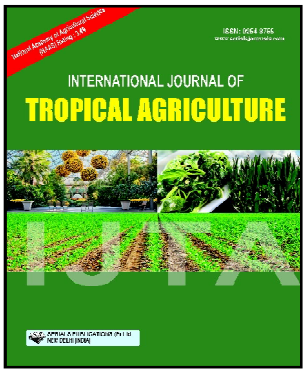

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Morphogenetic variability in *Colletotrichum capsici,* Genomic DNA isolation and PCR amplification of *Pseudomonas fluorescens* and biochemical characterization of brown seaweed algae and its efficacy on management of chilli anthracnose

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Abstract: Twenty (20) isolates of *Colletotrichum capsici,* the causal organism for fruit rot of chilli were collected from various chilli growing areas of Tamil Nadu. The genetic relationship between the 20 isolates of *C. capsici* was investigated using random amplified polymorphic DNA (RAPD) analysis and according to the Molecular polymorphism generated by RAPD the 20 isolates were grouped into two large clusters. No correlations were suggested between the isolates by the pathological and RAPD grouping. 10 different isolates of *Pseudomonas fluorescens* from various parts of Tamil Nadu were isolated and their identities confirmed by DNA sequence analysis. Among the selected seaweeds the antifungal compounds present in *Sargassum wightii* were evaluated by Gas Chromatography Mass Spectroscopy which revealed that, 6 compounds were present among which 9, 12-Octadecadienoic acid may have been responsible for the inhibition of the growth of *C.capsici.*

Key words: Chilli, *Colletotrichum capsici,* PCR amplification, RAPD, Biochemical characterization

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important spice crop of India. Among the several fungal, bacterial and viral diseases, affecting chilli, fruit rot caused by *Colletotrichum capsici* causes considerable damage, inflicting severe quantitative and qualitative losses. The loss due to this disease was estimated to range from 8 - 50% in different parts of India (Anand *et*

al., 2008). The fungus C*olletotrichum capsici* infects both unripe (green) and ripe (red) chilli fruits and survives on seed as acervuli (Chitra and Kannabiran, 2001). Infection of *C. capsici* is severe during mature fruit stage than in the early fruit stage. The fungal pathogen is both seed borne and air borne and affects the seed germination and vigour of the crop. Several fungicides have been reported to be effective in the management of fruit rot of chilli (Gopinath *et al*., 2006; Shovan *et al*., 2008). However, the indiscriminate use of fungicides leads to residual toxicity on the produce, development of fungicide resistance and also serves as a cause for environmental pollution (Suthin raj *et al.,* 2016b) and hence, there is an urgent need to develop alternative disease control measures. The organic control of plant pathogens is a potential alternative to the use of chemical pesticide (Jeyalakshmi and Seetharaman, 1999 and Hane graff and Suthin raj, 2018).

Plant growth promoting rhizobacteria *viz., Pseudomonas* spp and *Bacillus spp* have shown activity in suppressing the fungal infection in *invitro* and field applications (Domenech *et al*., 2005; Deshmukh *et al*., 2010; Ngullie *et al*., 2010). Seaweeds provide a rich source of antifungal compounds and biologically active secondary metabolites. Application of seaweed extracts is proved to be better to reduce the fungal diseases which ultimately increase its yield and help the growth of plants (Jayaraj *et al*., 2008 and Suthin raj *et al.,* 2018). Hence, various *in vivo* and *in vitro* experiments were conducted to evaluate the efficacy of different isolates of *P. fluorescens* and seaweeds against *C. capsici*.

MATERIALS AND METHODS

2.1. Survey of *Colletotrichum capsici*

A field survey was conducted to assess the extent of fruit rot occurrence of chilli in Tamilnadu state. The villages where chilli is traditionally grown are selected for assessing the prevalence of fruit rot disease caused by *Colletotrichum capsici*. Twenty locations representing rainfed and irrigated situations were selected for the survey. During survey plants affected due to fruit rot disease was found and also the total number of plants observed were counted and recorded. The percent disease incidence was worked out as per phytopathometry (Mayee and Datar, 1995). Also, the infected plants showing the typical symptoms of leaf spot and fruit rot due to infection with *C. capsici* were collected for isolation of the pathogen. Three replication were maintained for each treatment.

2.2. Collection and establishment of isolates of *C. capsici*

The diseased chilli fruits showing the typical symptoms of fruit rot disease were collected fresh from 20 conventional chilli growing areas of Tamilnadu. The pathogens isolated from each of these localities formed one isolate of *C. capsici*. The pathogens were isolated on potato dextrose agar (PDA) medium from the diseased specimen showing the typical symptoms. The infected portion of the fruit was cut into small bits, surface sterilized in 0.1 per cent mercuric chloride solution for 30 sec., washed in repeated changes of sterile distilled water and plated onto PDA medium in sterilized Petri dishes. The plates were incubated at room temperature (28 ± 2°C) for five days and were observed the fungal growth. The fungus was purified by single spore isolation technique (Rangaswami, 1958). Identification of the isolate was confirmed by comparing with the culture obtained from ITCC, IARI, New Delhi and the purified isolates were maintained on PDA slants for further studies.

2.3. Evaluation of pathogenicity of *C. capsici* isolates in pot culture condition

Five kilograms of top soil collected from chilli-growing field was steam pasteurized and filled in 30 cm diameter earthen pots. One month old seedlings of var. K. 2 were transplanted in pots. The spore

suspension (1×10^6 ml^{-1}) of *C. capsici* was prepared from 20 days old culture grown on PDA slants using sterile distilled water. Ninety day old plants were inoculated with spore suspension of *C. capsici* thoroughly over the plant canopy by pinpricking method. The inoculated plants were incubated in a growth chamber maintained at 28° C. The intensity of fruit rot was calculated as per cent disease index (PDI) as per the grade chart proposed by Ravinder Reddy (1982) using the formula proposed by Mc Kinney (1923).

Category value	*Per cent fruit area diseased*
0	0
1	1-5
2	6-10
3	11-25
4	26-50
5	51 and above

The per cent disease index (PDI) was calculated using Mc Kinney (1923) infection index.

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits observed}} \times \frac{100}{\text{Maximum category value}}$$

2.4. RAPD analysis of *C. capsici*

2.4.1. The isolation and identification of the pathogens from infected chilli

The Isolation and identification of the pathogens from infected chilli plants were described as earlier.

2.4.2. Genomic DNA isolation and PCR amplification of DNA (Suthin raj et al., 2012)

The total genomic DNA of *C. capsici* was isolated from mycelia. Isolates were incubated at 28°C for 4 days in tubes containing 20 ml of potato dextrose broth, agitated at 180 rpm. Mycelia were harvested by filtration through filter paper, dried between two layers of filter paper and stored at -80°C for further use. Dried mycelium was ground to fine powder with pestle and mortar using liquid nitrogen and transferred to 1.5 ml Eppendorf tube. 600 µl Cetyltrimetylammonium bromide (CTAB) was added and incubated at 65°C for 30 min, tubes were vortexed every 10 min. After cooling at room temperature equal volume (600 µl) of chloroform: isoamyl alcohol (24:1, v/v) was added in fume hood cabinet, gently mixed for 20-30 min and centrifuged at 7000 rpm for 5 min at 4°C. The aqueous phase was transferred to new tubes and repeat CIA extraction. After the second CIA wash, the DNA was precipitated by adding 300 l isopropanol, tubes were gently mixed and incubated at room temperature for 30 min. Tubes were centrifuged at 12000 rpm for 10 min and supernatant was decanted. The DNA pellet was dissolved in 50 µl of ddH_2O.

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) procedure described by Williams *et al.* (1990) was used by the following of a reaction mixture of 25 µl volume which consisting of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, dtp), 0.4 µl Taq DNA polymerase, 2.0 µl of primer, 1.0 µl of genomic DNA and 16.1 µl of sterilized double-distilled water. The reaction mixture was vortexed and centrifuged at 12000 rpm for 2 min. Amplification was carried out in a thermal cycle by using three temperature profiles, programmed for initial DNA denaturation at 94°C for 3 min, followed by 35 cycles consisting of DNA denaturation for 30 sec at 94°C, primer annealing at 35°C for 30 sec and polymerization for 1 min at 72°C with a final extension period of 10 min at 72°C. Amplification products were separated on 1.5% agarose gel in 1X TAE buffer at 110 V for about 3 h 100 bp + 1 kb DNA ladder mix (Fermentas) (0.5 µg/µl) was run for weight size comparison. Gels were stained with ethidium bromide for 30 min; they were visualized with UV light and photographed.

2.4.3. Analysis of RAPD profiles

Differences in fingerprinting patterns between isolates were assessed visually. Polymorphisms including faint bands that could be scored unequivocally were included in the analyses. Presumed homologous bands were scored as present (1) or absent (0) to create a binary matrix. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Cluster analysis was done with the Unweighed Pair Group Method with Arithmetic Mean (UPGMA) in the SAHN program of NTSYS-PC Package and a dendrogram was constructed based on genetic distances.

2.5. Evaluation of bacterial antagonists against *C. capsici, in vitro*

Ten isolate of *Pseudomonas fluorescens* obtained from the various areas of Tamilnadu*viz.,* (Tamilnadu Agriculture University -P.f_1), P. I_2 – Dharmapuri,P.I_3 – Marthandam, P.I_4 – Aduthurai, P.I_5 – Vallampadugai, P.I_6 – Vadalore, P.I_7 – Kovilpatti, P.I_8 – Annamalainagar, P.I_9 – Cuddalore, P.I_{10} – Sivapuri,(B.I_1) – Aduthurai Research Station, B.I_2 – Annamalainagar, B.I_3 –Sivapuri, B.I_4 –Vadalore and B.I_5 –Vallampadugai were tested for their antagonisticeffect on *C. capsici* by dual culture technique (Dennis and Webster, 1971).

P. fluorescens was multiplied on King's 'B' medium (20 g protease peptone, 1.5 g magnesium sulphate, 1.5 g dipotassium hydrogen phosphate, 15 g agar agar, 10 ml glycerol, 1000 ml distilled water and pH adjusted to 7.2). A 8 mm actively growing PDA culture disc of the pathogen was placed on PDA medium in sterilized Petri dish at one side 1.5 cm away from the edge of the plate, and incubated at room temperature (28 ± 2ºC). Forty eight h later, actively growing 48-h-old cultures of the respective test bacteria were separately streaked onto the medium at the opposite side of the plate, 1.5 cm away from the edge of the plate. The inoculated plates were incubated at room temperature (28 ± 2ºC). Three replications were maintained for each antagonist. Potato dextrose agar medium inoculated with the pathogen alone served as control. After 8 days the radial growth of the pathogen was measured. The results were expressed as per cent growth inhibition over control. The most effective isolates of *P. fluorescens* (P.I_1) was used for the further study.

2.5.1. PCR assay to identify P. fluorescens

Twenty isolates of *P. fluorescens* isolate from various parts of Tamilnadu and were grown in KB medium at 30°C for 48h and were routinely maintained at 4°C for long term maintenance, stock solution cultures were stored in 20% (v/v) glycerol (v/v) on an appropriate liquid medium at 20°C.

2.5.1.1. DNA extraction: Template DNA was prepared by boiling 200 l of bacterial suspension in milliQ (OD_{600}= 0.6) in safe lock Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000g x 10 min, 5°C; the supernatants were subsequently kept on ice or at-20°C. The microlitre of template DNA suspension was used foreach reaction.

2.5.1.2. PCR amplification: All the PCR were performed in a volume of 50 µl containing ≈50 to 100 ng of bacterial genomic DNA solution, 5 µl of 10 Ml PCR buffer, 200 µM of each dNTP; 2 mM of $MgCl_2$; 0.5 µM of each primer and 0.5 U of taq polymerase (obtained from Genei, Bangalore). DNA 16S specific region for *P. fluorescens* amplification was performedusing the primer set 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TGCATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3').The primer forward is species specific, while the reverse is family specific. The primers were developed and compared with partial regions 16S of *P. fluorescens* belonging to group 1 (NCBI, National Center for Biotechnology

Information) by software DNAsis 2.0 and the following thermal profile: 2 min at 94°C; 5 cycles consisting of 94°C for 45 s, 55°C for 1min, 72°C for 2min; 35 cycles consisting of 92°C for 45 s, 60°C for 45 s, 72°C for 2min; final extension of 72 °C for 2min; and final cooling at 4°C. The amplification was performed in a DNA thermal cycle (Yercaud Biotech, Salem).

Following amplification, 7 µl of product were analysed by electrophoresis at 100 V in (1% agrose gel 0.2 µg of ethidium bromide ml^{-1}) in TAE buffer. Photograph was taken with the Nikon COOLOIXSI0 VR Camera and Gel Documentation was done in DGelDAS Software analysis tool (Yercaud Biotech, Salem).

2.6. Evaluation of seaweed extracts against *C. capsici*

The efficacy of the various seaweeds listed in table was tested against *C. capsici*

Sl. No.	*Scientific Name*	*Anti-Microbial Property*	*Common Name*	*Collected from*
1.	*Sargassum muticum*	Phenol	Brown seaweed	Kanyakumari
2.	*Sargassum wightii*	Chloroform	Brown seaweed	Pamban
3.	*Caulerpaseal pelliformis*	Hexane	Green seaweed	Velankanni
4.	*Acanthophora spicifera*	Methanolic	Red seaweed	Pamban
5.	*Jania rubens*	Benzene	Red seaweed	Pondicherry

3.6.1. Preparation of seaweed extracts

3.6.1.1. Preparation of crude seaweeds extracts (Suthin raj et al., 2016a): Each 1 Kg of live, healthy and matured samples (Brown seaweeds, Green seaweeds and Red seaweeds) of each seaweed collected along the Coast of Pamban (Rameswaram (9°14'N; 79°14'E), Kanyakumari, Pondicherry, Velankanni and Gulf of Mannar, Tamil Nadu, India) were washed thoroughly in sea water followed by tap water to remove extraneous particles and epiphytes. Then they were air dried under shade in laboratory for 3 days. The shade-dried samples were chopped and pulverized. Each 50 g powdered sample was separately extracted for 7 days, thrice in 500 ml of 1:1(v/v) chloroform: methanol using a 1 litre Erlenmeyer conical flask under dark condition. The extractants were pooled and concentrated by using a flask evaporator under reduced pressure at 45°C, weighed and stored at 0°C.

2.6.2. Spore germination assay (Macko et al., 1977)

One drop of 5, 10, 15 and 20 per cent Seaweed extracts individually were placed in cavity slides and were allowed to air dry. A drop of the spore suspension (1×10^6spores ml^{-1}) of *C. capsici* prepared in sterile distilled water was added to each of the dried marine products and was thoroughly mixed. The prepared cavity slides were incubated in a moist chamber. Three replications were maintained for each treatment. The spore germination was observed and recorded after 48 h and the per cent germination was calculated. The spore suspension prepared in sterile distilled water served as the control.

2.6.3. Paper disc assay (Saha et al., 1995)

Spore suspension of the fungi was prepared from a ten days old culture with sterile distilled water. Various concentrations like 5, 10, 15and 20 per cent of seaweed extracts were made. Twenty ml of PDA medium was seeded with three ml of *Colletotrichum* suspension of the fungus and solidified. Sterile filter paper discs (10mm) were dipped separately in known concentration of treatments and placed equidistantly over the seeded medium. Three replications were maintained. The plates were incubated at 28±2ºC for 48 hr. The inhibition zone of the fungal growth

around the treated paper discs was measured and recorded. The paper disc dipped in sterile distilled water served as control.

2.6.4. Agar well method (Thongson et al., 2004)

Seaweed extracts like 5, 10, 15 and 20 per cent individually (10ml) were added to the sterilized potato dextrose agar medium and thoroughly mixed just before plating. Twenty ml of these mixtures individually were immediately poured into sterilized Petri plates and were allowed to solidify. A 9 mm of PDA disc was removed by using a cork borer to form wells; 1 ml of spore suspension was poured into a well. All these were carried out under aseptic conditions. The plates were incubated at 28±2°C for 10 days. Potato dextrose agar medium without natural product served as the control. Three replications were maintained. The radial growth of the colony was measured. The percent inhibition of the growth was calculated.

2.6.5. Identification of antifungal compounds

2.6.5.1. Analysis of antifungal compound through gas chromatography mass spectroscopy (GCeMS) (NIST Version. 2.0, 2005): Based on the growth inhibition studies, Seaweed extract was selected and chemical constituents were determined with a GC Clarus 500 Perkin Elmer Gas chromatography equipped with a mass detector. Turbo mass gold containing a Elite-1 (100% Dimethyl Poly Siloxane), 30 m × 0.25 mm ID employed were the following: Carrier gas, helium (1 mL/min); oven temperature program 110°C (2 min) to 280°C (9 min); injector temperature (250°C); total GC time (36 min). The water extract was injected into the chromatograph in 2.0 Ml aliquots. The major constituents were identiûed with the aid of a computer-driven algorithm and then by matching the mass spectrum of the analysis with that of a library (NIST Version. 2.0, year 2005). Software used for gas chromatography mass spectroscopy (GCeMS) was Turbo mass-5.1. This work was carried out in the IICPT, Tanjavur.

2.7. Evaluation of IDM *viz.*, antagonists, seaweed extracts, bacterial Biocontrol agents and fungicide for the management of fruit rot disease in the field condition.

The field trial was conducted at experimental farm, Annamalai University, Chidambaram, Cuddalore-Dist during December 2016 to March in 2017in a field with a history of chilli fruit rot incidence. The trials were laid out in plots (33 × 13 feet) arranged in a randomized block design. Thirty days old seedlings were planted in to the field plots in rows with row/plants spacing of 60 × 30 cm and a total population of 210 plants/plots. Three replicate plots were maintained for each treatment. Treatment application details and experimental observation were the same as in green house experiments with below mentioned treatment schedule. Regular cultivation practices were followed as per the recommendation. Three replication were maintained for each treatment.

2.7.1. Treatment details

T_1 – Application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT)

T_2 – Application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT)

T_3 – Application of *Caulerpaseal pelliformis* (prophylactic spray at 40 and 80 DAT)

T_4 – Application of *Jania rubens* (prophylactic spray at 40 and 80 DAT)

T_5 – $T_1 + T_2$

T_6 – $T_1 + T_3$

T_7 – $T_1 + T_4$

T_8 – Seed treatment with mancozeb + spraying 50 and 75 DAT

T_9 – Control

2.8. Experimental design and data analysis

The experiments were conducted using completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by Duncan's multiple range test (DMRT). Whenever necessary, the data were transformed before statistical analysis following appropriate methods.

3. RESULTS

3.1. Survey of fruit rot disease incidence

The data presented in Table 1 on the survey in different locations in Tamilnadu revealed the prevalence of fruit rot disease in all the villages surveyed. Among the different locations of Tamilnadu surveyed for fruit rot disease incidence, Kovilpatti (Cc1) registered the maximum incidence of the disease (19%) followed by Sattur (Cc2) with (17%). The other locations *viz.,* Villupuram (3.0%) and Naduthittu (4.0%) had lesser diseases incidence.

3.2. Pathogenicity of *C. capsici* on chilli

The isolate Cc1 was significantly the most virulent one which recorded the highest fruit rot intensity (69.8 PDI) and leaves infection (62.0 PDI). This was followed by Cc4 (67.4 PDI of fruit rot and 59.4 PDI of leaf infection), Cc2 (60.6 PDI of fruit rot and 57.8 PDI of leaf infection) and Cc3 (59.2 PDI of fruit rot and 56.0 PDI of leaf infection) while Cc12 and Cc15 were the least virulent one, which recorded 4.0, 4.0 PDI of fruit rot and 5.6, 5.6 PDI of leaf infection respectively.

Table 1
Survey of disease incidence of chilli *C. capsici* in different locality of Tamilnadu

S. No.	*Locality*	*Crop stage*	*Variety*	*Disease Incidence (%)*
1.	Cc1 – Kovilpatti	Fruiting	K-2	18* [a]
2.	Cc2 – Sattur	Fruiting	K-2	16[b]
3.	Cc3 – Vallampadugai	Vegetative	K-1	5.2[g]
4.	Cc4 – Sivapuri	Fruiting	CO-1	5.0[g]
5.	Cc5 – Naduthittu	Vegetative	CO-1	4.0[h]
6.	Cc6 – Perambalore	Fruiting	MDU-1	12.0[c]
7.	Cc7 – Thirunelveli	Fruiting	CO-1	9.2[e]
8.	Cc8 – Rajapalayam	Fruiting	Palur	10.0[i]
9.	Cc9 – Theni	Fruiting	Palur	8.1[f]
10.	Cc10 – Virurdhunagar	Fruiting	K-2	15.0[b]
11.	Cc11 – Ariyalur	Fruiting	Juwala	11.5[d]
12.	Cc12 – Pollachi	Fruiting	Palur	8.2[f]
13.	Cc13 –Sankarankovil	Vegetative	K-2	12.5[c]
14.	Cc14 – Periyakulam	Fruiting	Palur	8.0[f]
15.	Cc15 – Thirumangalam	Vegetative	Juwala	6.0[g]
16.	Cc16 – Musiri	Fruiting	MDU-1	7.5[f]
17.	Cc17 – Paramakudi	Fruiting	K-1	6.5[g]
18.	Cc18 – Viluppuram	Vegetative	CO-1	3.0[h]
19.	Cc19 – Dharmapuri	Fruiting	K-1	7.8[f]
20.	Cc20 – Aruppukottai	Fruiting	K-2	9.0[e]

* Values in the column followed by common letters do not differ significantly by DMRT (P=0.05)

Table 2
Evaluation of pathogenicity of *C. capsici* isolates in pot culture condition

S. No.	*Isolates*	*Per cent fruits infected*	*PDI*	*Per cent leaves infected*	*PDI*
1.	Cc1	87.00* [a] (4.47) **	69.8	78.00[a] (4.36)	62.0
2.	Cc2	76.00[a] (4.34)	60.6	72.00[a] (4.31)[a]	57.8
3.	Cc3	74.00[a] (4.31)	59.2	70.00[a] (4.26)	56.0
4.	Cc4	84.00[a] (4.44)	67.4	74.00[a] (4.28)	59.4
5.	Cc5	70.00[a] (4.26)	56.0	66.00[b] (4.20)	52.8
6.	Cc6	44.00[c] (3.80)	35.2	60.00[b] (4.11)	48.0
7.	Cc7	68.00[b] (4.23)	54.4	55.00[b] (4.02)	44.0
8.	Cc8	64.00[b] (4.17)	51.2	60.00[b] (4.11)	48.0
9.	Cc9	68.00[b] (4.23)	35.2	60.00[b] (4.11)	48.0
10.	Cc10	72.00[a] (4.29)	57.6	69.00[a] (4.24)	55.2
11.	Cc11	44.00[c] (3.80)	35.2	40.00[c] (3.71)	32.0
12.	Cc12	4.00[g] (1.49)	4.0	6.00[g] (1.84)	5.6
13.	Cc13	72.0[a] (4.29)	57.6	66.00[b] (4.20)	52.8
14.	Cc14	6.00[f] (1.86)	4.8	8.00[f] (2.11)	6.4
15.	Cc15	4.00[g] (1.51)	4.0	6.00[g] (1.86)	5.6
16.	Cc16	20.00[e] (3.04)	16.0	16.00[e] (2.82)	12.8
17.	Cc17	76.00[a] (4.34)	60.8	72.00[a] (4.29)	57.6
18.	Cc18	30.00[d] (3.43)	24.0	22.00[d] (3.13)	17.6
19.	Cc19	62.00[b] (4.14)	49.6	60.00[b] (4.11)	48.0
20.	Cc20	52.00[c] (3.96)	41.6	18.00[e] (2.93)	14.4

***** Values in the column followed by common letters do not differ significantly by DMRT (P=0.05),

*** *** Figures with in parenthesis are arcsin transformation

3.3. RAPD analysis of *C. capsici*

Among 20 random 10-mer primers, 6 primers exhibiting maximum polymorphism (Table A) produced easily scorable and consistent banding patterns by RAPD analysis, producing 4 to 8 bands of 0.3 to 2.5 Kb and the dendrogram was drawn from the RAPD patterns using UPGMA (Unweighed Pair Group Method with Arithmetic Mean).

The RAPD pattern obtained with primer code S1027 Base sequence ACGAGCATGG is shown in the Fig. 1 and 2. According to the Dendrogram Fig. 3, *C. capsici* can be divided into four main groups.

Table A
Nucleotide sequence of primers generating amplification of *C. capsici*

Primer code	*Base sequence (5'-3')*
S1027	ACGAGCATGG
S1063	GGTCCTACCA
S1089	CAGCGAGTAG
S1136	GTGTCGAGTC
S1155	GAAGGCTCCC
S1181	GACGGCTATC

The first group includes one isolate which was isolated from Sivapuri. The second group contains

other three isolates of *C. capsici* which were isolated from Virurdhunagar, Naduthittu and Sattur respectively. Third group contained other eleven isolates of *C. capsici* which were isolated from Thirumangalam, Periyakulam, Sankarankovil, Paramakudi, Aruppukottai, Rajapalayam, Pollachi, Ariyalur, Vallampadugai, Perambalore and Thirunelveli respectively. The last group includes five isolates which were isolated from Kovilpatti, Viluppuram, Dharmapuri, Musiri and Theni respectively. The RAPD analysis conducted in this study showed less variation between the groups. The results of the study support the hypothesis that variability exist at molecular level among the *C. capsici* isolates. There is no congruence between the RAPD pattern and the pathogenicity pattern of the test isolates. Isolates that was identical for pathogenicity most often dissimilar for RAPD markers. The clustering in the RAPD dendrogram was not associate with the pathogenicity, from which the isolates were obtained as two large clusters (3, 4) containing isolates from the all the pathogenicity pattern (Fig.3).

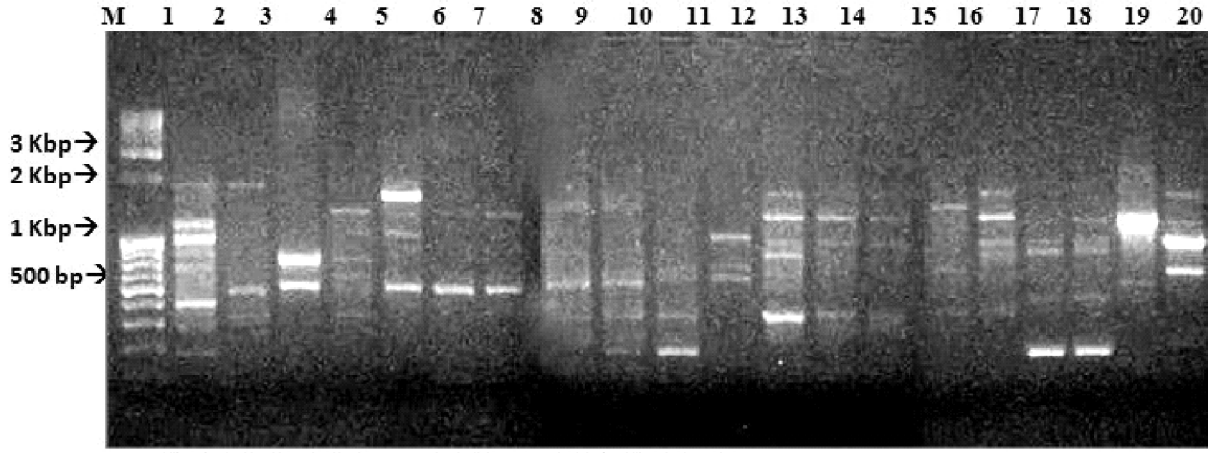

Figure 1: RAPD Profile for Primer No: RFu D

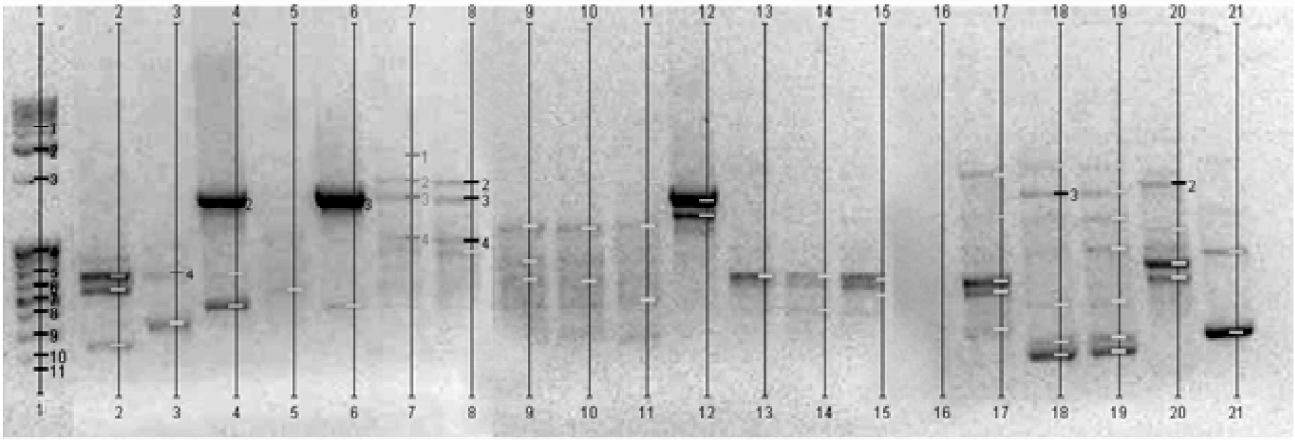

Figure 2: RAPD Pattern Analysis

Phylogeny (Neighbor Joining Method)

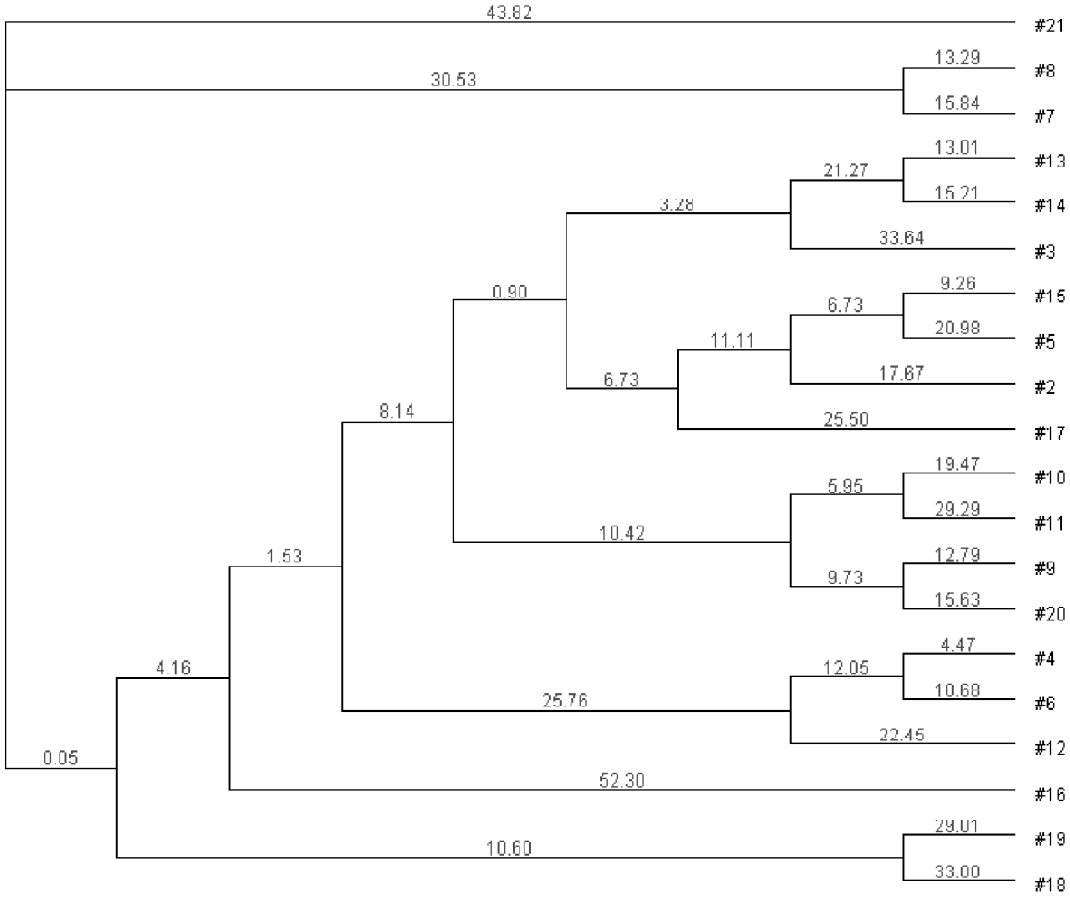

Figure 3: Dendrogram showing Phylogenetic analysis using RAPD Marker Data of Colletotrichum samples

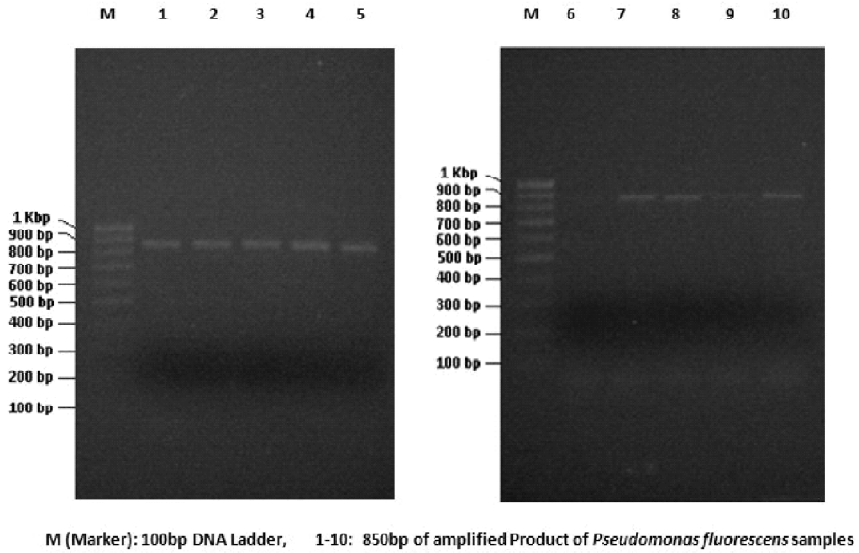

Figure 4: PCR Analysis of Pseudomonas fluorescens

Table 3

Evaluation of various isolates of *P. fluorescens* against *C. capsici* by dual culture technique

S. No.	*Isolates*	*Linear growth (mm)*		*% Growth inhibition*	*Mycelial dry weight (mg/50 m/broth)*				
		Antagonist	*C. capsici*		*10%*	*20%*	*30%*	*40%*	*Mean*
1.	*Pseudomonas fluorescens* (P. I_1) (TNAU-P.f_1)	68.00	22.00	76.11* [a] (4.33) **	240 (5.56)	180 (5.30)	102 (4.65)	18 (3.13)	5.66[a]
2.	P. I_2 – Dharmapuri	60.60	29.40	67.32[b] (4.22)	305 (5.72)	228 (5.43)	114 (4.74)	30 (3.43)	4.83[bc]
3.	P. I_3 – Marthandam	53.60	36.40	59.11[d] (4.05)	326 (5.79)	235 (5.46)	119 (4.78)	34 (3.55)	4.89[b]
4.	P. I_4 – Aduthurai	51.20	38.80	56.89[e] (4.50)	338 (5.82)	249 (5.52)	123 (4.82)	37 (3.63)	4.94[b]
5.	P. I_5 – Vallampadugai	54.50	35.50	60.55[d] (4.11)	330 (5.80)	239 (5.48)	121 (4.80)	36 (3.61)	4.92[b]
6.	P. I_6 – Vadalore	62.00	28.00	68.88[b] (4.24)	282 (5.64)	224 (5.41)	111 (4.71)	34 (3.55)	4.83[bc]
7.	P. I_7 – Kovilpatti	56.33	33.67	62.58[c] (4.15)	313 (5.74)	231 (5.44)	116 (4.76)	32 (3.49)	4.86[b]
8.	P. I_8 – Annamalainagar	66.70	23.30	74.10[a] (4.31)	260 (5.58)	200 (5.34)	109 (4.68)	25 (3.32)	4.72[ab]
9.	P. I_9 – Cuddalore	48.72	41.28	54.13[f] (4.00)	354 (5.87)	256 (5.54)	136 (4.91)	40 (3.70)	5.01[cd]
10.	P. I_{10} – Sivapuri	49.60	40.40	55.11[f] (4.02)	351 (5.86)	248 (5.51)	132 (4.89)	39 (3.68)	4.98[c]
11.	Control		90.00		540 (6.29)	540 (6.29)	540 (6.29)	540 (6.29)	6.29[d]
	Mean				5.79[d]	5.52[c]	4.91[b]	3.76[a]	

* Values in the column followed by common letters do not differ significantly by DMRT (P=0.05)

* * Figure*s with in parenthesis are arcsin transformation.

3.4. Isolation and identification of bioagents

A total of 10 isolates of *P. fluorescens* were isolated from rhizosphere soil of chilli in various part of Tamilnadu. All the collected isolates of *P. fluorescens* were identified and purified using streak method. After purification they were maintained as stock culture in kings B slant at 10°C (Table 3).

3.4.1. In vitro *evaluation of* P. fluorescens *against* C. capsici

The results of the screening of 10 isolates of *P. fluorescens* against *C. capsici* on PDA plates are presented in Table 3. Among the isolates *P. fluorescens* PI-1was appear to be most effective against the test pathogen showing 76.11 per cent inhibition of colony growth and minimum mean mycelial growth of pathogen (4.33 cm). It was followed by isolate PI-8 showing 74.10 per cent inhibition and minimum mean mycelial growth of pathogen (4.31), which where statistical on par each other. The isolate PI-9 and PI-10 gave minimum growth inhibition and maximum mycelial growth of pathogen. All the isolates significantly reduce the mycelial growth of the pathogen over the control.

3.4.2. Mycelial growth

The mycelial dry weight of the pathogen was also recorded on10, 20, 30 and 40 per cent concentration of culture filtrate of antagonistic. All the treatments were effective to reduce the growth of the pathogen. However, the treatment *P. fluorescens* isolate 1 maximum reduces the growth of 240, 180, 102 and 18 mg/50 ml broth at 10, 20,30 and40 per cent conc. respectively. Followed by *P. fluorescens* isolate 6 and *P. fluorescens* isolate 8 with growth of 260, 210, 109, 25 and 282, 224, 111 and 34 mg/50ml/broth respectively (Table 3). The results of the experiment showed the superiority of *P. fluorescens* PI-1 and hence the same was used for subsequent studies.

3.4.3. PCR assay to identify P. fluorescens

From the primer sets 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TG CATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3' used to amplify the DNA 16S specific region of *P. fluorescens* the size of the amplified single DNA fragment obtained was about 850 bp of 16SrRNA (Fig. 4).

3.5. Evaluation of various seaweed algae against *C.capsici*

3.5.1. Spore germination

Among the five brown seaweed extracts tested against *C.capsici,* extracts of *Sargassum wightii* at a high concentration (20%) was found to be the best in the reduction of spore germination (29.83 per cent). It was followed by a high concentration (20%) of *Jania rubens* (32.11 per cent). The rate of reduction was corborated with its concentration in case of all the tested brown seaweed extracts. *Sargassum wightii* and *Jania rubens* significantly reduced spore germination than other brown seaweed products in all the concentrations (Table 4).

3.5.2. Paper disc method and Agar well method

Various brown seaweed extracts were evaluated for their antimicrobial activities by paper disc and agar well method. The leaf extracts of *Sargassum wightii* at 20% conc. recorded 48.16 and 49.66 per cent inhibition zone in Paper Disc and Agar Well Method respectively followed by *Jania rubens* which recorded 46.15 and 47.50 per cent inhibition zone respectively (Table 4).

The result of the experiments revealed the superiority of *Sargassum wightii.* Hence the same was used for further studies.

3.5.3. Gas Chromatography Mass Spectroscopy (GCeMS) analysis

On the basis of performance the nature of chemical compounds present *Sargassum wightii* (Brown

Table 4
Evaluation of various seaweed algae against *C.capsici* under *in vitro* condition

S. No.	Seaweed	Spore germination (%)					Inhibition zone (mm)									
							Paper disc method					Agar well method				
		5%	10%	15%	20%	Mean	5%	10%	15%	20%	Mean	5%	10%	15%	20%	Mean
1	*Caulerpaseal pelliformis*	44.72	41.14	39.31	35.13	40.08^{c}	33.17	36.15	40.11	43.11	38.14^{c}	30.70	32.60	35.50	44.50	35.83^{c}
2	*Jania rubens*	41.12	39.23	36.73	32.11	37.30^{b}	37.11	39.13	43.02	46.15	41.35^{b}	32.00	34.80	37.80	47.50	38.03^{b}
3	*Acanthophora spicifera*	49.92	46.12	44.05	42.11	45.55^{e}	27.18	30.11	35.12	41.13	33.39^{e}	27.50	29.16	32.14	36.30	31.28^{e}
4	*Sargassum wightii*	38.20	36.12	32.52	29.83	34.17^{a}	40.12	42.02	45.11	48.16	43.85^{a}	34.50	36.15	40.41	49.66	40.18^{a}
5	*Sargassum muticum*	47.19	43.63	41.02	39.15	42.75^{d}	30.12	33.14	38.19	40.13	35.40^{d}	28.50	30.11	33.12	41.11	33.21^{d}
6	Control	96.00	96.00	96.00	96.00	96.00^{f}	0.00	0.00	0.00	0.00	0.00^{f}	0.00	0.00	0.00	0.00	0.00^{f}

* Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

seaweed) was determined. The results revealed that 6 compounds *viz.,* Asarone, Dihydroxanthin, Digitoxigenin, 6,9,12,15- Docosatetraeniic acid, methyl ester, cis- Vaccenic acid and 9, 12-Octadecadienoic acid were present in *Sargassum wightii*. The molecular weights, name of the compound, chemical formula, retention time and peak area percentage are given in fig 5 and 6. Among these, since the retention time, peak area and the copper nature of the compound, 9, 12-Octadecadienoic acid may have been responsible for the inhibition of the growth of *C.capsici*.

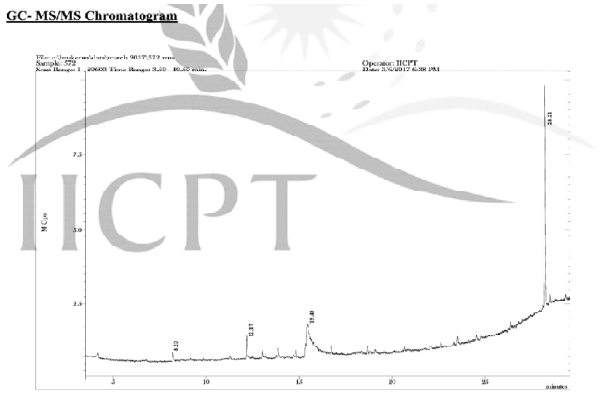

Figure 5: GC-MS analysis of *Sargassum wightii* (brown seaweed) extract

No.	RT	Name of the compound	Molecular Formulae	Molecular Weight	Peak Area %
1.	4.15	Asarone	$C_{12}H_{16}O_3$	208	0.11
2.	6.52	Dihydroxanthin	$C_{17}H_{24}O_5$	308	0.09
3.	10.30	Digitoxigenin	$C_{23}H_{34}O_4$	374	0.91
4.	10.80	6,9,12,15-Docosatetraenoic acid, methyl ester	$C_{23}H_{38}O_2$	346	0.25
5.	11.94	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	57.30
6.	13.83	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280	41.33

*Parameters tested are not covered under the scope of NABL accreditation

Figure 6: Compound identified in the *Sargassum wightii* (brown seaweed)

3.6. Effect of IDM formulation on fruit rot incidence under field condition

Efficacy of different IDM formulations was tested against fruit rot incidence under field condition. The fruit rot incidence was recorded at regular intervals and presented in Table 5. The application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT) plus application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT) (T_5) significantly reduce the fruit rot incidence of 68.71, 55.61 and 52.35 per cent increase over control at 100, 125 and 150 days after planting. Secondly, the seed treatment with mancozeb + spraying 50 and

Table 5
Effect of IDM formulation on fruit rot incidence under field condition

Treatments	*Fruit rot incidence on 100th day*	*% increase over control*	*Fruit rot incidence on 125th day*	*% increase over control*	*Fruit rot incidence on 150th day*	*% increase over control*
T_1 – Application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT)	4.48* [f]	42.56	8.50[c]	41.78	16.02[d]	34.93
T_2 – Application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT)	2.75[b]	64.74	6.92[b]	52.60	13.08[bc]	46.82
T_3 – Application of *Caulerpaseal pelliformis* (prophylactic spray at 40 and 80 DAT)	4.10[e]	47.43	7.85[bc]	46.23	13.72[bc]	44.27
T_4 – Application of *Jania rubens* (prophylactic spray at 40 and 80 DAT)	4.60[f]	41.02	8.57[c]	41.30	17.07[e]	30.66
$T_5 – T_1 + T_2$	2.44[a]	68.71	6.48[a]	55.61	11.72[a]	52.35
$T_6 – T_1 + T_3$	3.15[c]	59.68	7.06[bc]	51.64	14.11[cd]	42.68
$T_7 – T_1 + T_4$	3.45[cd]	55.76	7.96[c]	45.47	14.98[c]	39.10
T_8 – Seed treatment with mancozeb + spraying, 30 and 45 DAT	2.68[b]	65.64	6.89[b]	52.80	12.36[b]	49.75
T_9–Control	7.80[h]		14.60[e]		24.60[g]	

* In a row under each treatment, value in the column followed by common letters do not differ significantly by DMRT (P= 0.05), Three replication were maintained for each treatment.

75 DAT (T_8) recorded 65.64, 52.80 and 49.75 per cent increase over control on 100, 125 and 150 days respectively

3.7. Effect of IDM formulation on growth and yield attributes under field condition

All the treatments have significantly enhanced the growth and yield parameters of chilli, when compare control. The application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT) plus application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT) (T_5) recorded the mean plant height (109.55 cm) number of flowers/plant (188 nos), mean number of fruits/plant (112 nos), mean fruit length (9.01cm) and fruit yield (380 g/plant) Next, the seed treatment with mancozeb + spraying 50 and 75 DAT (T_8)with mean plant height (108.35 cm), number of flowers/plant (186 nos), mean number of fruits/plant (111 nos), mean fruit length (9.01 cm) and fruit yield (380 g/plant) (Table 5).

DISCUSSION

In the present study, The application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT) plus application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT) (T_5) treated chilli plants significantly reduced the incidence of *C. capsici*. Combined application of IDM formulation (viz., *P. fluorescens* and seaweed extracts, and

Table 6
Effect of IDM formulation on growth and yield attributes under field condition

Treatments	*Mean plant height (cm)*	*Mean no. of flowers/ plant*	*Mean no. of fruits/ plant*	*Mean fruit length (cm)*	*Fruit yield (g/plant)*
T_1 – Application of *P. fluorescens* (Seed treatment + prophylactic spray at 20 and 60 DAT)	100.66^{f}	168^{e}	96^{d}	7.22^{d}	354^{d}
T_2 – Application of *Sargassum wightii* (prophylactic spray at 40 and 80 DAT)	101.54^{f}	162^{f}	95^{d}	6.91^{e}	350^{d}
T_3 – Application of *Caulerpaseal pelliformis* (prophylactic spray at 40 and 80 DAT)	103.11^{e}	176^{c}	105^{cd}	7.01^{d}	360^{c}
T_4 – Application of *Jania rubens* (prophylactic spray at 40 and 80 DAT)	105.00^{c}	183^{b}	106^{c}	8.82^{b}	368^{c}
$T_5 – T_1 + T_2$	109.55^{a}	188^{a}	112^{a}	9.01^{a}	380^{a}
$T_6 – T_1 + T_3$	107.00^{bc}	184^{bc}	109^{b}	8.87^{b}	372^{b}
$T_7 – T_1 + T_4$	104.20^{d}	178^{c}	103^{c}	8.48^{bc}	369^{c}
T_8 – Seed treatment with mancozeb + spraying, 30 and 45 DAT	108.35^{ab}	186^{b}	111^{ab}	8.97^{ab}	376^{b}
T_9 – Control	100.54^{f}	161^{g}	90^{e}	6.01^{e}	349^{e}

* Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).Three replication were maintained for each treatment.

mancozeb) effectively control the disease incidence than the individual application of above components. Same phenomenon was observed by Vijay Pal *et al.* (2008), who reported that the minimum disease incidence (23.33 per cent) was recorded, with consortia of bio agents (*P. fluorescens*) + plant extracts + carbendazim compared with 60.22 per cent disease control over the untreated or water sprayed check. Similar conclusions on the management of plant diseases by different pseudomonads strains either as bacterial suspension or through different formulations have been reported by many workers (Viswanathan and Samiyappan, 1999).

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