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Research Paper

The ill effects of composite farming

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Abstract

Composite farming is a method of cultivation in which many layers of many crops are grown together such that no stretch of land remains uncultivated. Cultivation takes place in horizontal and vertical layers. Composite farming is practiced in various places where different kinds of crops are often grown alongside water bodies where cultivation of fish is also carried out. Composite farming is carried out in order to maximize agricultural productivity in different parts of India. In this investigation, a field in the outskirts of Cuttack was selected where both horizontal and vertical layers have been denoted with occasional weed plant infestation. Certain low lying areas have been used for culturing of fishes. *Brassica juncea* was grown as a common oil yielding plant which was contaminated by *Pseudomonas syringae* obtained from common weed *Solanum nigrum*. Simultaneously, Gram-negative coccobacilli were also isolated from *Brassica juncea*. Both the bacteria independently infected the host plant without any interaction. The percentage of infection in the diseased leaf sample was calculated to be 20%. Antibiotic sensitivity was tested for both *Pseudomonas syringae* and the coccobacilli and the antibiotics to which both showed maximum sensitivity were observed to be Tetracycline and Azithromycin. Thus, it can be concluded that in order to get high yield of oil, weed control and antibiotic dusting is necessary.

Keywords: antibiotic sensitivity, Brassica juncea, leaf-spot disease, *Pseudomonas syringae*, *Solanum nigrum*.

Introduction

The agricultural sector is the most important segment of the Indian economy. A major breakthrough in Indian agriculture has been brought about by the concept of 'Composite Farming', which entails growing many layers of crops simultaneously on a single stretch of land, both in vertical and horizontal layers, such that no area of the land remains uncultivated. While one area has trees, below it grown are the plants which can thrive without direct sunlight and below this layer, those plants are cultivated whose growth is not dependent on sunlight. Pisciculture can also be carried out along with it, as plants can be grown surrounding a fish tank. Composite farming is carried out on a routine basis in the fields surrounding the Central Rice Research Institute (CRRI), Cuttack. This method has resulted in improvement of the agricultural yield, thus contributing towards the country's economy.

On an agricultural land, along with the main crops which are cultivated, unwanted weeds may also grow due to the presence of several overlapping areas of cultivation. These weeds not only compete with the crops for space and nutrition, but may also harbor pathogenic species of microorganisms which cause disease in the crops. As different plants are grown in overlapping areas, there are possibilities that they may acquire pathogenic bacteria from the weeds and contract diseases. 'Leaf spot disease' is one such plant disease. It can be both bacterial and fungal.

Fungal ones are caused by species of *Alternaria, Pyrenophora, Drechslera,* etc, and the common bacterial genus for the same includes *Pseudomonas, Xanthomonas,* etc*.* This disease is characterized by appearance of spots on the foliage. The spots vary in size, shape and colour depending on the plant, the causative organism and the stage of the disease development. The spots may be yellowish or brownish, sometimes tan or black. Concentric margins may appear around them. Gradually, the spots may combine to form enlarged blotches. These anthracnoses are angular in shape and are mainly seen around the veins. Early symptoms of the disease include appearance of small, light coloured, translucent dots, while the older ones are brown centered. As the disease progresses from the leaf tip to the entire foliage, yellowing of the affected leaves occurs which ultimately fall off. This disease is most predominant in hot and humid climatic areas.

In our project, we particularly deal with the bacterial leaf spot disease in *Brassica juncea*. This is because no fungi were isolated from its spots. The pathogen isolated from the spots is *Pseudomonas syringae,* which was identified on the basis of disease symptoms, gram characterization, shape and physiological parameters*.*

Pseudomonas syringae is a Gram-negative, rod-shaped bacterium with polar flagella. It is a plant pathogen which can infect a wide variety of plants. Diseases by *Pseudomonas syringae* are favored in humid conditions, where the optimum temperature for it to cause disease is around 12-25^{0}C. The bacteria are seed-borne and are disseminated via rain splash. The bacteria's survival within the host is aided by a large number of its genes which help in its attachment and nutrient uptake. They may have siderophores which help in iron acquisition. The primary virulence factors produced by them are Type III Secretion System[1] and toxins.

While the former helps in injecting Type III effectors (the hop proteins) into the host, the latter suppresses the Salicylic-mediated defense system in the host plant.

Thus, the aim of this project is to study the limitations of composite farming in terms of the contaminating bacteria, to characterize these bacteria, and to prove its pathogenicity.

Materials and Methods

The two leaf samples (*S. nigrum* and *B. juncea*) were subjected to surface sterilization with 0.1% $HgCl_2$ solution, and their diseased portions placed on Nutrient Agar and Potato Dextrose Agar slants (2 slants of each for one sample). This was done to determine whether the leaf-spot disease had been caused by a bacterial plant pathogen (in Nutrient Agar) or fungal plant pathogen (in Potato Dextrose Agar). No growth was observed in case of Potato Dextrose Agar, which ruled out the presence of fungi in causing the leaf-spot disease.

The two distinct bacterial cultures corresponding to the two different leaf samples, obtained from the Nutrient Agar slants inoculated with the diseased leaf portions were sub-cultured in 2 Nutrient agar plates (one for each leaf sample) and thus, pure cultures of the bacteria were obtained.

Gram characterization of the bacteria was done. Also their size was measured using a standardised ocular micrometer. The antibiotic sensitivity against Ampicillin, Tetracycline, Streptomycin and Azithromycin using the disc-diffusion method (Kirby-Bauer test) was determined for the bacterial sub-cultures.

For identification and characterization of the bacteria, the standard biochemical tests were performed, namely Catalase test, Oxidase test, Indole production test, Methyl Red test, Voges Proskauer test and Citrate Utilization test. The results obtained from the biochemical tests were tabulated and compared with existing data to identify the bacteria unambiguously.

Following identification and characterization of the bacteria, cross-inoculation of the two bacterial isolates was carried out on the two leaf samples and the Koch's Postulates were verified. Glassware (conicals, test tubes, petriplates, slides, beakers, glass rod), inoculation loop, spirit lamp, physical balance, autoclave, Laminar Air Flow cabinet, Incubator, Compound microscope, Stage and Ocular micrometers, Gram Staining reagents (Crystal violet, Lugol's Iodine, 95% alcohol, Safranin)
Biochemical test reagents.

Gram Characterization procedure

1. The bacterial sample was taken in a drop of water on a clean grease-free glass slide with the help of a sterile inoculation loop. This was done within the Laminar Air Flow cabinet.
2. The bacterial smear was air-dried, followed by heat fixing on a spirit lamp.
3. Crystal violet stain was applied to the smear and kept for 30 seconds, followed by a distilled water wash.
4. Lugol's Iodine solution was applied to the smear and kept for 30 seconds.
5. The smear was washed with 95% alcohol.
6. Safranin was applied to the smear and kept for 1 minute.
7. The smear was washed finally with distilled water.
8. After drying the smear properly, the slide was observed under the compound microscope, first under low power then under high power.

Test for antibiotic sensitivity (Kirby-Bauer test) [2]

The Kirby-Bauer antibiotic sensitivity testing procedure made use of thin paper discs previously impregnated with pre-determined concentrations of four antibiotics namely, Ampicillin, Tetracycline, Streptomycin and Azithromycin. The paper discs were placed in petriplates in which the bacterial cultures had been individually pour-plated. If the bacteria were susceptible to a particular antibiotic, an area of clearing was observed around its paper disc in which the bacteria were unable to grow. This area of clearing is known as the Zone of Inhibition. Along with it, the rate of antibiotic diffusion is also used to determine the bacteria's sensitivity to that particular antibiotic.

Standard Biochemical Tests [3]

A. Catalase test procedure

1. A drop of 3% hydrogen peroxide was taken on a clean, grease-free glass slide and with the help of a sterile glass rod, bacterial culture from the isolated colony was transferred onto the drop.
2. Immediate appearance of effervescence constitutes a positive test for Catalase production.

B. Oxidase test procedure

1. A thin strip of filter paper was soaked with 1% oxidase reagent and then at once used by touching some of the bacterial culture on its surface with the help of a sterile glass rod.
2. A positive test for Oxidase production was indicated by the appearance of an intense deep blue colour within 10 seconds.

C. IMViC test

a) Indole production test procedure

1. The previously prepared Tryptophan broth was inoculated with the help of a sterile inoculation loop.
2. The inoculated broth was incubated for 24-48 hours at 37^0C.
3. After the incubation period was over, 0.5 ml of Kovac's reagent was added using a clean dropper by the walls of the culture tube, without shaking the tube.
4. The culture tubes were checked for the development of a reddish coloured ring in the upper alcohol layer of the Tryptophan broth, which was taken as a positive result for Indole production.

b) Methyl Red test procedure

1. The glucose phosphate broth was inoculated with the bacterial culture and incubated for upto 48 hours.
2. At the end of the incubation period, 5-6 drops of the reagent per ml of the culture were added to the culture tube.
3. The development of a bright red colour indicated a positive result.

c) Voges Proskauer test procedure

1. The glucose phosphate broth was inoculated with the bacterial culture and incubated for upto 48 hours.
2. At the end of the incubation period, 0.6 ml of α naphthol per 1ml of the broth culture was added along with 40% KOH solution and shaken well for at least 20 minutes.
3. The development of a copper red colour indicates a positive result.

d) Citrate utilization test procedure

1. On the surface of the Simmon's Citrate agar slant, an isolated bacterial colony was inoculated as a single streak.
2. The culture tubes were incubated for 24-48 hours.
3. At the end of the incubation period, the development of a deep blue colour indicates a positive result, else a negative result which is indicated by the colour of the medium remaining same i.e. green.

Proving the Koch's Postulates

The bacterium isolated from the diseased portion of *B. juncea* leaf was inoculated into a test tube containing Nutrient broth. After incubating the broth for 24 hours, the newly isolated bacterial culture was taken and inoculated onto a fresh *B. juncea* leaf after sterilizing it with 0.1% $HgCl_2$ solution. The leaf sample was kept in a sterilized petriplate and incubated at room temperature for 48 hours. At the end of the incubation period, it was seen that the same brown leaf-spots had developed on the surface of the leaf sample.

Procedure for Cross-inoculation

Cross-inoculation of the bacterium obtained from *S. nigrum* was carried out to determine whether it was this bacterium which was responsible for causing the leaf-spots in *B. juncea*. Here, the bacterium isolated from *S. nigrum* (*P. syringae*) was inoculated onto a fresh *B. juncea* leaf sample, after sterilizing the leaf sample with 0.1% $HgCl_2$ solution. This setup was kept in a sterile petriplate and incubated at room temperature for 48 hours. At the end of incubation, it was seen that the leaf sample developed brown spots.

Results and Discussion

Two distinct bacteria were isolated from the two diseased leaf samples- *Pseudomonas syringae* from *Solanum nigrum*, and Coccobacilli from *Brassica juncea*. Gram characterization revealed the *P. syringae* to be gram-negative short rods and the bacterium isolated from *B. juncea* was seen to be gram-negative coccobacilli. Furthermore, the length of the short rods was measured to be approximately 2.4 µm and the diameter of the coccobacilli was measured to be approximately 3 µm.
Antibiotic sensitivity testing showed that the Coccobacillus was almost resistant to Ampicillin and Streptomycin, but sensitive to Tetracycline and Azithromycin. In case of *P. syringae*, it was seen that the bacterium was showing more sensitivity to Tetracycline, Streptomycin and Azithromycin as compared to Ampicillin, to which it showed minimum sensitivity.

Pseudomonas syringae is the causal agent of the leaf-spot disease in *B. juncea*, whereas the Coccobacillus was assumed to be a member of its normal microbiota since it had no pathogenic effect on the said plant host. Thus, the standard biochemical tests and further testing procedures were carried out only on *P. syringae*. Performance of the standard biochemical tests showed the bacterium to be Catalase negative, Oxidase negative and Citrate utilization negative.

Verification of the Koch's postulates was carried out by re-inoculating *Pseudomonas syringae* onto a fresh *B. juncea* leaf sample. It was seen that the same leaf-spot disease was developed on the leaf surface, identical to the one on the original sample. Thus, it was proved that the bacterial pathogen responsible for causing the leaf-spot disease in *B. juncea* leaves was capable of again causing the disease upon re-inoculation on a fresh *B. juncea* leaf sample. Hence, *P. syringae* followed the Koch's postulates.

Cross-inoculation procedures showed that when *P. syringae* (isolated from *S. nigrum*) was inoculated onto a fresh *B. juncea* leaf sample, it led to the development of leaf-spot disease on the *B. juncea* leaf. This enabled the assertion that the leaf-spot disease in *B. juncea* was probably caused due to the presence of the weed plant *S. nigrum* (hosting the *P. syringae*) at its close proximity. As a result of this proximity, *P. syringae* was able to infect *B. juncea* and cause the disease.

Comparative studies with existing results based on the IMViC test results of *P. syringae*, led to the characterization of the bacterium as a member of the family Enterobacteriaceae. The members of the

Enterobacteriaceae family are mainly water-borne bacteria. In composite farming, it is a general trend to carry out Pisciculture alongside the agricultural field tract. It is possible for the water body used for Pisciculture to be contaminated with *P. syringae*. Weed plants (usually *S. nigrum*) growing near the water body could acquire the bacterium through the water which they absorb from the Pisciculture tank for their own metabolic needs. In this way the bacterium could spread among the weed plants. Furthermore, *S. nigrum* weed plants growing among *B. juncea* crop plants could result in the transfer of the bacterium from the former to the latter. Subsequently, this led to development of the leaf-spot disease in *B. juncea*.

Table 1: Gram characteristics, micrometry readings and antibiotic sensitivity tests

Bacteria isolated from	***Brassica juncea***	***Solanum nigrum***
Gram character	Gram negative	Gram negative
Morphology	Coccobacillus	Short rods
Number of stage divisions	8	8
Number of ocular divisions	23	23
Value of one ocular division (micron):	3.5	3.5
Length/Diameter of bacteria (micron):	3	2.4
Ampicillin sensitivity	Almost resistant	Less sensitive
Tetracycline sensitivity	More sensitive	More sensitive
Streptomycin sensitivity	Almost resistant	More sensitive
Azithromycin sensitivity	More sensitive	More sensitive

Table 2: Standard biochemical tests

Name of the test	**Result**
Catalase Test	Negative
Oxidase Test	Negative
IMViC test	
a) Indole Production Test	Negative
b) Methyl Red Test	Negative
c) Voges Proskauer Test	Negative
d) Citrate Utilisation Test	Positive

Hence, it was concluded that a major ill-effect of composite farming was this uncontrolled spread of plant pathogens due to the growth of excess weed plants among the crop plants and their possible acquisition of the pathogen from nearby sources or microbial reservoirs.

In the present investigation, the *Pseudomonas syringae* leaf spots were detected in *Brassica juncea*, but the carrier of this infection is *Solanum nigrum* growing as a contaminating weed in the compact environment. For years together, *Pseudomonas syringae* was proved to be a potent pathogen in different crop plants like tomato[4], maize[5], bean[6,7], coffee plants[8] and other crucifers[9] . The source of *Pseudomonas syringae* seems to be the Pisciculture tank also used in that area. The pathogenicity of *Pseudomonas syringae* was proved in both the leaf samples. In addition, the coccobacillus, growing as a natural flora in *Brassica juncea* did not cause any inhibition to the *Pseudomonas syringae*, but antibiotic sensitivity showed that the bacteria were most sensitive to Tetracycline and Azithromycin. So, it can be concluded that early infection of bacteria may not actually cause any major damage to the vegetables under consideration and the congregation of this bacteria prevents any major fungal infection.

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

Figure 1: Figures showing the area from where the diseased leaf samples were collected. (a) shows the area where composite farming is being carried out, (b) shows the crop plant *Brassica juncea*, (c) shows the weed plant *Solanum nigrum*

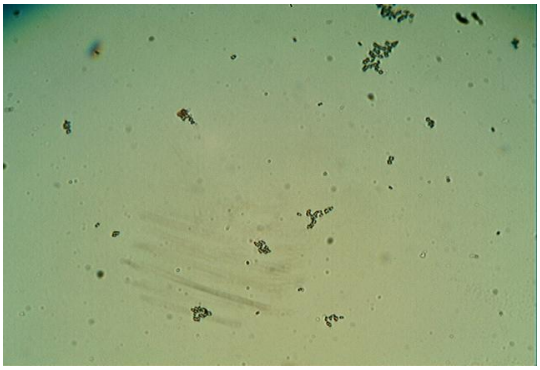

Figure 2(a): Figure showing gram negative coccobacilli isolated from *B. juncea*

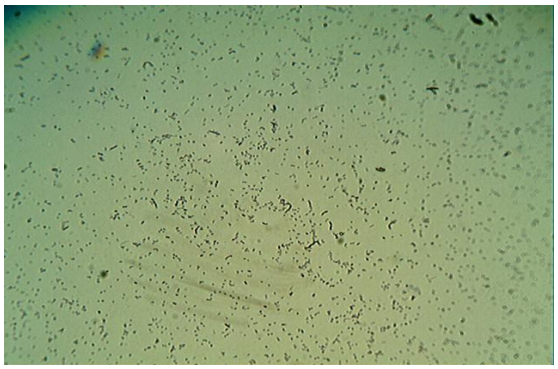

Figure 2(b): Figure showing gram negative short rods of *P. syringae*, isolated form *S. nigrum*

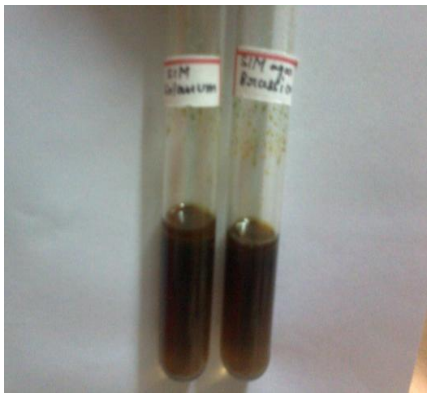

Figure 3(a): Figure showing the Indole production test results. The test tube on the left represents negative result for *P. syringae* (from *S. nigrum*) and the test tube on the right represents negative result for the coccobacillus (from *B. juncea).*

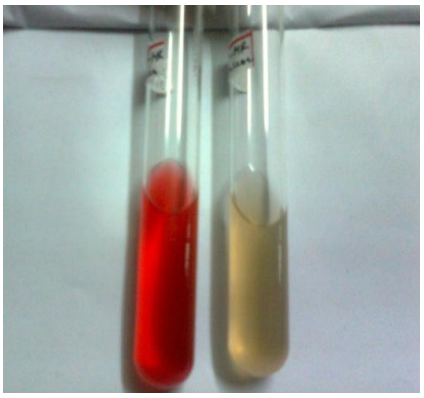

Figure 3(b): Figure showing Methyl Red test results. The test tube on the left represents positive result for the coccobacillus (from *B. juncea*) and the test tube on the right represents negative result for *P. syringae* (from *S. nigrum*).

Figure 3(c): Figure showing Citrate utilization test results. The test tube on the left represents positive result for *P. syringae* (from *S. nigrum*) and the test tube on the right represents negative result for the coccobacillus (from *B. juncea*).

Figure 4(a): Figure showing positive result for Koch's Postulates.

Figure 4(b): Figure showing positive result after cross-inoculation.

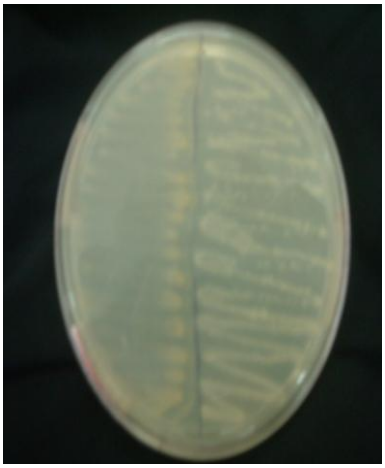

Figure 4(c): Figure Showing the petriplate where streaking (left side) and the cocobacillus (right side).

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