

Herbicidal Activity against the Common Weed Peperomia Wightiana and Synthesis of Chitosan Nanoparticals

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Abstract: The microbes and their secondary metabolites can be used instead of chemical herbicides as bioherbicides. Nanotechnology is one the promising field of research opens up in the present decade a wide array of opportunities in the present decade and is expected to give major impulses to technical innovations in a variety of industrial sectors in the future The present study was more focused on Mycoherbicides against the weed and wildy grown plant Peperomia wightiana which was vastly spreaded over Maharastra in India. And over the other commonly found weed Amaranthus retroflexus grows in gardens, railways and many other places. To improved the herbicidal activity of herbicidal protein with nanoencapsulation using chitosan maicrocapsules against weed. Characterization of this herbicidal protein, mass production, formulation and herbicidal activity on other economic weeds.

Keywords : Peperomia wightiana, Fusarium oxysporum 07, SDS PAGE and Chitosan nanoparticals

I. INTRODUCTION

Nanotechnology is one of the promising fields of research opens up in the present decade a wide array of opportunities in the present decade and is expected to give major impulses to technical innovations in a variety of industrial sectors in the future. The potential uses and benefits of the nanotechnology are enormous. These include agricultural enhancement involving nanoporous zeolites for slow release and efficient dosage of water and fertilizer, nanocapsules for herbicide delivery and vector and pest management [2].

Nanoencapsulation is a process through which substances such as an insecticide is slowly but efficiently released to a particular host plant for insect pest control. Nanoencapsulation with nanoparticles in form of pesticides allows for proper absorption of the chemical into the plants unlike the case of larger particles[7].

Revised Manuscript Received on April 10, 2019

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Microcapsules based on biodegradable polymer nanoparticles have attracted much attention for their potential in biomedical and agriculture applications. In the present study, improved activity of herbicidal protein with nano encapsulation using chitosan microcapsules against weed.

II. MATERIALS AND METHODS

A. Fungal strain: *F.oxysporum* 07 strain was isolated from local soil sample and diluted in 225ml of distilled sterilized water. From this sususpension 10ml was added to 990ml of water and from this 1ml was spread in petridishes in triplicates containing potato dextrose agar (PDA) supplemented with chloramphenicol 100mg/ml.

B. Extraction and purification of Necrosis inducing protein: For necrosis inducing protein production,500 ml of modified Fries media (sucrose 10g,casein hydrolysates 2g, sodium nitrate 1.5g, dipotassium hydrogen ortho phosphate 1g,potassium chloride 0.5g,Magnesium sulfatate 0.5g.Ferrous sulfate 0.01g, distilled water 1L.,pH 6.8) was prepared and sterilized by autoclaving. 0.1 ml of spore suspension derived from 10 days old PDA slant culture of *F.oxysporum* was inoculated and the inoculated flasks were kept at 28 C on a rotatory shaker (Scigene) at 150rpm for 21 days. After 21 days of growth, the broth was filtered through three layers of cheesecloth and the collected filtrate was extracted with methanol (1:5 ratios).

SDS PAGE was carried to assess the molecular weight of the protein. The gel was polymerized from a mixture of 17.5 ml of 30% acrylamide -0.8% methylene bis acrylamide- 17.5ml of 1.5M Tris hydrochloride (pH 8.8) – 35 ml of distilled water – 35ml of N,N,N',N' tetra methylene diamine -0.70ml of ammonium per sulfate (75mg/ml).

C. Herbicidal activity: Initially leaf necrosis assay was carried out with *F.oxysporum* fungal conidia. The expanded leaves of *Peperomia wightiana*; were detached from plant and cut into 6-9cm², surface sterilized with ethanol and washed with sterile distilled water to remove ethanol from surface. The cut pieces were inoculated with 10⁸ spores/ml of *F.oxysporum* 07 strain fungal conidia by wounding them with sterile needle on the surface of the leaf and transferred to Petri plate containing moistened cotton ball and filter paper. Later plates were incubated at 25°c for one week [1]. The leaf bioassay with protein was performed as descried earlier with 1.0mg/ml final concentration of lyophilized protein. A daily observation was made for the development of necrotic lecsions on the protein inoculated leaves. The protein was re extracted from necrotic lesion that developed on the tested weed.

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After one week, 30gms of necrotic leaves was collected, chopped and treated over night with 50 ml of methanol and chloroform at room temperature. Extracts were filtered through four layers of cheese cloth. Residues were then collected into sterile 10ml screw cap vials and partially purified by column chromatography as described earlier and the fraction obtained was identified TLC and bio leaf assay were done as explained earlier.

D. Effect of temperature on phytotoxicity: The effect of temperature on phytotoxic activity of herbicidal protein was studied. The protein with final concentration of 100mg/ml was dissolved in 5ml of Tris HCL buffer in a 10ml of test tube was heated at 40°C, 50°C, 60°C, 70°C and 80°C for one hour. After the heat treatment phytotoxic assay was performed using leaf bio assay as discussed earlier.

E. Preparation of Chitosan nanoparticles: Chitosan nanoparticles were prepared according to the procedure first reported by [3] based on the ionic gelation of CS with TPP anions. Chitosan was dissolved in acetic aqueous solution at 2.0 % concentrations, under magnetic stirring at room temperature, 4mL sodium tripolyphosphate TPP aqueous solution was added into 10mL chitosan solution, the reaction mixture was dried in hot air oven and the dried material was collected and examined under SEM (Scanning electron microscopy) to characterize chitosan nanoparticles.

F. Loading of herbicidal protein with chitosan: Herbicidal protein loaded chitosan nanoparticles were formed spontaneously upon dropwise addition of 12 ml of 0.4 % aqueous sodium tripolyphosphate solution to 20 ml of 0.35 %w/v chitosan solution containing 5mg/ml of the protein under magnetic stirring, followed by sonication. The resulting nanoparticle suspensions were centrifuged 4 times (15 min each) at 15000 rpm, washed with distilled water and dried at 257nm. Characterization of loaded chitosan with herbicidal protein was characterized by scanning electron microscopy.

G. Evaluation of herbicidal activity: Herbicidal activity of chitosan loaded herbicidal protein carried out with loaded protein with chitosan at 10, 25 and 50 µg concentration adopting leaf necrosis assay as described earlier.

H. Effect of necrosis inducing protein on seedlings emergence: The impact of the protein loaded chitosan nanoparticles on seedlings emergence of four economic important cereals was also carried out. Seeds of paddy, wheat, black gram and horse gram were dipped in protein (final concentration 100mg/ml) for 30 minutes, and the treated seeds were transferred to petridish containing moistened filter paper on cotton ball at temperature at 25°C for 48 hours. Seedling emergence

III. RESULTS AND DISCUSSION

A. Herbicidal activity of herbicidal protein

In the present study a phytotoxic protein with a molecular weight of 66KDa was isolated from methanol extract of culture filtrate of *f.oxysporium* 07 strain cultivated in unique liquid modified media. The lyophilized protein was evaluated for its herbicidal activity against a weed *Peperomia wightiana*.

The herbicidal activity of the culture filtrate was readily extracted into organic solvents such as methanol at pH 4 to 5. The methanol soluble extract was concentrated and partially purified by silica gel G₆₀ column. Among the 21 fractions, Fraction, 2, 7, 9, 13 and 15 showed phytotoxic activity and the fractions were pooled and show a high purity since as single major band appeared after SDS-PAGE which reveals a monomer with a molecular weight of 66Kda showed necrosis activity (Figure:1) confirms the active compound responsible for necrotic activity was protein. And the pooled fractions were lyophilized and stored at -20°C, used for bioassay.

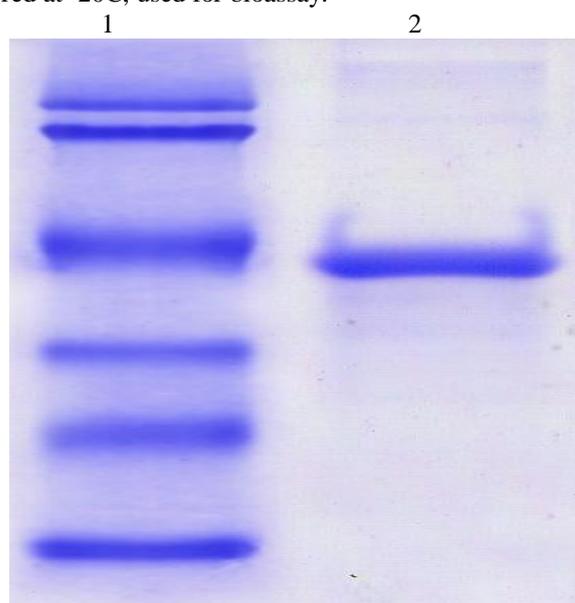


Figure 1: SDS PAGE

1. Standard Protein Marker
2. 66KD necrosis inducing herbicidal protein



Figure 2: Necrotic lesions

The partially purified protein showed phytotoxic effect on the tested weed *Peperomia wightiana*. First symptom appear within 24 hours as weak chlorotic marking which subsequently developed into well defined chlorotic spots which forms deep brown lesions. The diameter of necrotic area was 10.0, 7.0 and 5.0 mm² at 50, 25 and µg. concentration. (Table 1 and Figure 2) The fungal conidia also caused same phytotoxic effect and the diameter of necrotic lesions 7.2mm² (Figure 2).

Table 1: Surface necrotic area of respective treatments

S.No.	Treatment	Diameter of the necrotic area mm ²
1	F.oxysporum 07 strain conidia	
2	Necrosis inducing protein (50ug)	10.0
	Necrosis inducing protein (25ug)	7.0
	Necrosis inducing protein (25ug)	5.0
3	after re extraction from necrotic lesion	6.8±0.1 b

* Mean ± S.E

In column, the mean followed by same letter is not statistically significant (P>0.05) by DMRT.

The protein extracted from infected leaves also showed the same phytotoxic effect on fresh *Peperomia wightiana* leaves which shows the virulence of the protein after reextraction and the surface area of necrotic lesions is 6.8mm². Effect of temperature on phytotoxic activity reveals no distinct effect on phytotoxic activity was observed after heating the protein at 40, 50, 60, 70 and 80°C for 30 minutes. The toxin heated at respective temperature induced necrotic lesions with surface area of 7.0, 6.8, 6.8, 6.5 and 6.3 mm² (Figure: 3) respectively which clearly reveals the herbicidal protein could withstand at high temperature and retained its phytotoxic effect

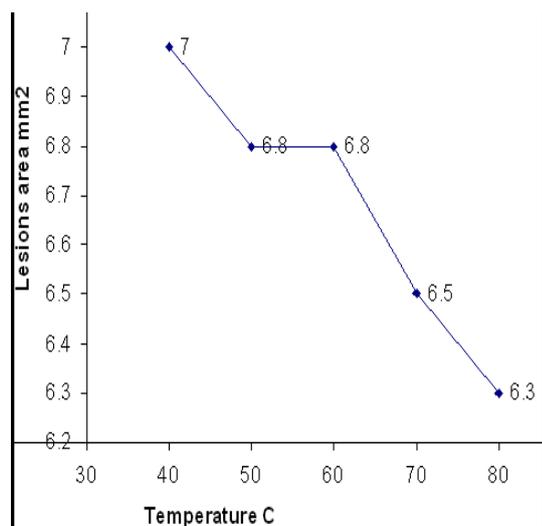


Figure 3: Surface area mm² of necrotic lesions produced by necrosis inducing protein at different temperature.

B. Effect on seedlings emergence:

Effect of herbicidal protein on seedling emergence of four important cereals reveals that maximum emergence of wheat (93.7%) followed by paddy (91.07%), 89.57 and 77.14 % seedling emergence was recorded in black gram and horse gram (Table: 2 & Figure: 4).

Table 2: Effect of necrosis inducing protein on seedling emergence (%)

S.No	Tested seeds	Seedlings emergence (%)	
		Control	Treatment
1	<i>Oryza sativa</i> (Paddy)	100.0	91.07
2	<i>Triticum. Aestivum</i> (Wheat)	100.0	93.70
3	<i>Vigna mungo</i> (Black gram)	100.0	89.57
4	<i>Macrotyloma uniflorum</i> (Horse gram)	100.0	77.14

In column, the mean followed by alphabet is statistically significant (P>0.05) by DMRT.

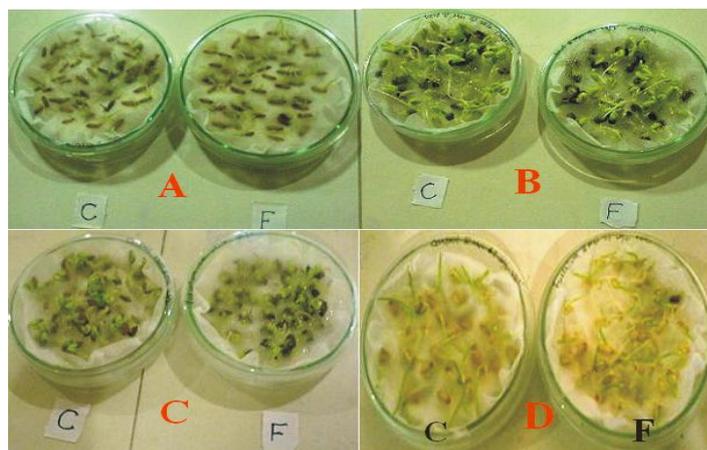


Figure 4: Effect of necrosis inducing protein on seedling emergence.

A) *Oryza sativa* B) *Vigna mungo* C) *Macrotyloma uniflorum* D) *Triticum aestivum*

C. Synthesis and characterization of chitosan nanoparticles: Spherical chitosan nanoparticles were formed after the immediate addition of sodium tripoly phosphate into the chitosan solution under stirred condition confirmed by scanning electron microscopy. The particle diameter (z-average) ranged from approximately 256-350 nm as seen in (Figure: 5 and Figure: 6) and the protein loaded chitosan were characterized by SEM with the diameter. SEM image showed that each particle unit exhibited a nanostructure confirmed the loaded chitosan with herbicidal protein.

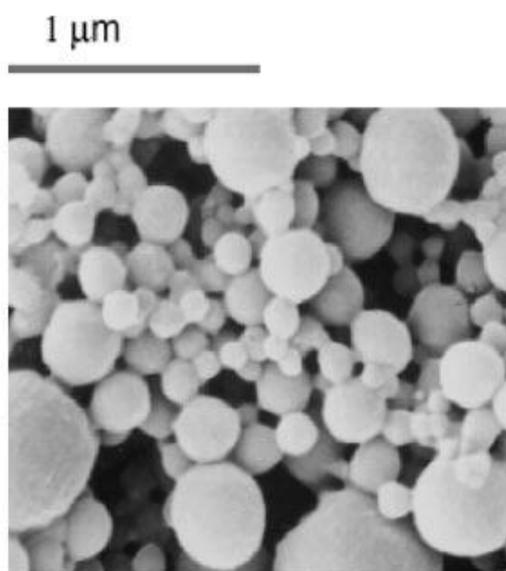


Figure 5: SEM of chitosan nanoparticles

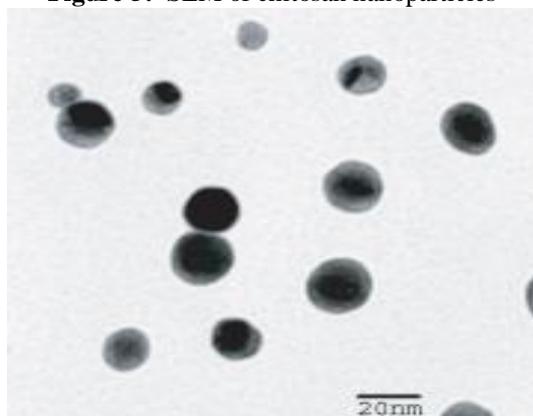


Figure 6: Chitosan loaded herbicidal protein

D. Herbicidal activity of chitosan loaded herbicidal protein: Improved herbicidal activity was recorded in all the tested concentration of chitosan loaded herbicidal protein. The diameter of necrosis formed on the leaflets of tested weed was increased from 5 to 10 mm in chitosan loaded herbicidal protein. The diameter of the necrotic lesion was found to be 18. mm at 50 μg concentration. 15mm and 15 mm of necrotic lesions was observed at 25 and 10 μg.(Table: 3).

Table 3: Surface necrotic area of chitosan loaded herbicidal protein

S.No.	Concentration	Diameter of the necrotic area mm ²
1	10	15
2	25	15
3	50	18

Seven different genera of fungi have been isolated from both soil and leaf samples from the environment. In which *Fusarium* and *Alternaria* shows phytotoxic activity that has the ability to kill the unwanted plants without any harm to the environment. Novel biological approach for the formation of copper nanoparticles using leave [4]. *Nigrospora oryzae* patch [5]. The antibacterial activity of

Bostrycin has been previously isolated from *bostryconema alpestre cesati*[6] and *arthrinium phaeospermum* [8].

The testing herbicidal protein did not cause any significant effect on seedling emergence of all the tested seeds. The mimicking of pathogenic necrotic symptoms produced by herbicidal protein isolated from *F.oxysporum* 07 strain on *Peperomia wightiana* suggest a herbicidal role for the protein in *Peperomia wightiana* necrotic lesions.

IV. CONCLUSION

Microbes and their secondary metabolites can be used instead of chemical herbicides as bioherbicides. The fore the thermo stability of the toxin reveals the toxin could withstand high temperature (up to 80°C). This retained the herbicidal activity up to 80°C and the diameter of surface area of necrotic lesions was found to be similar as at 30°C. The herbicidal activity of herbicidal protein can be improved with nanoencapsulation using chitosan. Characterization of this herbicidal protein, mass production, formulation and herbicidal activity on other economic weeds (Invitro and field trial) will be carried out in future study.

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