A Study on Compatibility of *Pseudomonas fluorescenes* L. and *Parthenium hysterophorus* L. as a Biocontrol agent to leaf spot by *Alternaria alternata* f. *sp. lycopersici* in Tomato

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Abstract

The weed, *Parthenium hysterophorus* (Congress grass) was found to be the world’s worst weed which, is taken along with *Pseudomonas fluorescens* to find out its biocontrol efficiency and to protect tomato plants from *Alternaria alternata* (leaf spot). Ten isolates of *Pseudomonas fluorescens* (AP1-AP10) were isolated from the tomato rhizosphere soil and its growth efficiency was determined in various parameters such as temperature, pH, carbon sources, nitrogen sources, amino sources and water. Secondary metabolites are produced by *Pseudomonas fluorescens* amended with various sugars, amino acids, nitrogen sources. Hot water extract was obtained from *Parthenium hysterophorus* and it was combined with *Pseudomonas fluorescens* in various concentrations and the secondary metabolites productions were evaluated. Antagonistic property was observed for isolates alone and also in the combinations of various concentrations of plant extracts against the plant pathogen *Alternaria alternata*. Green house studies were performed with various treatments such as root drenching, foliar spray, seed dressing and the combination of these treatments such as *Pseudomonas fluorescens* alone, *Parthenium hysterophorus* alone, *Pseudomonas fluorescens* and *Parthenium hysterophorus*, *Pseudomonas fluorescens* and pesticide, *Parthenium hysterophorus* and pesticide, *Pseudomonas fluorescens* and pesticide and control was maintained to all the treatments. Disease assessment was identified by giving challenge inoculation to the plants. The treatments given to the leaves were analysed by GCMS. Antifungal activity against *Alternaria alternata* and secondary metabolite production was effective in increasing concentrations of plant extracts. Among the different modes of application the combined mode was found to be most effective method based on morphological and physiological parameters of *Lycopersicon esculentum* in green house studies.

Key words: *Alternaria alternata*, Secondary metabolites, Antifungal activity, Pesticides, Drenching, Seed dressing.

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Introduction

Many factors are involved in low yield of tomato, among them are infestation by fungi, bacteria, nematodes or viruses and the competing weeds are predominant. Among the fungal diseases early blight, leaf spot and fusarial wilt are major. Both leaf spot and early blight can be controlled by using fungicides but it is costly. Alternaria alternata has a wide host range causing leaf spots and blights on many plant parts. Symptoms with dark brown to black cankers occur on foliage including leaves, petioles and stems. AAL produces host specific toxins (HST), AAL toxin which causes severe necrosis on stems and leaves in susceptible tomato cultivars, whereas resistant cultivars are tolerant and showed no symptoms. Alternaria alternata can also produce endo-polygalacturonase (endo-PG) and pectate lyase (PL) activities. These enzymes are responsible for the hydrolysis of pectic components of the plant cell wall. Due to the use of chemical pesticides, there is an increase in fungal resistance to the available chemical pesticides. Consequently some pest management has focused their effect on developing alternative inputs to synthetic chemicals for controlling pests and diseases. These alternatives based on microorganisms are referred as biological control agents.

Parthenium hysterophorus is otherwise called as bitter weed, false rugweed, fever few white top etc., which is an herbaceous ephemeral member of the asteraceae. It is an allelopathic weed and it inhibits the germination and growth of several crop plant and trees. The allelopathic potential of Parthenium hysterophorus weed result from the release of phytotoxic substances such as ferulic, caffeic, vanillic, chlorogenic, parthenin, ambrosin and coronopillin.

Fluorescent pseudomonads are able to improve plant health and are implicated in the natural suppressiveness of certain soils to many soil borne diseases. Some pseudomonas has been recognized as antagonist of plant pathogens and antibiotic producers. Siderophore, including salicylic acid, pyochelin and pyoveridine, which chelate the iron and other metals, also contribute the disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply essential trace minerals in natural habitats. These characteristics make Pseudomonas fluorescens as good candidate for using as seed inoculants and root dips for biological
control of plant pathogen. As a trial we have gone for Parthenium hysterophorus and Pseudomonas fluorescens as biocontrol substrates.

**Materials and Methods**

*Alternaria alternata* is a common pathogen of tomato plant. Infected leaf portion of *Alternaria alternata* that contains yellow necrotic spots after flowering stage were collected from the plant and it was surface sterilized with mercuric chloride. Then it was washed with sterile distilled water to remove the excess amount of mercuric chloride and the leaf was then placed in czapek dox agar plates and incubated at 28°C for 8 days. Lacto phenol cotton blue staining (LPCB) and microscopic studies were carried out for the confirmation of pathogen.

Suspected colonies from tomato rhizosphere soil samples obtained by serial dilution plating method with greenish agar nature were looked for further confirmation as Pseudomonas fluorescens. In Biochemical analysis, conventional tests like IMViC, Oxidase, Catalase, Nitrate reduction and carbohydrate fermentation were performed, expecting the cultures to be citrate, oxidase and catalase positive to confirm as *Pseudomonas fluorescens*. Further the growth of isolates AP1-AP10, were analysed by various parameters like temperature, pH, Carbon source, Nitrogen source and different water.

**Production of secondary metabolites in different Carbon, Nitrogen and Amino sources**

The antifungal metabolites produced by *Pseudomonas fluorescens* are salicylic acid, siderophore, indole acetic acid, hydrogen cyanide and enzymes such as lipase, protease and β-1, 3 glucanases. The standard succinate broth amended with different sugars, nitrogen sources and amino acids were inoculated with isolates (AP1-AP10) were incubated for 4 days and cells were separated by centrifugation at 8000 rpm for 10 min. All the harvesting procedures were carried out in dim light with samples maintained in covered ice baths. The SA was extracted from the acidified culture supernatant. The filtrate was adjusted to pH 2 with 1M HCl and extracted twice with double volume of ethyl acetate. To 1ml of the extraction, 2 ml of 2 M FeCl₃ and 1ml of distilled water were added. The SA reacts with 2 M FeCl₃ to form a purple iron +SA complex.
in the aqueous phase. The SA concentration in extraction was determined using a calibration curve of standard SA.

Cultures were grown for 40 hrs at 25°C with shaking (200 rpm) in 100 ml conical flasks. After incubation, the broth was centrifuged at 10000 rpm for 10 minutes and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 with 1N HCL and equal volume of ethyl acetate was added to it and mixed well and ethyl acetate fraction was collected. 5ml ethyl acetate fraction was mixed with 5ml of hathways reagent.

Isolates were inoculated in KMB liquid medium with carbon, nitrogen and amino sources for 3 days at 28°C at 220 rpm in a rotary shaker. The pH value of the broth was adjusted to 2.0 with 1 M HCl. Supernatant was collected after the broth was centrifuged at 10,000g for 10 minutes, and then it was extracted with equal volume of ethyl acetate with rigorous shaking. The collected organic layer was mixed with 1/10 volume distilled water and shaken rigorously. Finally, the organic phase was taken for evaporation under vacuum pressure.

The isolates were inoculated in nutrient broth with various carbon, nitrogen and amino sources were incubated at 28 ± 20°C for 1 week. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski’s reagent. The level of IAA produced was estimated by a standard IAA graph.

Log phase culture of the Pseudomonas fluorescens strain was added to Minimal media, incorporated with different substrates such as carbon, nitrogen and amino acid sources and incubated at 28±2°C with 150rpm for 72 hrs. Lipase activity was determined in an emulsifier free system using olive oil as substrate. Reactions were carried out in 100 ml conical plastic-stoppered flasks at 40°C by immersion in a water bath and shaking at 120 oscillations per min. The reaction mixture, consisting of 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, 1 ml of olive oil and 1 ml of culture supernatant, was incubated at 40°C for 30 min. The enzyme reaction was terminated by the addition of 5 ml of 96% ethanol and followed by titration with 0.05 N KOH solution using phenolphthalein as the indicator. One unit of lipase activity was defined as the amount which liberated 1µmole of fatty acid min⁻¹ at 40°C.
Maximum protease enzyme production was assayed using yeast extract casein medium incubating at 30°C for 48-72 h on shaker. At the end of fermentation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain the crude extract, which was used as enzyme source. Protein content was estimated by the method of Lowry et al., using bovine serum albumin as standard. β-1, 3 glucanases Crude enzyme extract of 62.5 ml was added to 62.5 ml of laminarin (4%) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 ml of dinitro salicylic acid and heated for 5 min. on boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with laminarin with zero time incubation served as blank.

**Preparation of aqueous extract from Parthenium hysterophorus**

Leaf of Parthenium hysterophorus was detached and it was surface sterilized with 1% mercuric chloride. Then it was mashed with mortar and pestil. Hot water extract was prepared by boiling 200g of the mixture/liter of water at 80°C for 10 minutes in hot water bath, the extract was filtered through muslin cloth and used as the standard stock solution.

The antifungal activity was studied against Alternaria alternata with Pseudomonas fluorescens (AP1-AP10) alone and in the combination of Parthenium hysterophorus in different concentrations from 5%, 10%....... 25% by dual culture method I and II.

Green House studies

**Pot preparation**

Red alfisol, sand and farmyard manure were mixed in a ratio 3:1:1 and they were subjected to complete sterilization. Sterilized soil was filled in pots and the tomato seeds were sowed. The temperature in the green house was maintained at 28 ± 20°C and the pots were adequately watered daily. The emergences of seedlings were recorded 7 days after sowing.

The treatments were given to sets of plants in the pots as Pseudomonas fluorescens alone (SET A), Parthenium hysterophorus alone (SET B), Pseudomonas fluorescens and Parthenium hysterophorus (SET C), Pesticide alone(SET D), Pseudomonas fluorescens and pesticide (SET E), Parthenium hysterophorus and pesticide (SET F), Parthenium hysterophorus, Pseudomonas fluorescens and pesticide (SET G) and control.
Application of Pseudomonas fluorescens alone (SET A) is done by: Seed treatment (A1), Root drenching (A2), Foliar application (A3), Seed treatment and root drenching (A1+A2), Seed treatment and foliar application (A1+A3), Root drenching and foliar application (A2+A3), Combination of seed treatment, root drenching and foliar application, (A1+A2+A3). The same is applicable for sets B, C, D, E, F and G.

In seed treatment the tomato seeds were surface sterilized with 0.02% of mercuric chloride and washed with distilled water to remove the traces. The seeds which are bacterized were sown in pots filled with soil. Temperature of the green house was maintained to 28 or 20˚C and adequately watered daily. Root dip method 10 days old plant was taken and the root was dipped in different culture suspension for 10-15 minutes and transplanted. Cells were harvested from mid-log phase culture in LB broth. These cells were resuspended in 10mm phosphate buffer at 7.0 and at a concentration of $10^8$ CFU/µl. The bacterial cells suspended in phosphate buffer were applied as foliar spray with a hand atomizer on to the 30 days old healthy tomato plants. After 24-48 hours the leaves were processed for analysis.

Physiological parameters

A Standard protocol was used for determining the total protein, carbohydrate and chlorophyll of treated leaves\textsuperscript{13}. Based on the morphological and physiological parameters leaf samples of tomato plants treated with Pseudomonas fluorescens alone (A1+A2), Parthenium hysterophorus alone (B1+B2), Pseudomonas fluorescens and Parthenium hysterophorus (C1+C2), Pesticide alone (D1+D2) and Control that had the combinations of seed drenching + root dip + foliar application (C) were analysed by GCMS.

Challenge inoculation

50 ml of CDA agar was prepared and inoculated with Alternaria alternata, and incubated in total darkness for 6-7 days. Then 10-15 ml of distilled water was added and shaken gently to liberate the conidia. It was filtered through a double layer of cotton to remove the mycelia bits. The concentration of conidia was determined by using haemocytometer. Spore suspension was mixed with tween 80 in 1% (polyoxy ethylene sorbitan monooleate) and sprayed onto 3 week old plants. It was sprayed with distilled water in the evenings, covered with plastic bags for 5
Consecutive nights of 16 hours and allowed to dry during the day to provide intermittent wetness for 80 hours. The temperature was maintained at 24±2°C.

**Results and Discussion**

In CDA agar plates *Alternaria alternata* colony was first grayish, white, wooly and later become greenish black or brown with a light border. It is eventually become covered by a short grayish, aerial hyphae. The reverse of the petri plate appeared black color. Thus with all macroscopic and microscopic evidences, it was confirmed as *Alternaria alternata*. From the 25 soil samples from different tomato rhizosphere soil, *Pseudomonas fluorescens* were isolated and they were named as AP₁, AP₂, and AP₃…AP₁₀. Isolates had maximum growth in temperature 35°C, pH 7.0, glucose sugar, yeast extract, threonine and tap water. According to De Meyer and Hofte, the optimal temperature assessed was 30°C but our isolates had maximum growth at 35°C because our isolates were indigenous population of tropical country soil that favors the optimum to 35°C. Other than temperature pH at 7.0, glucose as carbon source, threonine as an enhancing amino acid, tap water yielding maximum growth, yeast extract as best nitrogen source, were the results obtained that made to hold with the study of Sayyed et al.,

Various carbon, nitrogen and amino sources were tested for the production of salicylic acid was found to be higher in mannose, beef extract and histidine. Highest expression of SA levels in *Pseudomonas putida* were observed when they were grown on glucose, succinic acid, and citric acid; Similarly high level of HCN production in lactose, valine and peptone. HCN, a volatile metabolite is thought to play a major role in biological control of some soil borne and air borne diseases by significantly increasing the Peroxidase activity, that enhances the plants disease resistance potential; Siderophore production in maltose, beef extract and histidine. According to Barbeau et al., carbon and nitrogen sources greatly influence the siderophore production. Among the carbon and nitrogen sources, mannitol (2%) and glutamine (0.1%) were found to increase the siderophore production; Maximum level of auxin and phosphatase in mannitol, tryptophan and yeast extract. Secretion of growth promoting substances in the form of auxins and phosphatase along with other secondary metabolites will surely elevate growth of the plant. Synonymous findings were reported by Gupta et al.,
The maximum protease enzyme production was found in galactose, beef extract and isoleucine by AP$_5$ of 45.2, 47.7 and 47.9 U/ml respectively. The maximum lipase enzyme production was found in dextrose, peptone and lysine by AP$_5$ of 2.8, 4.9 and 5.1 U/ml respectively. According to Van Lon and Bakker,$^{22}$ the highest lipase activity was observed in plant supplemented with yeast extract and protease-peptone (5.58 U ml$^{-1}$), while the lowest lipase activity was obtained with tryptone + lactose (2.81 U ml$^{-1}$). It has been reported that some micro-organisms showed higher activities when grown in medium containing glucose. It creates strong support to our study that glucose, yeast extract are increasing lipase activity. Chitinase and glucanase induction in plants by PGPR's could help plants to digest the fungal cell wall, extending to the release of saccharides that result in the production of Phytoalexins$^{23}$. The maximum production of β-1, 3-Glucanase enzyme was found in the dextrose, beef extract and serine as 1.4 µMol/ml, 1.2 µMol/ml, and 1.2 µMol/ml respectively by AP$_5$.

Among the Pseudomonas fluorescens isolates tested AP$_4$ and AP$_8$ showed good inhibition activity for antifungal activity against with Alternaria alternata by dual culture method.$^I$. According to Rachna et al.,$^{24}$ antifungal activity of different strains of Pseudomonas fluorescens were tested against some plant pathogens such as Alternaria cajani, Curvularia lunata, Fusarium sp., Bipolaris sp. and Helminthosporium sp. in in vitro. Different concentrations (1000, 2000, 3000, 4000 and 5000 µg/mL) of Pseudomonas fluorescens were used and maximum spore germination of fungus was inhibited at 4000 and 5000 µg/mL. The result indicated that all the strains of Pseudomonas fluorescens presented a most significant value against Alternaria cajani and Curvularia lunata, which similar to our findings.

The aqueous extracts of Parthenium hysterophorus was more compatible with the antagonist Pseudomonas fluorescens (AP$_5$) at 20% among all other concentrations, by yielding maximum level of salicylic acid, siderophores and hydrogen cyanide. Similarly maximum level of growth promoting molecules, IAA and Phosphatase were produced of 8.0mg/l and 81µg/ml respectively. Compare to the production by antagonist alone, along with plant extract yielded high, which gives positive direction for opting the weeds as a substrate for biocontrol agent. Synchronously the identical results were obtained for protease, lipase, β-1,3 glucanase and
chitinase of 51 U/ml, 5.7 U/ml, 1.7 μMol/ml and 3.1 μMol/ml, remarkably a drastic increase in the level of defense enzymes that goes parallel to secondary metabolites and growth parameters but above the level of isolates alone.

The pots were prepared according to the formulations; based on delivery it is segmented into seven groups with replica of eight and each having seven types of formulations along with control. The tomato seeds were sowed and after 7 days of sowing, the percentage of seed germination was calculated. From the results it is evident that, morphometric parameters like, the maximum seed germination, root length, shoot length, root biomass, shoot biomass, no of primary roots, and leaf surface area were 100%, 24 cm, 15 cm, 0.35 g/plant, 0.25 g/plant, 13/plant and 11.03 sq.cm respectively (Table 1). All the best outcomes were obtained from both seed dressing and root drenching in the combination of Pseudomonas fluorescens (AP5) and Parthenium hysterophorus extract (20%) (Cp1+Cp2).

Table 1: Effect of Antagonistic Bacteria and Hazardous Weed extracts on Morphological Parameters of Lycopersicon esculentum (Tomato)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatments</th>
<th>Seed Germination (%)</th>
<th>Root length</th>
<th>Shoot length</th>
<th>Root biomass</th>
<th>Shoot biomass</th>
<th>No. of Primary Roots/plant</th>
<th>Leaf Surface area/ plant Sq.cm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>80</td>
<td>19</td>
<td>12</td>
<td>0.21</td>
<td>0.29</td>
<td>10</td>
<td>10.32</td>
</tr>
<tr>
<td>2</td>
<td>A1+A2</td>
<td>90</td>
<td>20</td>
<td>13</td>
<td>0.22</td>
<td>0.31</td>
<td>11</td>
<td>10.55</td>
</tr>
<tr>
<td>3</td>
<td>B1+B2</td>
<td>85</td>
<td>21</td>
<td>12</td>
<td>0.21</td>
<td>0.31</td>
<td>12</td>
<td>10.22</td>
</tr>
<tr>
<td>4</td>
<td>Cp1+Cp2</td>
<td>100</td>
<td>25</td>
<td>15</td>
<td>0.25</td>
<td>0.35</td>
<td>13</td>
<td>11.03</td>
</tr>
<tr>
<td>5</td>
<td>D1+D2</td>
<td>95</td>
<td>22</td>
<td>14</td>
<td>0.24</td>
<td>0.34</td>
<td>13</td>
<td>10.36</td>
</tr>
</tbody>
</table>

Estimation of biochemical components in test plants, treated especially by the combination of seed dressing and root drenching was, supporting evidence to the morphometric results with maximum level of total protein, carbohydrate and chlorophyll production by the SET (Cp1+Cp2) with values of 0.051, 0.042 and 0.13 mg/gm of leaf
respectively. The growth promoting effect of antagonistic bacteria and plant extract could be correlated with induction of defense enzymes and secretion of growth factors.

Fresh plant treated produced many compounds (Fig-1) like Cyclobutanol (RT-12.954), Glycine (RT-16.972), Etanamine (RT-23.441), N-Ethyl-N-Methyl (RT-30.659), Diethyl methylamine (RT-32.527) etc., where the most important and well-known compound was Cyclobutanol that induces resistance, followed by Benzenethanamine that increases the potential of the plant collectively and N-octadecane that give anti-oxidant property to plants.

**Fig - 1** GC MS analytical figure for tomato leaf extract of control plant.

Pesticide treated alone produced compounds (Fig-2) like Acetic acid (RT-4.60), Cyano-acetic acid (RT-6.52), Melonic Ketone (RT-14.19), Propanamine (RT-17.71) etc., acetic acid produced is an inducer of resistance, Propanamine is a pesticide product and Decan-1-ol is a factor that prevents tumor and necrosis. The plant was treated with Pseudomonas fluorescens (APs) that produces compounds (Fig-3) like Piperidine (RT-2.150), 2- Amino hexamine (RT-2.867), Butylcycloheptanone (RT-27.408), Tetradecane (RT-27.958) etc., among which Piperidine is having fungicidal activity on a wide range of phytopathogenic fungi. This Piperidine is a most convincing and concluding compounds that will play a key role when Pseudomonas fluorescens is adopted as a Biocontrol agent.
Parthenium hysterophorus and Pseudomonas fluorescens (Cp1+Cp2) has produced 12 compounds (Fig-5) which are Dodecanal (RT-2.11), N-Dodecanal (RT-2.134), 1-dodecanal (RT-2.991), Lauraldehyde (RT-6.50), Aldehyde C-12 (RT-17.71) etc., where 1-Dodecanol is a plant protection chemical that has been elicited. From above study 61 compounds were identified among which most of them are adopted for production of commercial pesticides and fungicides which is an emulating aspect based on SAR. Among 61, property of them is known only for 10 compounds that reveals the need for IR and NMR studies. It is puzzling where
property of so many compounds needs to be defined in a refined mode of study through obtaining their fractions.

Fig – 4 GC MS analytical graph for tomato leaf extract with *Parthenium hysterophorus* alone.

![GC MS graph](image)

Fig – 5 GCMS analytical graphs for tomato leaf extract with *Parthenium hysterophorus* and *Pseudomonas fluorescens* alone.

![GCMS graphs](image)

In supporting our view on biocontrol agents as more suitable one than commercial fungicides, a study by Pablo et al.,\textsuperscript{26} has revealed that application of Carbendazim inhibited pathogens related protein and enzymes Polyphenol oxidase (PPO), Peroxidase (POD). This effect of fungicides could be harmful to the resistance of tobacco plants to pathogen.
According to the study of Vann peer et al., there was increased accumulation of phytoalexins in stems of bacteria and inoculated plants compared with non-bacterial fungal-infected plants.

The severity of disease by Alternaria alternata was high with more than 70%, so it should be considered as a serious disease and there was an essential need to control it. But the plants treated with the combination of Pseudomonas fluorescens (AP5) and Parthenium hysterophorus extract (20%) (Cp1+Cp2) showed less disease scoring symptoms.

**Conclusion**

Comprehensive biochemical and genetic approaches are now starting to reveal the complex signaling pathways that mediate plant disease resistance. Initiation of defense signaling often involves specific recognition of invading pathogens by the products of specialized host resistance (R) genes. Both in-vivo and in-vitro morphological and physiological parameters of Lycopersicon esculentum in greenhouse studies supported this, which gives the direct evidence for the use of Pseudomonas fluorescens (AP5) and Parthenium hysterophorus amendments that benefits the weed and also clears the pathogen, Alternaria alternata. This may be a basic platform in preparing biofertilizers with Pseudomonas fluorescens, which can be used in the field to induce systemic acquired resistance in controlling diseases of many commercial crops.

**References**


