Original Research Article

Isolation and characterization of plant growth promoting rhizobacteria *Enterobacter hormaechei* and their suppression efficacy against *Colletotrichum falcatum* combination with chitosan

ABSTRACT

Aims: This study aimed to explores the suppression efficacy of plant growth promoting rhizobacteria (PGPR) *Enterobacter hormaechei*, chitosan and its oligomers either singly or in combination on red rot disease causing pathogen *Colletotrichum falcatum* in sugarcane.

Methodology: The study was conducted to isolate twenty nine PGPRs from sugarcane rhizosphere and investigate their potential for plant growth activities. Isolated PGPRs were characterized by biochemical and molecular identification by 16S rRNA sequencing. The study was further preceded for *in vitro* screening of plant growth promoting traits viz., production of indole acetic acid (IAA), hydrogen cyanide (HCN) ammonia (NH_3) production and antifungal activity against *C. falcatum*.

Results: Twenty nine PGPRs were isolated from sugarcane rhizosphere and characterized by biochemical and molecular identification. Five isolates PSC1, PSC3, KSC1, P13 and P35 showed highest plant growth promoting traits viz., indole acetic acid, hydrogen cyanide, ammonia production and antifungal activity against *C. falcatum*. Nucleotide 16S rRNA sequence analysis using clustalW program revealed that isolate PSC3 showed phylogenetic affiliation and maximum homology (99%) with *E. hormaechei*. *In vitro* assays, chitosan and chitooligosaccharides (COS) caused differential growth inhibition. Among three treatments of chitosan, COS and chitosan + *E. hormaechei*, two treatments showed significant antifungal activity ($P<0.05$). Chitosan treatment showed radial growth range from 2.53±0.07 to 1.87±0.03 cm against *C. falcatum* in comparison with control (9.13±0.09cm). The significant growth inhibition 79.6% was observed in chitosan at concentration 0.6% but the combination of chitosan with PGPR *E. hormaechei* PSC3 showed highest growth suppression of *C. falcatum* (86.6%) whereas fungal treated with only *E. hormaechei* showed growth radial inhibition 41.3%.

Conclusion: This research work explores new antifungal combination to overcome on red rot disease of sugarcane using PGPR and chitosan.

Keywards: *Enterobacter hormaechei*; *Colletotrichum falcatum;* Sugarcane; Chitosan, Chitooligosaccharides; Antifungal activity.

1. INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are plant-associated microorganisms that are known to induce plant defenses and confer beneficial effects such as increased plant growth and low susceptibility to diseases caused by pathogens[1]. Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers[2]. PGPR have been proven to counteract the activities of other harmful soil borne microorganisms, thus promoting plant growth[3]. Some PGPRs also elicit physical or chemical changes related to plant defense, a process called ‘‘induced systemic resistance’’ (ISR)[4]. Although it is well known that ISR triggered by PGPR confers resistance against pathogen-induced plant diseases[5].

The red rot caused by *Colletotrichum falcatum* Went is the most ruinous disease of sugarcane and a big menace to both cane growers and sugar industry[6]. Conventional control of disease depends on the use of chemical inputs and resistant varieties. Development of new variants of the fungus, health hazards and environmental pollution concerned with the excessive use of agro-chemicals have resulted in adopting the biological control using native strains of PGPRs as a supplemental approach to minimize pesticide usage[7]. Certain strains of PGPRs have been used as ingenious weapon to protect plants from various soil borne pathogens. These bio-antagonists adopt single or multiple mechanisms of action to suppress these pathogens which include antibiosis[8], production of iron chelators, secretion of hydrolytic enzymes, synthesis of hydrogen cyanide thus disease control can be obtained by applying bacterial cells or their metabolic products[9].

Chitosan is derived from chitin, a polysaccharide found in exoskeleton of shellfish such as shrimp, lobster or crabs and cell wall of fungi[10]. Chitosan, poly (1, 4)-2-amino-2-deoxy-β-D glucose is a deacetylation product of chitin, a polysaccharide second by the prevalence in nature after cellulose[11,12]. It is a nontoxic, biodegradable biopolymer of high molecular weight. Recent studies on chitosan have attracted interest for converting chitosan to oligosaccharides[13]. In this respect, chitosan oligosaccharides, because of their shorter chain length, display a reduced viscosity and are soluble in aqueous media at pH values close to neutrality, which increases their bioavailability and opens a wide

range of new potential applications[14]. Due to its properties, various studies shown that chitosan has antifungal and antibacterial activities in different diseases [15, 16].

In view of this, the focus of the work presented in this paper is directed towards isolation and identification of PGPRs from sugarcane rhizosphere. Subsequently, *in vitro* screening of the potential antagonists that control red rot disease causing pathogen. Further, this research work proceeded to check antifungal activity of chitosan and their combination with *E. hormaechei*. Therefore such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers association with chitosan is an efficient approach to replace fungicides.

2. MATERIALS AND METHODS

2.1 Processing of soil samples for isolation of Phosphate solubilizing microrganisms

Phosphate solubilizing rhizobacteria (PSB) were isolated from sugarcane rhizospheric soil by dilution plate technique using Pikovskaya's medium[17]. Appropriate soil dilutions were plated on Pikovskaya's agar medium by spread plate technique and incubated at 30 ± 1 ºC for 2-3 days. The colonies forming halo zone of clearance (Pikovskaya's medium) around them were counted as P-solubilizers.

2.2 Morphological and Biochemical characterization

The efficient PSB were identified on the basis of morphological, physiological and biochemical characteristics according to the standard methods described in Bergey's manual of systematic bacteriology[18] and laboratory manual of basic microbiology[19].

2.3 Molecular characterization of efficient strains

Molecular characterization of most efficient bacterial isolates was done by sequencing of their 16S rRNA gene. Bacteria PSC3 showed efficient plant growth promoting mechanism among the all other strains. Molecular characterization of bacteria PSC3 has been completed after DNA isolation of selected bacteria PSC3 followed by quantification of DNA sample; amplification of DNA by using primer > 27F aga gtt tga tcc tgg ctc ag > 1492 tac ggt tac ctt gtt acg act; choosing the PCR product based on concentration and processed for sequencing. Sequences have been submitted to NCBI GeneBank by Sequin.

2.4 Detection of Indole acetic acid (IAA) production

Indole acetic acid production was quantitatively measured by the method given by Gordon and Weber (1951)[20]. Bacterial cultures were grown in a nutrient broth amended with tryptophan (5mM) for 3-4

days. Cultures were centrifuged at 10,000 rpm for 20 min. Two ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent. Pink colour indicates presence of IAA.

2.5 HCN production

All isolates were subjected for the production of hydrogen cyanide (HCN) by amending 4.4 g glycine/ l media. Whatman No.1 filter paper was soaked in 2 % sodium carbonate and 0.5% picric acid solution was placed in the upper lid of the plate. The plates were sealed with parafilm and incubated at 28 ± 30°C for 5 days. The formation of orange to red colour indicates the production of hydrogen cyanide[21].

2.6 Siderophore assay

The isolates were screened for the siderophore production by adapting the universal methods explained by Schwyn and Neilands (1987)[22].

2.7 Detection of ammonia production

Qualitative detection of ammonia production was done by the method given by Bakker and Schippers, (1987)[23]. Bacterial isolates were grown in peptone water for 2-3 days at optimum growth temperature. After incubation, 1ml of Nessler's reagent was added in each tube. Tubes showing faint yellow color indicated small amount of ammonia, and deep yellow to brownish color indicated maximum amount of ammonia.

2.8 Peptone dextrose agar media preparation:

The experiment was conducted 20.0 g potato, 2.0 g dextrose, 2.0 g agar were mixed in 100 ml distilled water in a conical flask and was make a air tight with the cotton plug and wrapped with silver foil. And it is placed in the autoclave for 1 hour at 121^0C at 15 lbs. Subsequently, it was taken out from the autoclave and allowed to cool for solidify down in the laminar air flow.

2.9 Preparation of chitooligosaccharides

The enzymatic method was used for preparation of COS from chitosan [24].

2.9.1 Immobilized papain preparation

Chitin flakes (1.0 gm) were suspended in phosphate buffer (20 ml, 0.1 M, pH 6.5) and added 5mM cysteine; 2mM ethylene diamine tetraacetate (EDTA); 17.5 mg freeze-dried papain (EC3.4.22.2). The solution was kept at 5°C for 15 min in refrigerator. Then, 5% glutaraldehyde (3.1 ml) was added and the suspension was kept under mild stirring at 5°C for 14 h. The chitin–papain was filtered and washed with the same phosphate buffer trice, then stored in distilled water[24,14].

2.9.2 Activity determination method of papain for chitosan

An immobilized papain (3 gm is equals to 28.5 mg papain) was added to chitosan solution (1%, 10 ml) prepared by acetate buffer (0.1 M, pH 4.0). The suspension was kept under mild stirring at 45^0C for 1 h. The viscosities of the solution before and after reaction were determined by viscometer at 20°C. Under these conditions, the papain activities decreased the viscosity of substrate chitosan.

2.9.3 Determination of Chitosan and chitooligosaccharides content

The COS contents were determined by 3, 5-DNS colorimetry[25].

2.9.4 UV-Vis Spectroscopy

UV-Vis spectra of chitosan derivatives are usually recorded in aqueous acid (acetic acid) solutions in a 1.0 cm quartz cell at ambient temperature. The Diffuse Reflectance UV-Visible (DRUV) spectra of powdered or film samples are measured. Analysis in the vacuum ultraviolet through the near-infrared range has also been applied.

2.10 Effect of Chitosan on mycelia radial growth

Antifungal activity was determined by a radial hyphal growth of *C. falcatum*. Mycelium Growth Inhibition *in vitro* was performed on growth medium treated with 0.2%, 0.4%, 0.6% chitosan, COS concentration and combination of *E. hormaechei*. After 48 hr of incubation, agar piece of uniform size (diameter, 8 mm) containing fungi were simultaneously inoculated at the centre of each petri dish containing the various concentration of chitosan followed by incubation at 25 ± 2° C for 14 days. After incubation of fungi on culture medium containing chitosan, radial growth of fungal mycelium was recorded. Radial inhibition was calculated when growth of mycelia in the control plate reached the edge of the petri dish. The fungicidal effect to growth of fungi, in terms of percentage inhibition of mycelial growth was calculated by using the formula % inhibition = dc – dt/dc x 100 Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment[26].

3. Results and Discussion

3.1 Characterization of Chitooligosaccharides

Chitosan treated with papain releases COS. COS were preliminary confirmed by 3, 5-DNS method and with formation of brown coloured complex with sugars. The results showed that the viscosity of COS decreased upto 51.47% of the beginnings chitosan solution. This was also confirmed presence of COS.

147 **3.2 UV-Vis Spectrum**

148 Structure of COS was confirmed by UV-vis spectroscopy. UV-vis spectrum was recorded on Perkin
149 Elmer Lambda 3B UV-vis spectrometer. Ultraviolet protection factor (UPF) was measured using UV
150 Shimadzu 3101 PC spectrophotometer. UV-Vis spectra of chitosan derivatives are usually recorded in
151 aqueous acid solutions in a 1.0 cm quartz cell at ambient temperature[8]. The Diffuse Reflectance UV-
152 Visible (DRUV) spectra of powdered or film samples are measured[27]. Chitosan include various ratios
153 of two far-UV chromophoric groups, N- acetylglucosamine (GlcNAc) and glucosamine (GlcN); as a
154 result, their extinction coefficients for wavelengths shorter than approximately 225 nm are non-zero.
155 Because GlcNAc and GlcN residues show no evidence of interacting within the chitosan chain, the
156 monomer units contribute in a simple, additive way to the total absorbance of these polymers at a
157 particular wavelength[28]. The UV spectra of mixtures of N-acetyl-glucosamine and glucosamine
158 hydrochloride are quite similar to the spectra of chitosan, and the λmax is 201 nm in 0.1 M HCl
159 solution UV-vis absorbance spectra of chitosan exhibits characteristic peak at 230 nm. After
160 preparation of chitoligosaccharides, this peak undergoes a characteristic peak at range 360–348nm is
161 which observed (Fig.1).

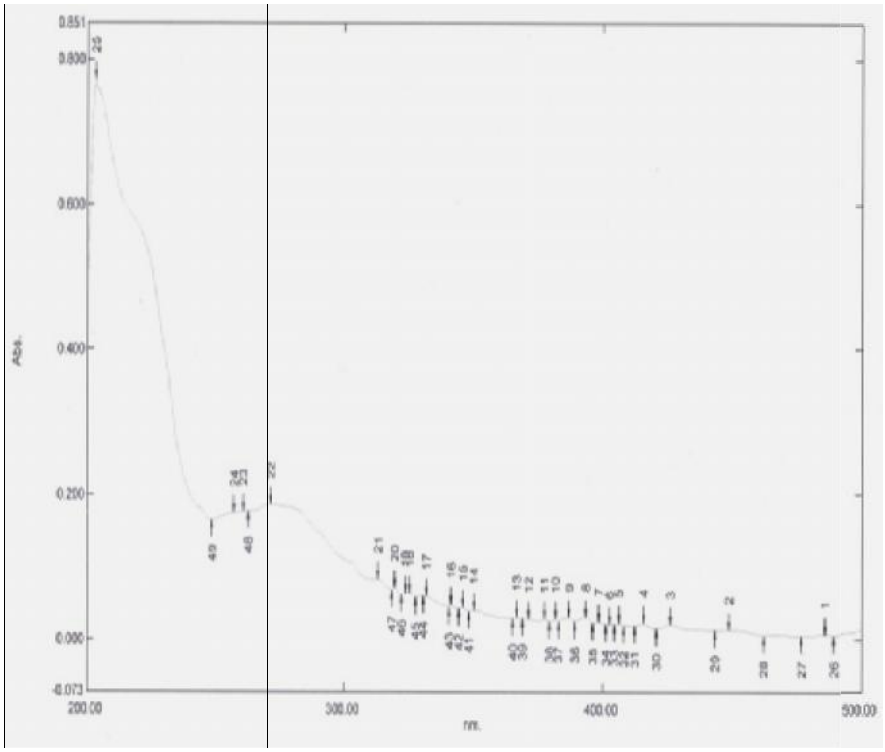
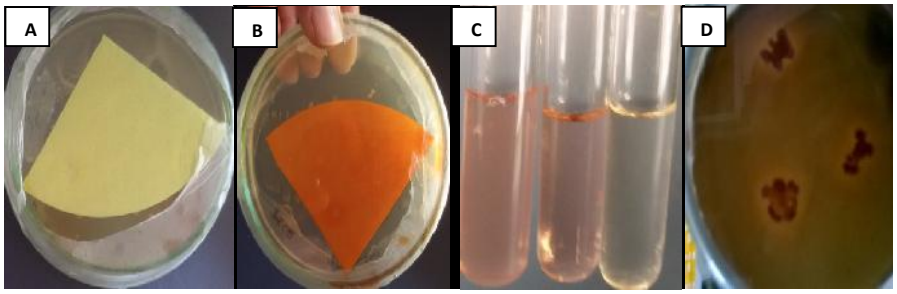


Fig 1: Characterization of chitooligosaccharides using UV- Vis spectroscopy

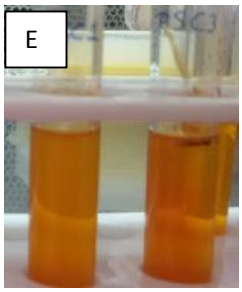
3.3 Isolation and Biochemical characterization of Isolates

The study was conducted to isolate PGPRs from sugarcane rhizosphere and investigate their potential for plant growth activities. Twenty nine PGPRs were isolated by serial dilution in selective media from two places of Uttar Pradesh. Isolated PGPRs were characterized by morphological, physiological and biochemical method. The study was further preceded for molecular identification of bacteria by 16S rRNA sequencing, and *in vitro* screening of plant growth promoting traits viz., production of Indole acetic acid (IAA), Hydrogen cyanide (HCN) Ammonia (NH_3) production and antifungal activity against *C. falcatum*. Five isolates PSC1, PSC3, KSC1, P13, P35 showed highest plant growth promoting activities. Gene sequencing of 16S rRNA (1492 bp long) to identify and decipher their phylogenetic affiliation of these bacteria. Nucleotide sequence analysis of test isolates using clustalW program revealed that isolate PSC3 showed maximum homology (99%) with *Enterobacter hormaechei*.

176 *E. hormaechei* strain is gram-negative rods which are motile, catalase positive, and oxidase
177 negative and ferment D-glucose. The strain show negative Voges-Proskauer reactions. A detailed
178 biochemical profiling of the isolate is given in Table 1. Acid is produced from the compound D-sorbitol.
179 Test for indole production was also negative.
180



181



182
183

184 **Fig 2:** Screening of Plant growth promoting traits of *E. hormaechei* PSC3: (A and B) HCN production,
185 (C) IAA production (D) siderophore production and (E) NH_3 production.

186 **Table 1:** Biochemical characteristics of *Enterobacter hormaechei* strain isolated from sugarcane
187 rhizosheric soil

Biochemical test	***E. hormaechei* PSC3**	**Biochemical test**	***E. hormaechei* PSC3**
Colony shape	Irregular spreading	Methyl Red test	+
Colony colour	Yellow	Voges-Proskauer test	-
SIM (Motality)	+	Sucrose	-
Oxidase	-	D-Lactose	-
Catalase	+	Mannitol	-
TSI	R/R	D-Sorbitol	+
Citrate	+	Innositol	-

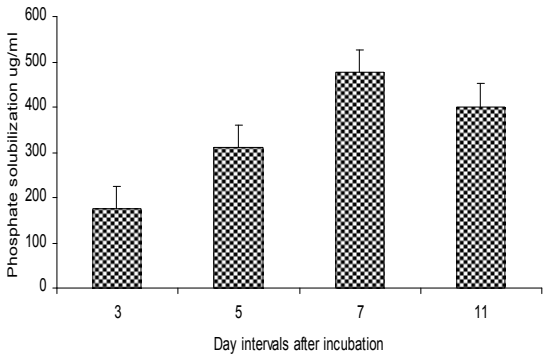
Nitrate	+	Maltose	-
Gelatin	+	Dextrose	-
Starch	-	Galactose	-

188

189 **3.4 Evaluation of isolates for their Plant growth promoting activities and physiological traits:**

190 The phosphorus solubilizing activity was evaluated (Fig 3). Phosphate solubilizing activity of
191 bacterial isolates PSC3 showed the highest phosphate solubilizition efficiency 475.51 µg/ml at 7th day
192 of intervals and lowest at 3rd day of intervals. *E. hormaechei* KU196780 was showing plant growth
193 promoting activities like indole acetic acid production hydrogen cyanide production and ammonia
194 production (Fig 2).

195 PGPR isolate PSC3 grew up to 250 mM and none grew at 300 mM of NaCl conditions, but
196 the concentrations of 50 to 100 mM NaCl were critical as the isolate showed discriminatory
197 performances in these NaCl concentrations. At 50 mM NaCl, isolate exhibited very luxuriant good
198 growth comparison with other concentration. At 250 mM and 300 mM NaCl, the isolates show very
199 less or no any growth respectively. PEG of 20, 40 and 60 % were found high for the growth of isolate
200 PSC3 (Table 2). The isolate PSC3 showed greatest growth at pH 7.0, 9.0. There was no any growth
201 on pH 5.0, 11.0. The finding showed that this strain might be help in drought and saline stress in
202 plants.



203

204 **Fig.3:** Quantification of Phosphate solubilization in g/ml by *E. hormaechei* PSC3 strains in different
205 day intervals (Data are expressed as mean ± SE, n=3)

206 **Table 2:** Plant growth promoting characteristics of *Enterobacter hormaechei* strain isolated from
207 sugarcane rhizospheric soil (+ Good, ++ Strong, +++ Very strong)

PGP traits	*E. hormaechei* PSC3	Stress tolerance traits	*E. hormaechei* PSC3
Phosphate solubilization	+	pH	+
NH_3 Production	++	NaCl	+
HCN Production	+++	PEG	+
IAA Production	+++	Cu	-
Siderophore Production	+	Hg	-

3.5 Molecular identification of isolate PSC3

Molecular tools for the identification of soil bacteria were used and 16S rDNA gene analysis was intensively used to understand the phylogenetic relationships. The accession numbers of the 16S rDNA sequences is KU196780. Bacterial phylogenetic classification is based on sequence analysis of the 16S rRNA molecule or its genes. For further identification at genus level, bacterial isolates were identified through homology search with BLAST and FASTA using partial sequence of 16S rDNA[29]. Sequencing data showed that the isolates belonged to genus, *Enterobacter spp*. being a dominant species. Nucleotide sequence analysis of test isolates using clustalW program revealed that isolate PSC3 showed maximum homology (99%) with *Enterobacter hormaechei* (Fig. 5).

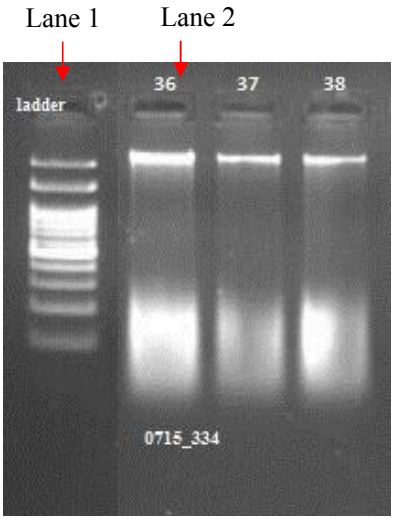


Fig. 4 : Agarose gel electrophoresis of the 16S rDNA PCR products of bacterial isolate .Lane 1: 1kb DNA ladder; Lane 2: bacterial isolate PSC3

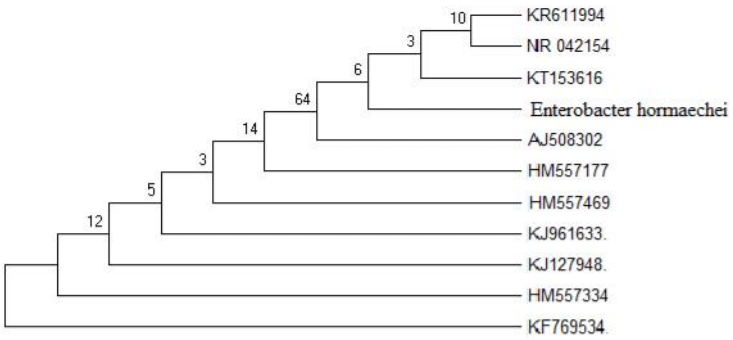


Fig 5: Neighbor-joining phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of some of their closest phylogenetic taxa.

3.6 Growth Inhibition

Antifungal activity of chitosan, COS and their combination with *E. hormaechei* were evaluated based on the diameters of growth inhibition percentage against *C. falcatum*. If there is no inhibition, it is assumed that there is no antifungal activity. Fig.7 shows representative radial growth plates with red rot causing fungus *C. falcatum* after 7 day incubation. Initially we found the significant result of individual effect of chitosan, COS with 0.5 % and *E. hormaechei* after 10 days of incubation (Fig.6). In which *E. hormaechei* showed 41.3 % growth inhibition of fungal pathogen *C. falcatum*. The diameter of radial growth of *C. falcatum* is larger in 0.6 % than that of other concentration of chitosan 0.2%, 0.4%, and indicating *C. falcatum* is susceptible to chitosan at the dose of 0.6%. Fig 8 showed the antifungal activity of chitosan with different concentration against *C. falcatum* by radial growth pattern. With regards to diameters of the radial growth, chitosan with various concentrations all demonstrated effective inhibition on the growth of fungi ($P < 0.05$). The average mean of radial growth is 1.87 to 9.13 cm against *C. falcatum*. Microscopic analysis of lactophenol blue stained fungal mycelia showed structural aberration in chitosan treated *C. falcatum* (Fig.9). The present study also revealed potential antifungal activity of the plant growth promoting a rhizobacteria *E. hormaechei* (Fig. 6). The validation of potential antifungal activity has been validated against known organisms, such as *C. falcatum*. *In vitro* prescreening showed noticeable antagonistic activity of isolate PSC3 against *C. falcatum* with a variable range of percentage inhibition. These findings support the fact that, virtually, all the agricultural soils possess some suppressive effect on various soil borne pathogens causing diseases in plants which may be because of the antagonistic activities of microbes existing in soil. This phenomenon is also known as “general suppression” or “general antagonism”. This may be possible

247 due to production of HCN. Michelsen and Stougaard, 2012 reported that HCN is a secondary
248 metabolite produced by many antagonistic *Pseudomonas* species[30]. He also found that production of
249 HCN inhibited growth of hyphae of *Rhizoctonia solani* and *Pythium aphanidermatum*.

250 One way ANOVA analysis indicated significant difference among the treatments (F3,
251 11=2.298 P<0.05). The treatment T2 (Chitosan 0.2%), T3 (Chitosan 0.4%), T4 (Chitosan 0.6%),
252 showed significant result compare with T1 (Control) somewhat treatment T4 showed greatest
253 significant in this treatment. Which indicate 0.2% chitosan solution showing very efficient antifungal
254 result against *C. falcatum.* Chitosan at the rate of 0.6 % showed 79.6% growth inhibition of *C.*
255 *falcatum* (Fig.10). Also our study coincided with those of Meng *et al.,* 2012 who demonstrated that
256 Chitosan and COS had stronger inhibitory effect on mycelia growth of two fungal pathogens *A.*
257 *kikuchiana* and *P. piricola*[31]. Numerous studies on antifungal activity of chitosan against plant
258 pathogens have been carried out and reviewed[32]. Chitosan's inhibition was observed on different
259 development stages such as mycelial growth, sporulation, spore viability and germination, and the
260 production of fungal virulence factors. It has been commonly recognized that antifungal activity of
261 chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution and, of
262 course, the target organism. Mechanisms proposed for the antifungal activity of chitosan focused
263 mainly on its effect on fungal cell wall[33] and cell membrane[34].

264 Analysis of variance was used to determine whether levels of significant with chitosan treated
265 in *C. falcatum* fungal strain different among control. The analysis showed no significant difference
266 among the treatment (F 3, 11= 3.89 P<0.05). The treatment T2 (COS 0.2%), T3 (COS 0.4%), T4
267 (COS 0.6%), showed significant result with other treatment somewhat treatment T2 showed lowest
268 radial growth in this treatment which indicate 0.2% showing no efficient antifungal result against *C.*
269 *falcatum fungal* (Fig.10).

270 Chitosan and its derivatives offer a great potential as natural biodegradable nontoxic
271 substances which have anti-microbial and eliciting activities. In the present study, the Chitosan was
272 showing antifungal activity and highly effective in managing the red rot disease in sugarcane. The
273 0.6% of Chitosan was showing significant dose compared control. The Chitosan was showing more
274 effect than COS. That indicate chitosan was efficient antifungal agent and highly effective in
275 managing the complication associated with red rot disease. The 0.2% chitosan and COS was
276 showing significant dose compared 0.2%, 0.4%., 0.6%. The study revealed that chitosan was

effective in inhibiting mycelial growth of *C. falcatum*. However, when compared to chitosan and *E. hormaechei* is relatively more effective than chitosan (Table 3). Furthermore, our results indicated that both chitosan and plant growth promoting rhizobacteria were effective in controlling diseases caused by *C. falcatum*.

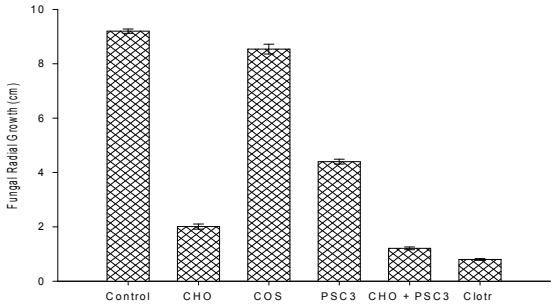


Fig.6 Growth inhibition of *Colletotrichum falcatum* in presence of initial chitosan COS *E. hormaechei* and their combination (Data are expressed as mean ± SE, n=3)

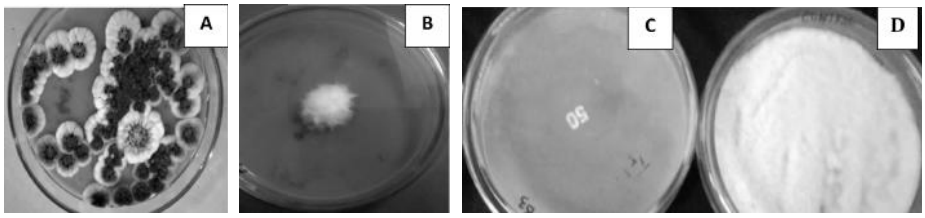


Fig.7 Growth of *Colletotrichum falcatum* in presence of initial screening of different concentration of chitosan after 7 days. (A) 0.1% Chitosan (B) 0.5% Chitosan(C) 1.0% Chitosan (D) without chitosan control

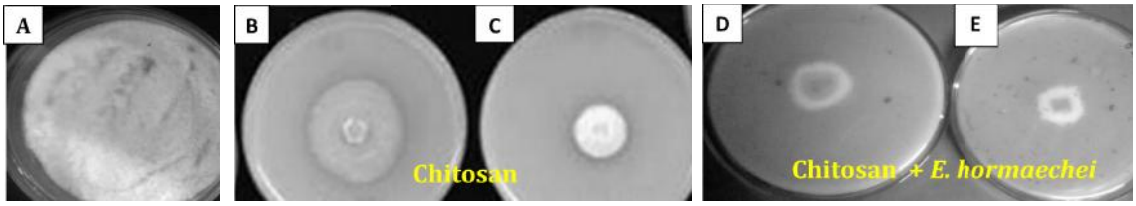


Fig.8 Growth of *Colletotrichum falcatum* in presence of chitosan and chitosan with *E. hormaechei* and their combination after 7 days of inoculation (A) Control, (B) 0.2% Chitosan, (C) 0.6% Chitosan,(D) 0.2% Chitosan with *E. hormaechei,* (E) 0.6% Chitosan with *E. hormaechei*

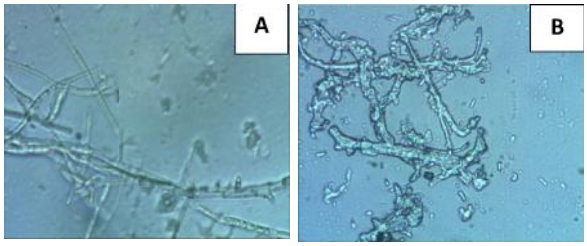


Fig.9 Microscopic analysis of lactophenol blue stain mycelia (a) Control (b) Chitosan treated mycelia showing deformed structure.

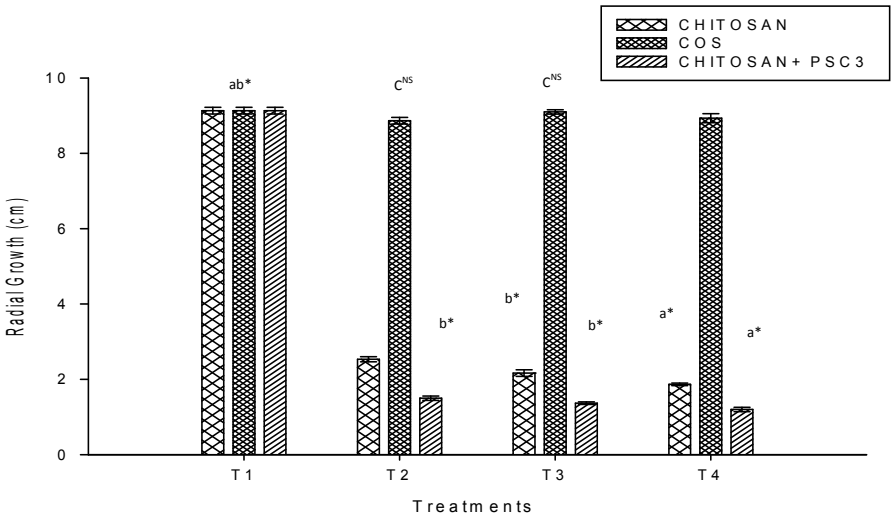


Fig.10 Antifungal Activity of different concentration of chitosan against *Colletotrichum falcatum* (Data are expressed as mean ± SE, n=3).

Values are statistically significant at *p<.05. Significance determined by ANOVA was compared within the treatments as follows: a Control vs 0.6% chitosan and chitosan+ *E. hormaechei*; b 0.2%, 0.4%, 0.6% Chitosan vs. Control and ^{CNS} Not significant

Table 3. Percentage of radial growth inhibition of *Colletotrichum falcatum*

Concentration of / COS Chitosan	Growth Inhibition % by Chitosan	Growth Inhibition % by COS	Growth Inhibition % by Chitosan + *E. hormaechei*
0.2 % Chitosan/COS	72.3%	2.85%	83.5%
0.4% Chitosan/COS	76.3%	1.0%	84.99%
0.6% Chitosan/COS	79.6%	3.0%	86.85%

4. Conclusion and Future Prospects

This present research findings proved that this study is helpful for developing new biocontrol combination from chitosan and plant growth promoting rhizobacteria for managing fungal diseases and associated complications. The study reveals that chitosan solution and their concentration have significant effect of antifungal activity but their combination with plant growth promoting rhizobacteria *E. hormaechei* showed greatest growth inhibition of *C. falcatum* (86.85%). The chitosan and *E. hormaechei* seems promising for the development of a new formulation for fungal infection in plants.

A further investigation of the best antifungal result of chitosan and *E. hormaechei* like time of application, concentration, combination with other components, physiological changes in plants and molecular mechanism are needed and provide future line of work for controlling red rot disease of sugarcane for sustainable agriculture.

REFERENCES

1 Dimkpa C, Weinand T, Asch F. Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ. 2009; 32, 1682–1694.

2 Kloepper JW. Plant growth promoting bacteria (other systems). In: Okon J, editor. Azospirillum/Plant Association. Boca Raton, FL: CRC Press, 1994; pp. 137–54.

3 Glick BR. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology. 1995; 41,109–117.

4 Van Loon LC, Glick BR. Increased plant fitness by rhizobacteria. In: Sandermann H (ed) Molecular ecotoxicology of plants.Springer, Berlin, pp 2004; 177–205.

5 Yang J, Kloepper J, Ryu C. Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sciences. 2009; 14:1–4.

353 6 Alexander KC and Viswanathan R. Major diseases affecting sugarcane production in India and
354 recent experiences in quarantine. In: Sugarcane Germplasm Conservation and Exchange. ACIAR
355 1996; 67: 46 - 48.
356 7 Muthamilan M, Jeyarajan. Integrated management of Sclerotium root rot of groundnut involving
357 *Trichoderma harzianum, Rhizobium and carbendazin*, Indian Journal of Mycology Plant Pathology.
358 1996; 26:204-209.
359 8 De Souza HKS, Bai G, do Pilar Gonçalves M, Bartos M, Whey protein isolate–chitosan interactions:
360 A calorimetric and spectroscopy study. Thermochim. Acta. 2009; 495,108–114.
361 9 Benizri E, Baudoin E and Guckert A. Root colonization by inoculated plant growth-promoting
362 rhizobacteria. Bio-control Science Technology. 2001; 11, 557-574.
363 10 Wojdyla AT. chitosan in the control of rose disease: six years trials. Bull Polish Acad Sciences
364 Biological Sciences 2001; 49:233-252.
365 11 Rinaudo M. Chitin and chitosan: Properties and application. Prog Polymer Science. 2006; 31(7),
366 603-632.
367 12 Katiyar D, Hemantaranjan A, Singh B and Nishant Bhanu A. A Future Perspective in Crop
368 Protection: Chitosan and its Oligosaccharides. Advances in Plants Agricultural Research. 2014; 1(1):
369 06.
370 13 Kim SK, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides
371 (COS): A review, Carbohydrate Polymer 2005; 62: 357–368.
372 14 Katiyar D, Singh B, Lall AM, Haldar C. Efficacy of chitooligosaccharides for the management of
373 diabetes in alloxan induced mice: A correlative study with antihyperlipidemic and antioxidative activity.
374 European Journal of Pharmaceutical Sciences. 2011; 44: 534–543.
375 15 Katiyar D, Hemantaranjan A, Singh B. Chitosan as a promising natural compound to enhance
376 potential physiological responses in plant: a review. Indian Journal of Plant Physiology. 2015; 20
377 (1):1–9.
378 16 Hadrami AE, Adam LR, Hadrami IE and Daayf F. Chitosan in Plant Protection. Maine Drugs.
379 2010; 8, 968-987
380 17 Pikovskaya RI, Mobilization of phosphorus in soil connection with the vital activity of some
381 microbial species. Microbiologiya. 1948; 17, 362–370.

382 18 Kreig NR and Holf JG. Bergeys Manual of Systematic Bacteriology. William and Wilkins, 1984.
383 Baltimore, USA.
384 19 Kanwar SS, Gupta MK, Punj V, Biochemical reaction and their significance. In: Laboratory Manual
385 of Basic Microbiology, Department of Microbiology, College of Basic Sciences, Himachal Pradesh
386 Krishi Vishvavidyalaya, Palampur. 1997; pp. 54-64.
387 20 Gordon SA,Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiol, 1951; 26,192-
388 197.
389 21 Lorck H. Production of hydrocyanic acid by bacteria. Physiol Plant 1948; 1142–146.
390 22 Schwyn B and Neilands JB. Universal chemical assay for the detection and determination of
391 siderophores. Anal Biochem. 1987;160, 47–56.
392 23 Bakker AW and Schippers B. Microbial cyanide production in the rhizosphere in relation to potato
393 yield reduction and Pseudomonas spp.- mediated plant growth-stimulation. Soil Biology and
394 Biochemistry. 1987;19:451–457.
395 24 Hong L, Haiying W, Changhu X, Mei Y, Preparation of chitosan oligomers by immobilized papain.
396 Enzyme and Microbial Technology. 2002;31,588–592.
397 25 Miller GL.Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical
398 Chemistry. 1959; 31: 426-428.
399 26 Singh J, and Tripathi NN, Inhibition of storage fungi of blackgram (vigna mungo) by some
400 essential oils". Flavour and Fragrance. J. 2009; 14,1-4.
401 27 Ramaprasad AT, Rao V, Sanjeev G, Ramananic SP, Sabharwal S, Grafting of polyaniline onto
402 the radiation crosslinked chitosan. Synth. Met. 2009;159:1983–1990.
403 28 Singh J, Dutta PK, Dutta J, Hunt AJ, Macquarrie DJ, Clark JH. Preparation and properties of
404 highly soluble chitosan–L-glutamic acid aerogel derivative. Carbohydrate Polymers. 2009; 76:188–
405 195.
406 29 Tamura K, Dudley J, Nei M and Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis
407 (MEGA) software version 4.0. Molecular Biology and Evolution. 2007; 24,1596-1599.
408 30 Michelsen CF, Stougaard P. Hydrogen cyanide synthesis and antifungal activity of the biocontrol
409 strain Pseudomonas fluorescens In5 from Greenland is highly dependent on growth medium.
410 Canadian Journal of Microbiology. 2012; 58(4):381-390.

411 31 Meng X, Lingyu AB, Yang A, John F, Kennedy C, Shiping Tian B. Effects of chitosan and
412 oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit
413 .Carbohydrate Polymers. 2010; 81,70–75.
414 32 BenShalom N, Ardi NR, Pinto R, Aki C, Fallik E. Controlling gray mould caused by *Botrytis cinerea*
415 in cucumber plants by means of chitosan. Crop Protection. 2003; 22,285–290.
416 33 Elghaouth A, Arul J, Wilson C. Benhamou, N., Ultrastructural and cytochemical aspects of the
417 effect of chitosan on decay of bell pepper fruit. Physiology Molecular Plant Pathology. 1994; 44, 417–
418 432.
419 34 Zakrzewska A, Boorsma A, Brul S, Hellingwerf SJ, Klis KM. Transcriptional response of
420 Saccharomyces cerevisiae to the plasma membrane-perturbing compound chitosan, Eukaryotic Cell.
421 2005; 4:703–715.