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Laboratory Management of Plant Pathogenic Fungi

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Abstract: ***Plant pathology is that the scientific study of diseases in plants caused by pathogens (infectious organisms) and environmental conditions (physiological factors). Organisms that cause communicable disease embody fungi, bacteria, viruses, viroids. Plant pathology conjointly involves the study of infectious agent identification, illness etiology, illness cycles, economic impact, disease medical specialty, disease resistance; however plant diseases influence human and animals, pathosystem, genetic and management of disease. In the present article we have collected infected leaves of the different plants from the field and isolated fungus and treated with respective biocontrol agent. The result showed the Rhizobium inhibits the growth of Aspergillus spp. and Penicillium spp. (+), whereas on the Fusarium spp. (-) it does not show any effect. In case of Azatobacter, it inhibits the growth of Penicillium and fusarium spp. (+) whereas the Azatobacter (-) does not inhibits the growth of Aspergillus spp..B. subtilis inhibits the growth of the Fusarium spp. (+) but does not inhibits the growth of the Aspergillus and Penicillium(-).***

Keywords: Plant pathogen, Plant fungi, Plant infections

1. Introduction

Plant pathology is the scientific study of diseases in plants caused by pathogens (infectious organisms) and environmental conditions (physiological factors). Organisms that cause infectious disease include fungi, bacteria, viruses, viroids. Plant pathology also involves the study of pathogen identification, disease etiology, disease cycles, economic impact, plant disease epidemiology, plant disease resistance, how plant diseases affect human and animals, pathosystem, genetic and management of plant disease.

Plant in both natural and cultivated populations carry inherent disease resistance, but there are numerous examples of devasting plant disease impacts. However, disease control is reasonably successful for most crops. The food and agriculture organization estimate indeed that pests and diseases and pests early, such as novel sensors that defat plant odours and spectroscopy and biophotonics that are able to diagnostic plant health and metabolism.

Plant Pathogen Fungi:

The fungi reproduce both sexually and asexually. Many soil inhabiting fungi are capable of living saprotrophically , carrying out the art of their life cycle in the soil. These are known as facultative saprotrophs. Fungal disease may be control through the use of fungicides and other agriculture practices. However, new races of fungi often evolve that are resistant to various fungicides. Biotrophic fungal pathogens colonize living plant tissue and obtain nutrients from living host cells.

Fungi cannot make their own food, so they must somehow get it from other organisms, living or dead. Some fungi can digest things like leaves and wood. These 'pathogenic' or disease-causing fungi get inside the plant either by making a hole in its skin (epidermis), or by growing in through the plant's breathing holes (stomata).

Then they either poison or kill the plant cells before absorbing food them. The spore of some fungi come through the air and attack leaves, making dead spots or even killing the whole leaf. Some fungi live in the soil and enter roots. They can either block the water conducting cells or kill them, causing the plant to wilt. About 85% of plant diseases are caused by fungi.

Fungi that live in soil can move from plant to plant by growing along intermingled roots or out from infested plant debris in the soil. Some fungi can survive on their own for long periods of the without a host by living in plant debris or soil**.**

Alternariaalternata is a fungus which has been recorded causing leaf spot and other diseases on over 380 host species of plant. It is an opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts. Boxwoods caused by the fungus *Cylindrocladiumbuxicola*. *Cercosporaapii* is a fungal plant pathogen, who causes leaf spot on celery, and found on other plants, including Impatiens.

Grey leaf spot is a foliar fungal disease that affects maize, also known as corn. There are two fungal pathogens that cause GLS, which are *Cercosporazeaemaydis* and *Cercosporazeina*. Collar rot is symptomatically described disease that is usually caused by any one of various fungal and oomycete plant pathogens. It is present where the pathogen causes a lesion localized at or about the collet between the stem and the root. In botany and mycology, a haustorium is the appendage or portion of a parasitic fungus or of the root of a parasitic plant that penetrates the host's tissue and draws nutrients from it.

Leaf spot disease of mulberry is an important fungal disease of mulberry plantation causing considerable damage to the rearing and ultimately to the cocoon crop parameters. Various methods for the management of the disease have been studied by various workers in other states. There are reports that foliar spray of carbendazim and Mancozeb 70% WP in the ratio of 0.1% and 0.2% were most effective in reducing the disease. However due

to residual effect of synthetic fungicides, there is demand for more eco-friendly substances like bio pesticides. As such exploration of plant resources for their antifungal potential against the pathogen is quite inevitable for a sustainable and eco-friendly management of the pathogen. Further these plant extracts could be readily used by the farmers to lessen the impact of the pathogen on their mulberry plantation. Using plant resources for its antifungal activity is an attractive avenue for the development of sustainable mode of moriculture in organic farming system. Hence, new plants especially locally available need to be explored for their antifungal property.

Control of plant diseases is crucial to the reliable production of food, and it provides significant reductions in agricultural use of land, water, fuel and other inputs. Plants in both natural and cultivated populations carry inherent disease resistance, but there are the numerous examples of devastating plant disease impacts (see Irish potato famine, chestnut blight), as well as recurrent severe plant diseases (see rice blast, soybean cyst nematode, citrus canker). However, disease control is reasonably successful for most crops. Disease control is achieved by use of plants that have been bred for good resistance to many diseases, and by plant cultivation approaches such as crop rotation, use of pathogen-free seed, appropriate planting date and plant density, control of field moisture, and pesticide use. Across large regions and many crop species, it is estimated that diseases typically reduce plant yields by 10% every year in more developed settings but yield loss to diseases often exceeds 20% in less developed settings. Continuing advances in the science of plant pathology are needed to improve disease control, and to keep up with changes in disease pressure caused by the ongoing evolution and movement of plant pathogens and by changes in agricultural practices. Plant diseases cause major economic losses for farmers worldwide. The Food and Agriculture Organization estimates indeed that pests and diseases are responsible for about 25% of crop loss. To solve this issue, new methods are needed to detect diseases and pests early, such as novel sensors that detect plant odours and spectroscopy and biophotonics that are able to diagnostic plant health and metabolism.

1.1*Fusarium*

Scientific Classification

Kingdom: Fungi
Division: Ascomycota
Class: Sordariomycetes
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*
Role in disease:

Fusarium is a large genus of filamentous fungi, part of group often referred to as hyphomycetes, widely distributed in soil and associated with plants. Most species are harmless saprobes and are relatively abundant members of the soil microbial community. Some species produce mycotoxis in cereal crops that can affect human and animals health.

The main toxins produced by these *Fusarium* species are fumonisins and trochothesens. *Fusarium spp*. Are important plant pathogens causing various disease such as crown rot, head blight and scab on cereal grains and they may occasionally cause infection in animals. *Fusarium spp*. Causes diseases to the tomato, tobacco, legumes, sweet potato, etc.

Fusariumwilt:

Fusariumwilt is a common vascular wilt fungal diseases. The pathogen that causes Fusarium wilt is *Fusariumoxysporum*.*Fusarium* wilt is a disease caused by a fungus. *Fusarium*oxysporum f. sp. *Lycopersici*, which lives in the soil. It is often confused with Verticillium wilt because both produce similar symptoms in tomatoes.

The fungus works its way up through the plant's roots, clogging water-conducting tissue in the stem. That prevents water from reaching branches and leaves, starving the plant. Affected plants produce very few tomatoes. Often the entire plant dies.

Host and Symtpoms:

The fungal pathogen *Fusariumoxysporum* affects a wide variety of hosts at any age. *Fusarium* infects tomato, sweet potato, tobacco, legumes, cucurbits, and banana, but it will also infect other herbaceous plants.

Symptoms:

- First sign are yellowing and wilting on one side of the plant, - a leaf, single shoot, branch, or several branches. Yellowing and wilting move up the plant as the fungus spreads.
- Wilted leaves dry and drop prematurely.
- If the plant does not die, it will be weak and produce inferior products.

1.2 Penicillium species:

Scientific Classification

Kingdom: Fungi
Phylum: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: *Penicillium*

Penicilllium is a genus of ascomycetous fungi of major importance in the natural environmental as well as food and drug production. Some members of the genus produce penicillin, a antibiotic which kills the bacteria. Some *penicillum* species affects the fruits and bubs of plants, including: *P. expansum*– apples and pears *P. digitatum* - citrus fruit *P. allii* - garlic

Blue Mould Disease:

Blue mould disease in garlic is associated worldwide with various *Penicilliumspecies*. And has been attributed to significant annual crop losses in India.

Symptoms:

- Stunted the growth of plant and also reduced the size of bulb.
- Chlorotic plants with withered leaves.

1.3 Biocontrol Agent:

It is defined as the reduction of pest population by natural enemies and typically involves an active human role. This is frequently referred to as the natural control also known as biological control agent includes predators, parasitoids, and pathogens. Biological control agent of plant diseases are most often referred to as antagonists.

Rhizobia:

Rhizobium is a genus of Gram-negative soil bacteria that fix nitrogen. It forms an endosymbiotic nitrogen fixing association with roots of legumes and Parasponia. The bacteria colonize plant cells within root nodules where they convert atmospheric nitrogen into ammonia and then provide organic nitrogenous compounds such as glutamine or ureides to plant. *Rhizobia* promotes the growth of plants either directly through N_2 fixation, supply nutrients, synthesis of phytohormones and solubilization of minerals, or indirectly as a biocontrol agent by inhibiting the growth of pathogens. The bio-control effects of Rhizobiais due to the secretion of secondary metabolites such as antibiotics.

Azatobactor:

Azatobacter is a genus of usually motile, oval or spherical bacteria that form thick walled cysts and may produce large quantities of capsular slime. They are aerobic, free-living soil microbes which play an important role in the nitrogen cycle in nature, which is inaccessible to plant, and releasing in the form of ammonium ions into the soil.

Azatobacter is one of the dominant non-symbiotic nitrogen fixing heterotrophic bacteria. The most dominant non-symbiotic nitrogen fixing heterotrophic bacteria in Indian soil is *Azatobacterchroococcum*. The beneficial effects of *Azatobacter* are not only due to its ability to fix atmospheric nitrogen, but also to secrete growth substances and antifungal antibiotics, which improve plant stands in inoculated fields by inhibiting root pathogens. Apart from nitrogen fixing ability. *Azatobactor* usually produces considerable amounts of biologically active substances such as vitamins of B-group like nicotinic acid, biotin, auxins and gibberllins. It also produces antifungal antibiotics i.e. fungistatic substances with a broad active spectrum but inactive against bacteria. It inhibits growth of *Fusarium, Aspergillus, Alternaria, Rhizotonia*.

***Bacillus subtilis*:**

Bacillus subtilis also known as the hay bacillus or grass bacillus. It was originally named vibrio *subtilis* by Christian Gottfried Ehrenberg, and renamed *Bacillus subtilis* by Ferdinand Cohn in 1872.It is a Gram-positive, catalase-positive bacterium, this species is commonly found in upper layer of soil. A member of the genus *Bacillus*, *Bacillus subtilis* is rod-shaped, and can form a rough, protective endospore. *Bacillus subtilis* has histrorically been classified as an obligate aerobe, though evidence exists that is a facultative aerobe. It is one of the bacterial champions in secreted enzyme production and used on an industrial scale by biotechnology companies.

2. Materials and Methods

Requirements:

2.1 Collection of infected plant

Collect infected leaves of the different plants from the field in the clean polythene bag.

2.2 Chemicals, media and reagent

2.2.1 PDA (Potato Dextrose Agar) Medium: Water (1000ml), Potatoes (200gm), Dextrose (20gm), Agar powder (20gm).

2.2.2 Sodium Hypochloride (0.5%).

2.2.3 Cotton Blue Stain: Phenol (200gm), Cotton Blue (0.5gm), Glycerol (400ml), Lactic acid (200ml), Distilled water (200ml).

2.3 Glass Ware: Petri dishes, beakers, flasks, stirrer, pipettes, forcep, cutter, filter paper disc, wireloop and spreader**.**

2.4 Sterilization: All glass ware and media were autoclaved at 15 psi for 20 minutes before use.

2.5 Isolation of fungi from infected leaves:

Figure 1: Infected Leaf

The fungus was sensitive to chemical disinfection of leaves, making difficult the spore's removal from the leaf surface.

Extraction of spores from lesions found on leaves: leaves with more than three false mildew lesions were randomly selected, washed with sterile water, immersed is sodium hypochloride solution 0.5% (v/v) for 2 minutes and the excess of disinfectant was removed with sterile distilled water. Disinfected leaves were placed on petri dishes containing PDA agar and incubated at temperature. After 48 hours, the mycelium developed on the agar plate.

2.6 Subculture of Fungi:

Select pure colony on the surface of the petri dish. Take a loopful colony of isolated fungi on a potato dextrose agar petri plate. Incubated at room temperature for 8-10 days.

2.7 Identification of isolated fungi:

2.7.1 Lactophenol Cotton Blue Staining:

Take a clean slide and add a drop of lactophenol cotton blue stain in the centre of clean slide. Remove a fragment of fungus colony teasing the needle. Place a fragment in a drop of stain and tease gently. Then apply coverslip and observe under 40X microscope.

2.8 Isolation of Bio-control Agent:

2.8.1 Isolation of *Azatobacter* from soil.

Requirements:

1. Nitrogen free mannitolagar (for 1000ml)

a) Mannitol - 10gm
b) K_2HPO_4- 0.5 gm
c) $MgSO_3$. $7H_2O$- 0.2 gm
d) NaCl- 0.2gm
e) $MnSO_4$- 0.0005gm
f) $CaCO_3$ -10 gm
g) pH - 6.8-7.0

Procedure

From soil sample 1gm of soil was dissolved in 10ml of sterile distilled water and loopful suspension were streak on nitrogen free mannitol agarand incubated at 28^0c for 3 days.

Identification of *Azatobacter spp.:*

Isolated *Azatobacter* were identified on the basis of morphology Gram staining motility. Monochrome staining of faint yellow pellicle and Gram's staining of isolated colonies showed the presence of cyst and gram negative rods in the enrichment media.

2.9 Isolation of *Rhizobium*

Requirement:

1. Root nodules 2. YEM agar medium 2. Test tube with nylon mesh 3. Petri dishes 5. 0.1% acidified HgCl , 5ml conc..HCL, 1 lit. Distilled water. 6. Sterile tap water. 7. Nichrome blade. 8. Plates containing YEM agar medium.

YEM agar (yeast extract mannitol agar) medium

1. K_2HPO_4- 0.5 gm.
2. $MgSO_3.7H_2O$ – 0.2 gm.
2. NaCl – 0.1 gm.
3. Mannitol – 10 gm.
5. Yeast extract – 1 gm.
6. Distilled water – 100 ml.
7. Agar – 15 gm.
8. pH – 6.8**.**

Procedure:

The fresh leaves and plump root nodules of fenugreek were collected from the plants grown in pots. The collected nodules were surface sterilized with 75% ethanol and 0.1% mercuric chloride and washed thoroughly with distilled water. *Rhizobium* strain was obtained by streaking the crushed root nodules on YEM agar plates and incubated at 29.4 c. After 2 days of incubation, *Rhizobium* colonies were obtained. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures of *Rhizobium*. Pure isolates were used for further analysis and all tests were performed in triplicates.

Identification of *Rhizobium spp*.:

Rhizobium colonies were observed on YEM agar plates which were characterized as gram negative rods.

2.9.1 Isolation of *Bacillus subtilis*

Requirements:

Nutrient agar composition:

1. Peptone – 1 gm
2. Beef extract – 0.33 gm
2. NaCl-0.5 gm
3. Distilled water – 100ml
5. pH– 7.3.

Procedure

1 gm of soil sample was dispensed in the first test tube containing 10ml sterile saline. The dilution was prepared. The suspension spread on nutrient agar medium plates by using spreader, was sterilized by dipping it in alcohol and incubated at room temperature (37^0c) for 24 hours.

Identification of *Bacillus subtilis*

Isolated *Bacillus subtilis* were identified on the basis of morphology Gram staining motility. We can also identify the *Bacillus subtilis* bacteria by using simple technique such as staining methods.

Applying Method of Bio-control Agent:

Procedure:

1) Effect of different bio-control agent against *Fusarium species*, *Penicillium species*, *Aspergillus species* was determined by dual culture method.
2) A plug of mycelium of isolated pathogen (5mm diameter) was placed at the center of PDA plate.
3) Then the bacterium was streaked 2cm away from the agar plate at both sides of the plate.
4) Incubated at room temperature for 7 days and antagonists' effect was determined by measuring the longest and shortest free growth zone between the bacteria and fungi.
5) Mycelialdisc (5mm diameter)of fungi and that of isolated pathogen were placed at a two opposite sides of PDA plates, incubated at room temperature.
6) In case of control, only a plug of mycelium of a selected pathogen was placed at center of PDA plate.

3. Results and Discussion

3.1 Isolation of Pathogen and Identification

Pathogenic fungi are isolated from the infected plant parts and identified on the basis of variety of characteristics including like colour of colony, shape, and also by staining methods and observing under microscope**.**

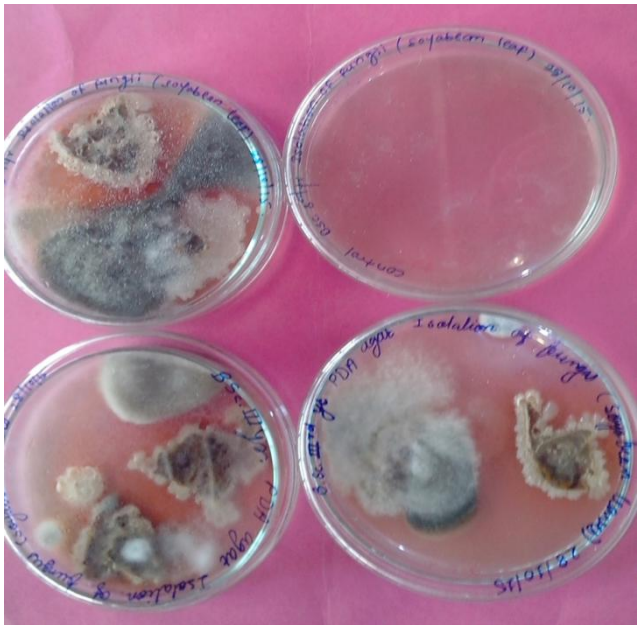


Figure 2: Piece of Infected Plant on PDA

3.2 Identification of fungus

By using the staining process, we identified the pathogenic fungus. For staining process, we were used the Lactophenol Cotton Blue staining. So, we were easily identified the pathogenic fungi.

Figure 3: Fusarium spp.

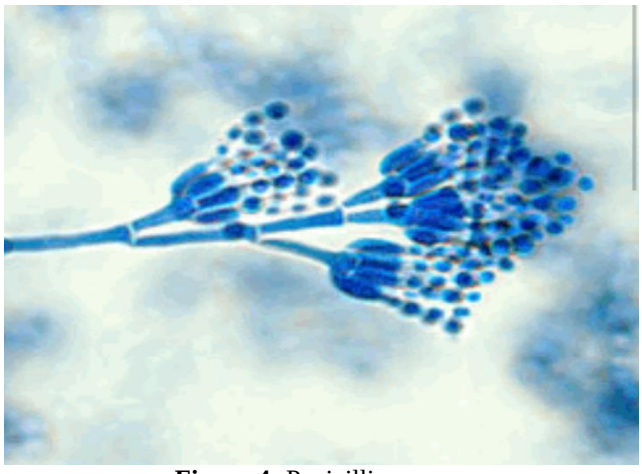


Figure 4: Penicillium spp.

Isolation of Bio-control Agent

We were isolated the Bio-control agent such as *Azatobacter*, *Rhizobium* and *Bacillus subtilis* from the soil.

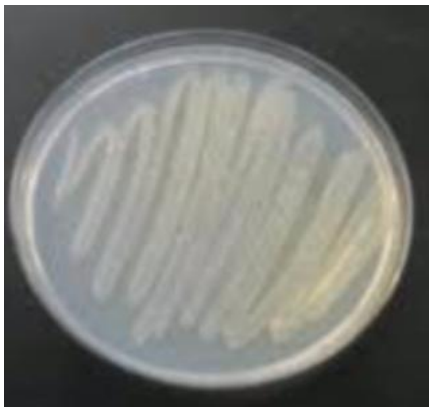
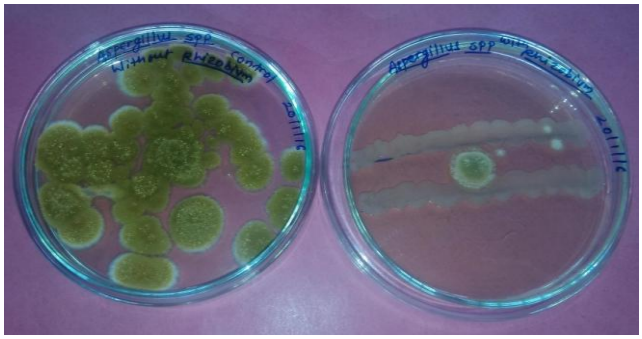


Figure 5: *Azatobacter*

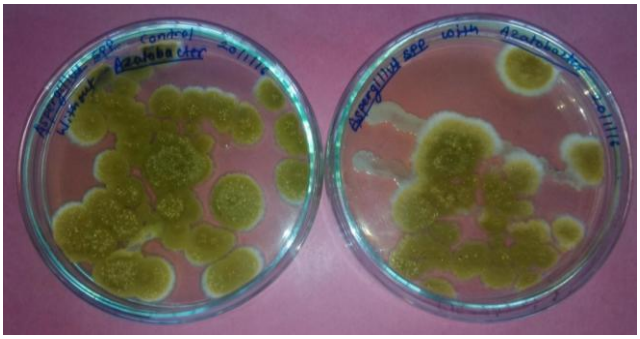
Figure 6: *Rhizobium*

Figure 7: *Bacillus subtilis*

Effect of Bio-control agent on pathogenic fungi:



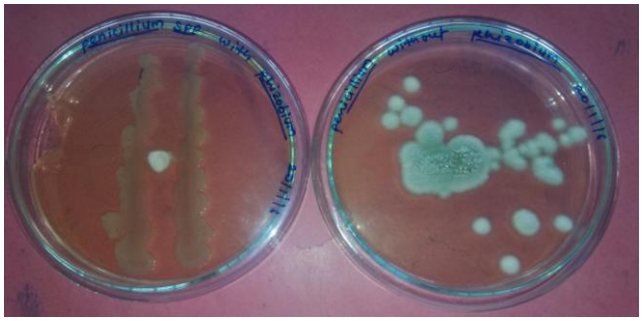
Aspergillus spp. with *Rhizobium* inhibits the growth of*Aspergillus*fungi.



Aspergillus spp. with *Azatobacter*does not inhibit growth of*Aspergillus* fungi.

Aspergillus spp. with *Bacillus subtilis* doses not inhibit the growth of *Aspergillus*fingi.

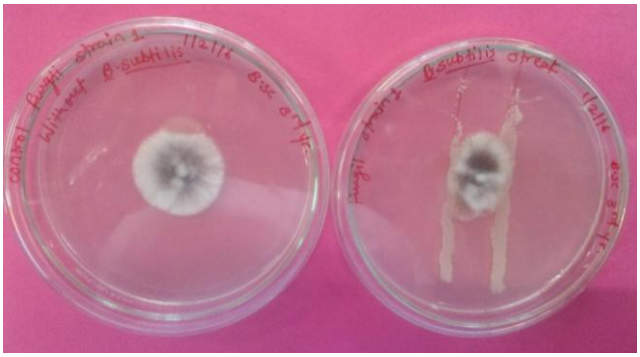
Penicillium spp. with Azatobacter inhibits the growth of Penicillium fungi.



Penicillium spp. with *Rhizobium* inhibits the growth of *Penicillium* fungus.

Penicillium spp. with *Bacillus subtilis* does not inhibit the growth of *Penicillium* fungi.

Fusarium with *Azatobacter* inhibits the growth of *Fusarium* fungi.



***Fusarium spp*. with *Bacillus subtilis* inhibits the growth of *Fusarium* fungi.**

Fusarium spp. with *Rhizobium* does not inhibit the growth of *Fusarium* fungi.

4. Conclusion

1) From the above observation and result it was concluded that *Rhizobium*, *Azatobacter*, *Bacillus subtilis* were found effective against isolated pathogenic fungi
2) Hence these bio-control agents can be used to control the disease caused by fungal isolate to the plant.
3) *Rhizobium spp*. inhibits the growth of *Penicillium*, *Fusarium* as well as *Azatobater spp*. inhibits the growth of *Penicillium*and *Fusarium* whereas *Bacillus subtilis* inhibits the growth of Fusarium only.
4) By using the bio-control agent, hence it is concluded that, the *Rhizobium* inhibits the growth of *Aspergillus spp*. and *Penicillium spp*. (+), whereas on the *Fusarium spp. (-)* it does not show any effect. In case of *Azatobacter*, it inhibits the growth of *Penicillium* and *fusarium spp*. (+) whereas the *Azatobacter (-)* does not inhibits the growth of *Aspergillus spp.*. *B. subtilis* inhibits the growth of the *Fusarium spp*. (+) but does not inhibits the growth of the *Aspergillus* and *Penicillium(-).*

Fungal spp.	*Rhizobium*	*Azatobacter*	*B. subtilis*
Aspergillusspp.	+	-	-
Penicillium spp.	+	+	-
Fusarium spp.	-	+	+

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