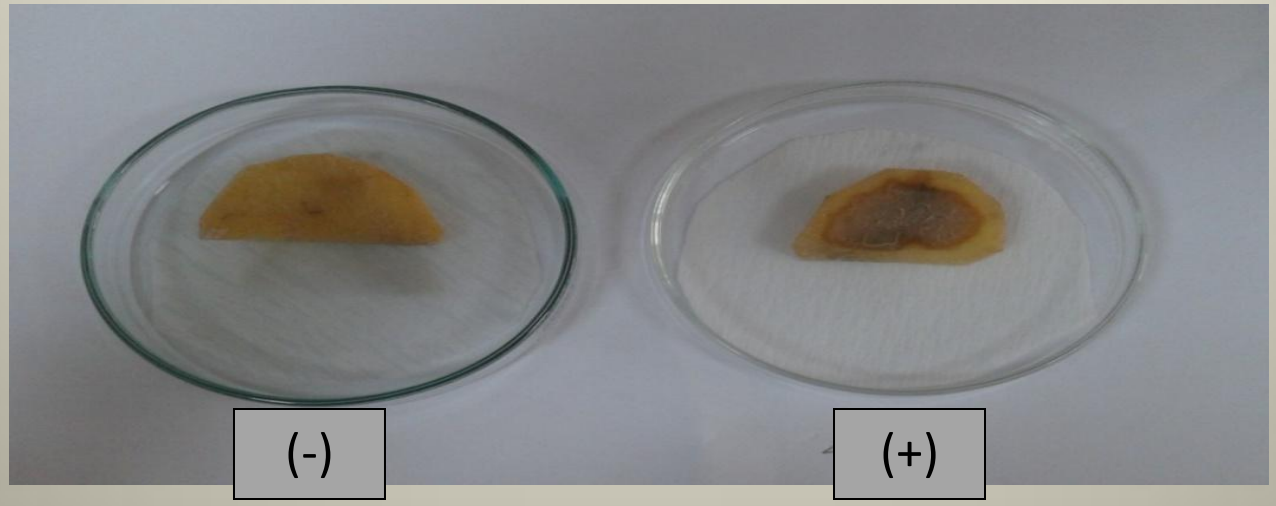


Fig. 10. Pectolitic activity test on potato ---- (-) control; (+) HK18B-3 isolate

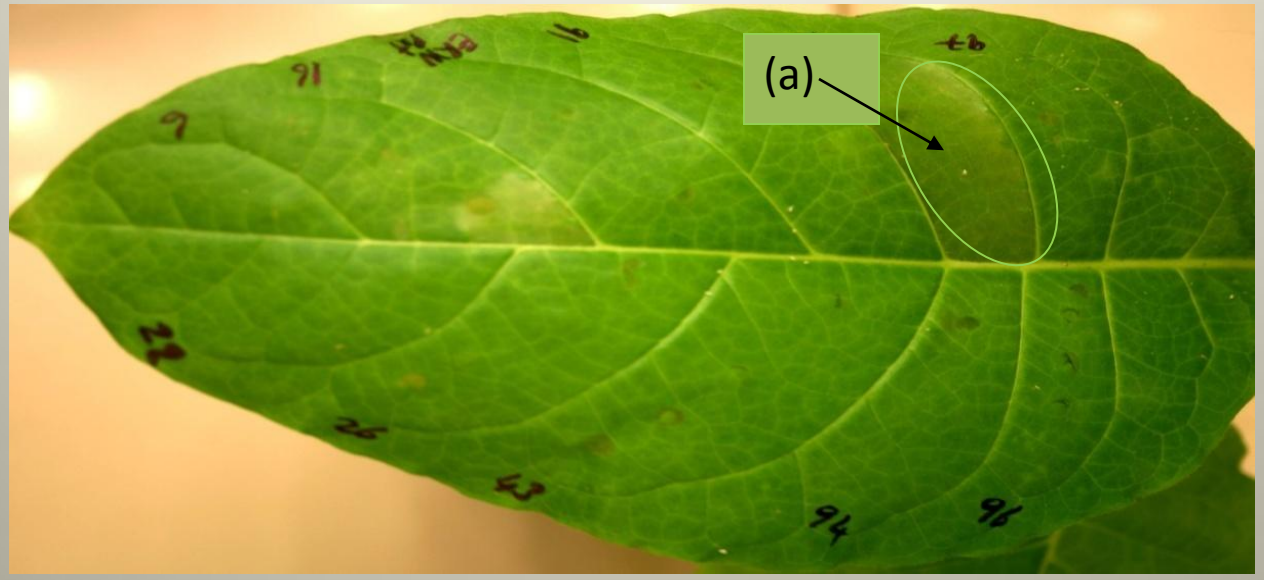


Fig. 11. Hypersensitivity symptom caused by reference culture Psp (Pseudomonas savastanoi pv. Phaseolicola on tobacco leaf (a)

Selection of Candidate PGPR Isolates

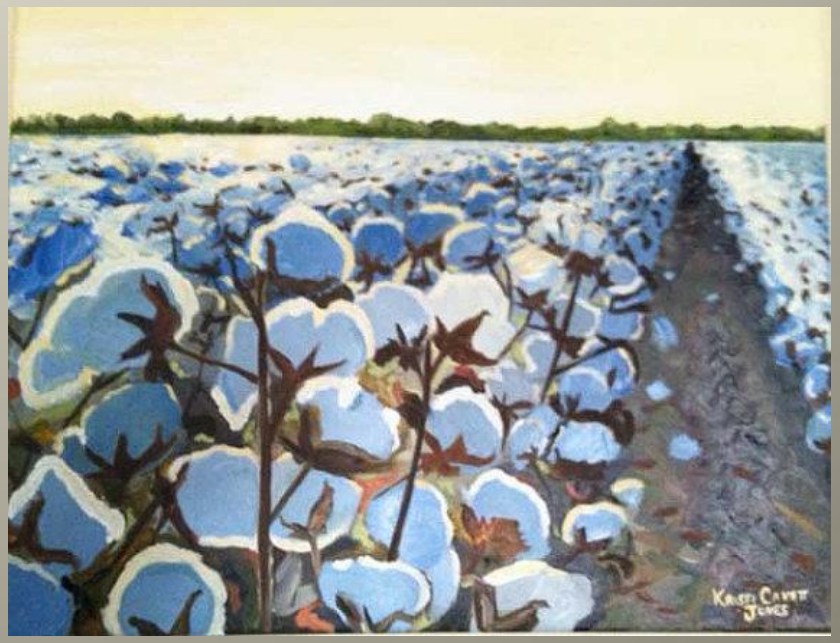
Selection was applied among candidate PGPR isolates obtained from soil according to their phosphorus reduction and antagonistic properties.

4.1. Selection of Candidate PGPR Isolates According to Phosphorus Reduction Properties

Isolates that have positive response to pectolitic activity and hypersensitive reaction on tobacco tests which have higher patogenity risk were eliminated.

The abilities of phosphorus dissolving of the rest isolates were determined. Fort this purpose were used two different methods. First is solid NBRIP (National Botanical Research Institute Phosphate-Bromo Phenol Blue broth medium) (Nautiyal, 1999); second is liquid NBRIP (Nautiyal et al., 2000).

In first method bacteria purely isolated from the soil were cultivated on Nutrient Agar medium and then they were sown to sterile petri dishes contain solid NBRIP nutrient medium by using sterile toothpick. There were 9 isolates in each petri dish (Fig. 5).



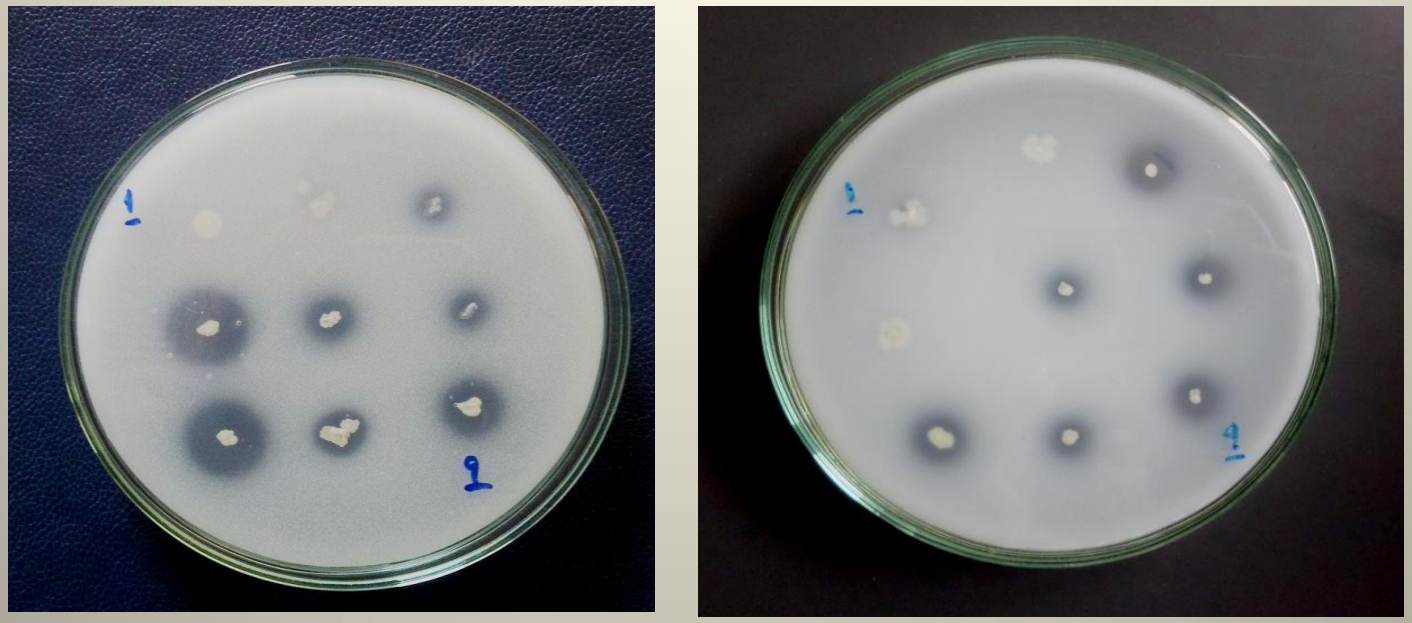


Fig. 12. Colonies of bacteria and phosphorus dissolving zones by bacteria isolated from the soil on solid NBRIP nutrient medium.

Trial was established with three replication. After one week diameter of transparent zone around colony of bacteria on nutrient medium and diameter of colony of bacteria were measured. Obtained values were proportioned and phosphorus dissolving index was calcuated (Fig. 6). Bacteria having the highest phosphorus dissolving index were selected (Johri et al., 1999). Only phosphorus dissolving index is not enough at selection of phosphorus dissolving bacteria. At the same time diameter of colony of bacteria is also important. Therefore it is necessary that determination of phosphorus amount dissolved by bacteria on liquid medium (Kusek, 2007).

Firstly 238 phosphorus dissolving isolates were ordered according to phosphorus dissolving index from the highest one to the lowest one. First 70 isolates were selected. Then the rest isolates were ordered according to diameter of colony. From these isolates were selected 30 ones. Thus, total 100 isolates dissolving phosphorus on the liquid nutrient medium were determined.

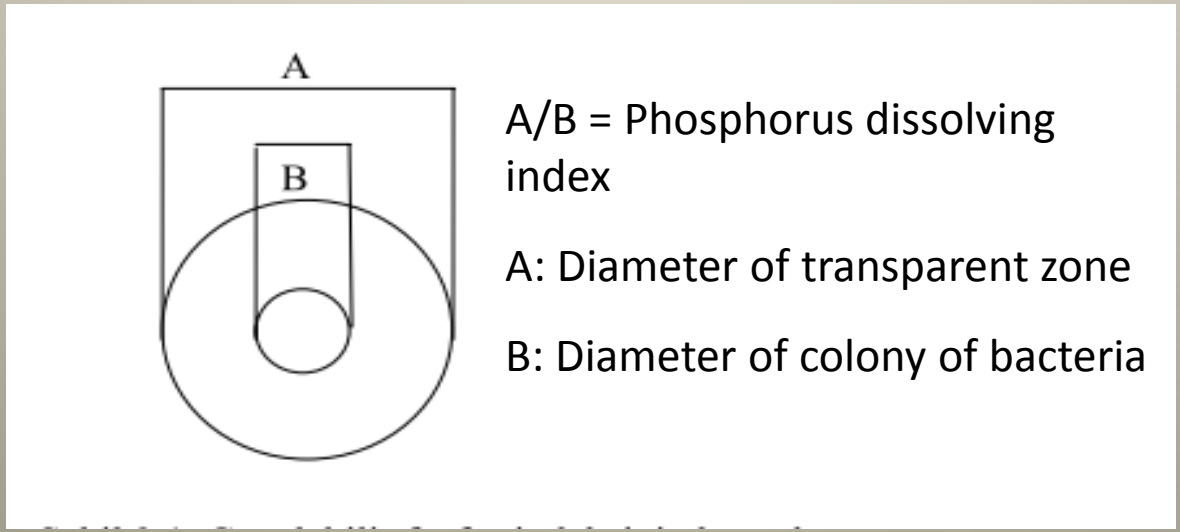


Fig. 13. Calculation of phosphorus dissolving index

100 isolates that they can dissolve phosphorus on liquid NBRIP nutrient medium were selected according to phosphorus dissolving index and diameter of colony of bacterium and amount of phosphorus (Nautiyal et al., 2000) were determined according to Barton (1948). Barton solution is yellow and as dissolved phosphorus amount is increasing in the solution, it increases yellowness of the solution to which is added

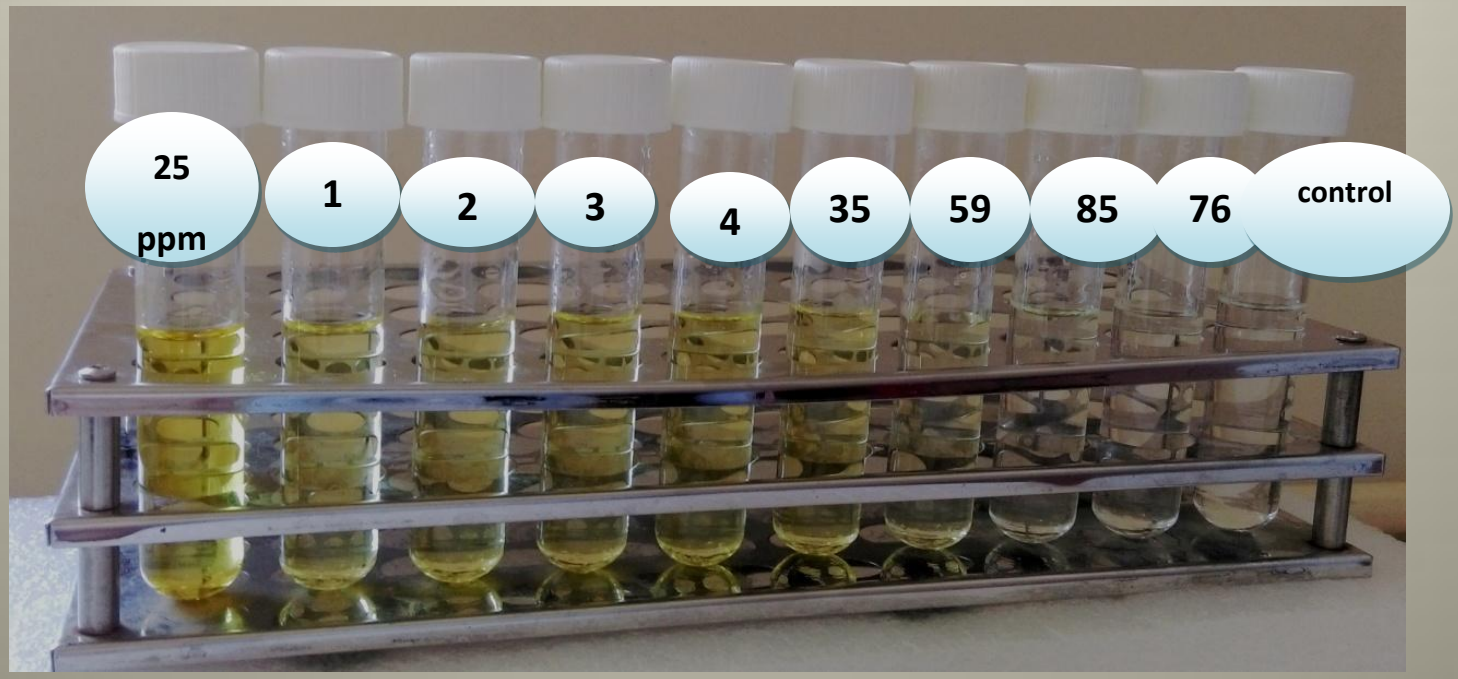
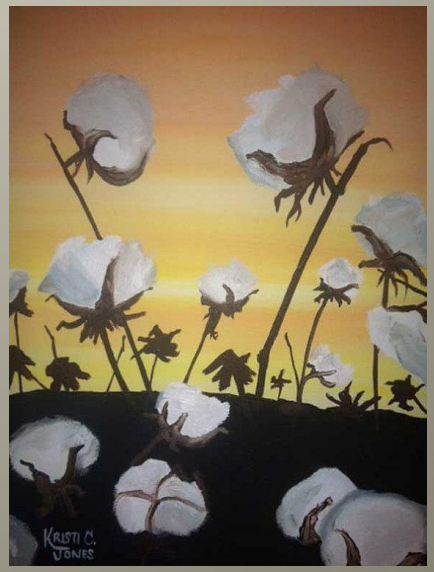


Fig. 14. Colour differences among PGPR isolates on NBRIP nutrient media. First (25 ppm) and final tubes (control). In the other tubes have been seen colour differences of different isolates.

4.2. Selection of Candidate PGPR Isolates According to Antagonistic Properties

In tests of *in vitro V. dahliae* Kleb. inhibition zone of isolates of bacteria from the soil were determined according to dual culture method. Isolates which responded positively to pectolitic activity and hypersensitive reaction on tobacco tests having higher patogenity risk were eliminated. The rest isolates were pre-eliminated with cot.vd 110 using dual culture method on PDA nutrient medium whereby pith and it was determined that 30 bacteria isolates formed inhibition zone (Fig. 8).

In further studies a trial will established with 3 replication at PDA nutrient medium under *in vitro* conditions in order to determine in which rates 30 bacteria isolates form inhibition zone.



$$\text{Inhibition}(\%) = \frac{\text{Diameter of fungus in control petri dish} - \text{Diameter of colony of fungus in petri dish with bacterium}}{\text{Diameter of fungus in control petri dish}} \times 100$$

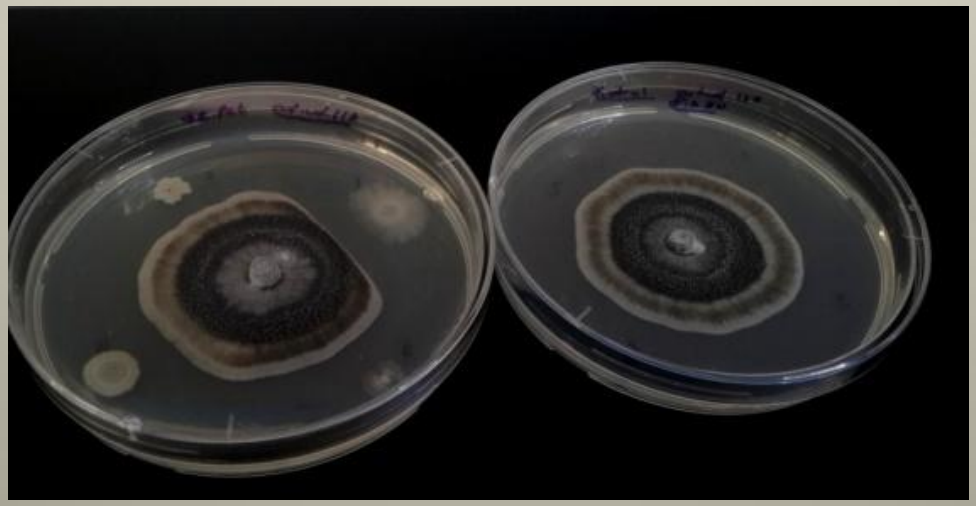


Fig. 15. Inhibition zone caused by isolates on pre-experimentdual culture antagonistic effect.

Results and Discussion

Properties of bacteria dissolving phosphorus were determined. At the same time, antagonistic properties of these isolates were determined. According to obtained means of diameters of colonies, diameters of zones and phosphorus dissolving index values of first 40 bacteria isolated from the soil on solid NBRIP nutrient medium were given in Table 1.

Table 1. Means of diameters of colonies, diameters of zones and phosphorus dissolving indexes of 40 candidate bacteria isolated from the soil on solid NBRIP nutrient medium

Isolates	Means of Zone Diameter (mm)	Means of Colony Diameter (mm)	Phosphorus Dissolving Index
HK16-4	9,50	1,98	**4,80**
HK16-3	9,38	2,17	**4,32**
HK16-7	9,39	2,25	**4,17**
HK16-6	9,58	2,36	**4,06**
HK16-1	9,22	2,34	**3,94**
HK16-5	8,62	2,21	**3,89**
HK4-5	12,24	3,30	**3,71**
HK13-3	8,16	2,28	**3,59**
HK2-7	11,47	3,44	**3,33**
HK15-1	8,42	2,64	**3,19**
HK16-9	8,16	2,61	**3,13**
HK16-8	8,27	2,84	**2,91**
HK12-4	9,36	3,47	**2,69**
HK2-12	11,77	4,69	**2,51**
HK4-8	8,81	3,54	**2,49**
HK4-6	10,21	4,21	**2,42**
HK3-4	6,19	2,65	**2,34**
HK2-14	7,90	3,45	**2,29**
HK3-3	6,30	2,92	**2,15**
HK20B-26	3,21	1,50	**2,14**

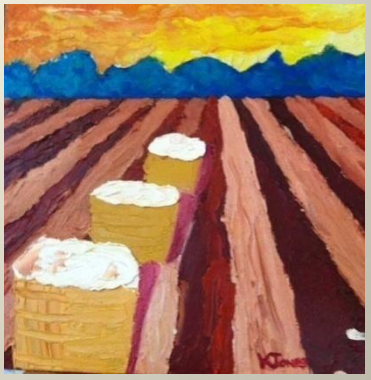
Isolates	Means of Zone Diameter (mm)	Means of Colony Diameter (mm)	Phosphorus Dissolving Index
HK21B-13	2,54	1,23	**2,07**
HK5-61	4,91	2,49	**1,98**
HK16-2	8,64	4,46	**1,94**
HK10-56	18,95	9,86	**1,92**
HK4-7	8,73	4,75	**1,84**
HK5-55	4,66	2,65	**1,76**
HK2B-12	4,43	2,52	**1,76**
HK3B-18	2,57	1,45	**1,76**
HK10-2	11,01	6,40	**1,72**
HK2B-21	2,54	1,49	**1,70**
HK10-58	9,07	5,42	**1,67**
HK8B-12	3,84	2,33	**1,65**
HK10-43	9,12	5,57	**1,64**
HK19B-18	2,61	1,60	**1,63**
HK10-55	8,61	5,31	**1,62**
HK10-40	8,22	5,09	**1,61**
HK10-16	10,59	6,64	**1,60**
HK10-1	9,76	6,13	**1,59**
HK10-35	8,18	5,18	**1,58**
HK3B-12	3,72	2,35	**1,58**

Table 2. The absorbance and ppm values in spectrophotometer (430 nm) of 25 solublizing phosphorus at maximum level of bacteria isolates selected on liquid NBRIP nutrient medium according to phosphorus dissolving index

Isolates	Means of Absorbance	Means of ppm
HK10-60	0,33	307,49
HK10-12	0,31	289,33
HK10-14	0,30	282,52
HK10-24	0,30	279,80
HK10-59	0,30	277,07
HK10-16	0,29	272,08
HK10-22	0,29	269,81
HK10-20	0,29	269,36
HK10-5	0,29	267,54
HK10-58	0,29	267,54
HK10-53	0,28	263,46
HK10-2	0,28	260,28
HK10-13	0,28	259,82

Isolates	Means of Absorbance	Means of ppm
HK10-4	0,28	258,50
HK10-6	0,27	258,01
HK26-16	0,27	258,01
HK10-43	0,27	257,55
HK10-15	0,27	254,83
HK10-1	0,27	253,92
HK10-38	0,27	253,92
HK10-41	0,27	253,92
HK10-34	0,27	251,20
HK10-55	0,27	249,84
HK10-40	0,27	249,38
HK10-36	0,26	248,93

It was determined that 30 bacteria have antagonistic property against fungus according to dual culture test applied bacteria and fungus.



Conclusion

Phosphorus dissolving properties of 238 of 650 bacteria isolated from the soil were investigated. It was determined that 238 bacteria isolates have phosphorus dissolving ability. At the same time, antagonistic properties of 30 isolates were determined.

In the next part of the study obtained these isolates will be chracterize and they will be inoculate to cotton seeds and then they will be used in pot trials. By pot trials possibilities of using of these isolates asbiological control agents in order to suppress of disease agent (*Verticillium dahliae*) and effects of morpfological traits releated growth and development of cotton plant (plant height, node number, simpodia number), seed cotton yield, yield components (boll number per plant, boll weight, seed cotton weight per boll), ginning outturn, fiber characters (fiber length, fiber fineness, fiber strength, fiber elongation, fiber uniformity index) will be investigated.

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