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Antifungal activity of oligochitosan against purple blotch pathogen (*Alternaria porri* (Ellis) Cif) of onion

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Abstract
Purple blotch (*Alternaria porri* (Ellis) Cif) is one of the severe onion diseases in Maharashtra causing higher yield losses. Oligochitosan is obtained by gamma irradiation of chitosan and attempted to explore its effectiveness against the pathogen *in vivo* as well as *in vitro*. Present study indicated that the fungicide mancozeb @ 0.2% inhibited maximum mycelial growth and this was followed by treatment of chitosan @ 0.24%. The spore germination assay indicated that as the concentration of chitosan increased from 0.02 to 0.24%, the inhibition of spore germination was increased from 51.69 to 79.18%. The disease intensity was minimum (7.81 PDI) on the treatment chitosan @ 0.04% sprayed 30, 45, 60 DAT as compared to untreated control treatment (water spray) as 37.25 PDI. The oligochitosan is a safe natural polymer to reduce the losses caused by fungal infection and it will be helpful in hazardous chemical free disease management option.

Keywords: Onion, *Allium cepa*, *Alternaria porri*, oligochitosan, gamma irradiation

Introduction
The onion (*Allium cepa*) also known as the bulb onion or common onion, often called as "queen of kitchen" is used as a vegetable. India is the largest producer of vegetables in the world, the productivity is very low as 12.5 tons/ha as against to 15.8 tons/ha in China and 44.21 tons/ha in Japan (Singhal, 1999). India ranked second in production of onion after China in 2012 and produced 133.721 lakh metric tons. Area under onion was 9.59 lakh ha and productivity was 17.01 tons/ha. Maharashtra ranked first in production in 2013-14 by producing 5864040 metric tons. Area under cultivation was 468000 ha and productivity was 12.53 tons/ha (Anonymous, 2015) [2].

Diseases are major problems in onion productivity and which affect the foliage showing purple blotch, rust, smut, downy mildew and white tip disease symptoms. The bulbs may be affected by splitting, white rot and neck rot. Among the diseases, purple blotch is a major concern. The purple blotch is managed by following clean cultivation, good drainage and use of drip irrigation along with seed treatment of thiram @ 3g/kg seed followed by foliar sprays of chemical fungicides as mancozeb @ 0.2%, tricyclazole @ 0.1% and hexaconazole @ 0.1% (Anonymous, 2013) [2].

Chitosan is an organic natural biopolymer modified from chitin, which is the main structural component of squid pens, cell walls of some fungi and shrimp and crab shells (Suchada *et al*., 2010) [19]. Chitin is the second most abundant polymer in nature after cellulose (Cohen-Kupiec and Chet, 1998) [4]. Chitosan has also been extensively utilized as a foliar treatment to control the growth, spread and development of many diseases involving viruses, bacteria, fungi and pests (Rabea *et al*., 2003) [15]. Oligochitosan has shown higher mycelial growth inhibition of *Phytophthora capsici* than native chitosan although both are equally effective in controlling different phytopathogens. Thus, the molecular weight of chitosan influences the antifungal activity of this polymer (Xu *et al*., 2007) [24]. Thus it was thought worthwhile to evaluate the antifungal potential of oligochitosan against purple blotch causing pathogen of onion.

Material and Methods
Material
Diseased samples of onion leaves were collected from Vegetable Improvement Project, ZARS, Ganeshkhind, Pune-411067.

Culture media
The common laboratory medium PDA (Potato Dextrose Agar) was used for isolation of organism responsible for the purple blotch of onion and culture of isolated organism was maintained on PDA slants for further investigation. The PDA medium was also used for Poison Food Technique. Procedure was carried out in laboratory at Plant Pathology Section, College of Agriculture, Pune and NARP, Ganeshkhind, Pune.

Fungicide
For the study of sensitivity of fungicide against *A. porri*, Mancozeb 75% WP of Indofil Company make was used.

Chitosan
The oligochitosan (Low Molecular Weight Chitosan) was prepared by irradiating normal chitosan with electron Beam 100 KGy dose at BRIT, BARC, Mumbai.

Methods
Isolation, purification and maintenance of pathogen
Isolation
The fresh samples showing typical purple blotch symptoms were brought to the laboratory. These samples were washed in tap water to remove the extraneous material and then placed in paper. Then small bits of affected leaf portion along with the healthy tissue were cut and surface sterilized in 0.1 per cent mercuric chloride ($HgCl_2$) solution for one minute. Then these bits were washed thricce in sterilized water to remove traces of mercuric chloride. These bits were then aseptically transferred to sterilized Petri plates which were already poured with PDA medium. The plates were then incubated at room temperature (27± 1^0 C) for 3-6 days to obtain fungal growth. This growth was sub cultured on PDA slants for obtaining pure culture.

Purification and maintenance of pathogen
The cultures were purified by single spore technique (Johnston and Booth, 1983) [9]. In case of single spore technique 2 to 3 drops of spore suspension prepared from 10 days old culture were used to spread on the surface of plain agar medium in Petri plates and incubated at 27±1^0 C for 24 hours. The plates were observed for germinating spores under stereoscopic microscope and finally germinating spores were lifted by inoculating needle and transferred aseptically to potato dextrose agar slants for further growth. The pure cultures thus obtained from above isolates of *A. porri*, were maintained by repeated sub-culturing at an interval of 30 days for further studies. The stock culture in PDA slants was stored at 4^0 C in refrigerator.

***In vitro* assessment of fungal sensitivity to chitosan**
In vitro assessment of fungal sensitivity to mancozeb and chitosan was studied by two methods:
1) Inhibition of the mycelial growth by poison food technique.
2) Spore germination assay.

Inhibition of the mycelial growth by poison food technique
The fungicide and chitosan were evaluated to suppress the growth of the pathogen *in vitro* by adopting Poisoned Food Technique (Mathur and Shekhawat, 1986). The principle involved in the Poisoned Food Technique is to poison the nutrient medium with a fungitoxicant and then allowing the test fungus to grow on it. Glasswares used in the study were sterilized prior to their use. PDA was prepared and distributed in 100 ml lots, in 250 ml of flasks and sterilized as usual. Required quantities of mancozeb (0.2%) and chitosan (0.02, 0.04, 0.08, 0.16, 0.20, and 0.24%) were added to each of the flasks. The flasks were shaken thoroughly and medium was poured in sterilized Petri plates in triplicate for each chemical. Seven days old culture of fungus was used for the inoculation of these Petri plates. The fungal disc of 5 mm diameter was cut with the help of sterilized cork borer and transferred aseptically at the center of each Petri plate containing poisoned PDA medium i.e. fungitoxicant. The PDA plates without mancozeb and chitosan were also inoculated with fungal culture to serve as control treatment. The plates were incubated at room temperature (27± 10^0 C). Observations were recorded after seven days for colony diameter of the fungus. The percentage of inhibition of mycelial growth was worked out by the formula given by Vincent (1947) [22].

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition of fungal growth.
C = Growth of fungus colony diameter (mm) in control.
T = Growth of fungus colony diameter (mm) in treatment.

Spore Germination Assay
To evaluate inhibition of spore germination, spore suspension of *A. porri* (10^5 spores per ml) was prepared from a ten days old fungal culture. The slides were coated with cellulose nitrate to provide a uniform surface in the depression well. The solution of test chemical mancozeb, chitosan and spore suspension was prepared in double the concentration and it was mixed in equal quantities. Using a one ml pipette, two drops of the mixture were placed in the well of the cavity slide. These slides were kept in moist chambers prepared by placing a fold of water soaked filter paper in both base and lid of Petri plates and the plates were incubated at 27±1^0 C for 24 hrs. Each treatment was replicated three times (Palma-Guerrero *et al*., 2008) [13]. The per cent spore germination (SGI%) was recorded using the formula given by Kiraly *et al*. (1974) [10]. Per cent inhibition in spore germination was determined by comparing spore germination percentage between control and treated slides.

$$\text{No. of spores germinated} = \frac{\text{Per cent spore germination}}{\text{Total no. of spores examined}} \times 100$$

***In vivo* evaluation of chitosan and mancozeb on disease development**
The experiment was conducted in the research field of Vegetable Improvement Project, NARP, Ganeshkhind, Pune-67 during *kharif*, 2015. Seedlings of onion variety Baswant 780 released by MPKV, Rahuri were raised on nursery beds. Thirty days old seedlings of the variety were transplanted in 2.7 m^2 of each plot containing well decomposed farm yard manure (FYM) mixed soil. Watering was done as per the requirements to maintain relative humidity between 80-90 per cent. All plants were inoculated with spore suspension of the fungus using knapsack sprayer. Then oligochitosan and mancozeb were sprayed with different concentrations at different growth stages. The triplicate treatments were as,

T1 - Untreated control,
T2 - Mancozeb @ 0.2% at 30 DAT (Days After Transplanting)
T3 - Chitosan @ 0.02% at 30 DAT

T4 - Chitosan @ 0.04% at 30 DAT
T5 - Chitosan @ 0.02% at 30 & 45 DAT
T6 - Chitosan @ 0.04% at 30 & 45 DAT
T7 - Chitosan @ 0.02% at 30, 45 & 60 DAT
T8 - Chitosan @ 0.04% at 30, 45 & 60 DAT

Plants were observed weekly to record the disease severity until physiological maturity of crop using 0-9 scale (Datar and Mayee, 1986).

0 to 9 grade disease scale

0 to 9 grade disease scale		
1. Leaf area free from infection	(free)	grade 0
2. Leaf area infected up to 1%	(very light)	grade 1
3. Leaf area infected up to 10%	(light)	grade 3
4. Leaf area infected up to 25%	(medium)	grade 5
5. Leaf area infected up to 50%	(heavy)	grade 7
6. Leaf area infected more than 50%	(very heavy)	grade 9

The per cent disease index (PDI) was calculated by using the formula,

$$\text{Percent disease Index (PDI)} = \frac{\sum \text{numerical ratings}}{\text{Total no. leaves observed}} X \frac{100}{\text{Maximum grade}}$$

Statistical analysis
The complete data under the research experiments was statistically analyzed as per the procedure laid by Panse and Sukhatme (1954) [14].

Results and discussion

Inhibition of the mycelial growth by poison food technique
It was observed that under absolute control treatment (T_1) the fungus (*A. porri*) grown profusely (Table 1). On eighth day of inoculation, the mean colony diameter of fungus was 7.2 cm. Rest of all the treatments were significantly differed over absolute control in inhibition of the fungus mycelial growth. The fungicide mancozeb @ 0.2% inhibited maximum mycelial growth (81.5% over absolute control). This was followed by treatment of chitosan @ 0.24% (T_8) wherein inhibition was 80.61%. However, these two treatments were statistically at par with each other. It was also observed that, as the concentration of chitosan lowered or minimized the inhibition percentage was lowered. At the concentration of 0.02%, the inhibition was only 28.53% (Table 1).
El Hassni *et al.* (2004) [6] stated that chitosan was reported to exert an inhibitory action on the hyphal growth of numerous pathogenic fungi, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternata* and *Pythium aphanidermatum.* Hernández-Lauzardo *et al.* (2011) [7] stated that recent studies of chitosan-fungal cell interactions showed that the polymer penetrates the cell and cause intracellular affectations. Stössel and Leuba (1984) [18], Sudarshan *et al.* (1992) [20], Wang (1992) [23], Tsai *et al.* (1999) [21] and Rhoades and Roller (2000) [16] found that the minimal growth-inhibiting concentrations of chitosan varied between 10 and 5,000 ppm.

Table 1: Per cent inhibition of growth and spore germination of *A. porri* with chitosan concentrations.

Sr. No.	Treatments	Mycelial colony growth		Spore germination (%)	
		Mean diameter (cm)	Per cent inhibition	Mean	Inhibition
1	Control	7.2 (15.55)	00.00	99.33 (87.28)	00.00
2	Mancozeb @ 0.2%	1.33 (6.62)	81.5	16.00 (23.54)	83.88
3	Chitosan @ 0.02%	5.1 (13.05)	28.53	48.00 (43.84)	51.69
4	Chitosan @ 0.04%	4.1 (11.67)	42.68	44.67 (41.92)	55.05
5	Chitosan @ 0.08%	3.57 (10.88)	50.09	36.67 (37.24)	63.10
6	Chitosan @ 0.16%	3.07 (10.08)	56.96	32.67 (34.80)	67.07
7	Chitosan @ 0.20%	2.43 (8.95)	65.62	26.00 (30.63)	73.81
8	Chitosan @ 0.24%	1.4 (6.78)	80.61	20.67 (27.02)	79.18
SE(m)±		0.31			1.42
CD(0.05)		0.96			4.34
CV		5.19			6.02

Values in parentheses are arc sin transformed.

Spore germination assay
In absolute control treatment (T_1), the spores of fungus (*A. porri*) germinated abundantly (Table 1). After 24 hrs, the spore germination was 99.33%. Remaining all treatments were significantly differed over absolute control in inhibition of spore germination. The fungicide mancozeb @ 0.2% inhibited maximum spore germination (83.88% over absolute control). This was followed by treatment of chitosan @ 0.24% (T_8) in which inhibition was 79.18%. From spore germination assay. It was observed that, as the concentration of chitosan increased from 0.02 to 0.24%, the inhibition of spore germination increased from 51.69 to 79.18%.
Hernández-Lauzardo *et al.* (2011) [7] stated that the spores are more sensitive than the hyphae to chitosan application. Meng *et al.* (2010) [12] stated that chitosan and oligochitosan inhibited the mycelial growth and germination of spores of the fungal pathogens, *Alternaria kikuchiana* and *Physalospora*. Hernández-Lauzardo *et al.* (2008) [8] specified that the low molecular weight chitosan was more effective for inhibition of mycelial growth of *R. stolonifer*, while the high molecular weight chitosan affected spore shape, sporulation and germination. Palma-Guerrero *et al.* (2008) [13] showed that chitosan completely inhibited spore germination of *Fusarium oxysporum* and *Verticillium dahlia*.

Per cent Disease Index (P.D.I.)
Disease severity was recorded since initiation of the disease i.e. at 30 DAT (Days After Transplanting). During last i.e. terminal observation which was recorded at 58 DAT, in absolute control PDI was 37.25. The lowest mean PDI as 6.21 was observed in treatment of chitosan @ 0.04% applied at 30, 45 & 60 DAT (T_8). All values under imposed treatments were statistically superior over absolute control. It is interesting to note here values of PDI under all attempted treatments excluding absolute control were statistically at par with each other. The chemical as well as non-chemical treatments had statistically similar effect in retaining the disease intensity at minimum. Per cent change over chemical treatment mancozeb

spray application was compared and found that all chitosan spray treatments excluding chitosan @ 0.02% spray 30 DAT (T_2) were with positive values. It means, these six treatments were superior over mancozeb sprays in reducing the purple blotch of onion. Among these six treatments chitosan @ 0.04% sprayed at 30, 45, 60 DAT had 24.83 per cent superiority over mancozeb spray treatment (Table2).

El Hadrami *et al.* (2010) [5] stated that chitosan is known to induce reactions locally and systemically that involve signaling cascades, and the activation and accumulation of defenses-related antimicrobial compounds and proteins. Bautista-Banos *et al*. (2003) [3] through *in situ* study on papaya fruit reported to control anthracnose disease at 1.5% chitosan applied before *C. gloeoporioides* inoculation.

Table 2: Per cent Disease Index of purple blotch of onion under influence of chitosan concentrations and number of sprays

Sr. No.	Treatments	Terminal PDI	Per cent change over	
			Untreated control (T_1)	Mancozeb (T_2)
1	Control (Water spray)	37.25 (37.60)	0.00	-258.52
2	Mancozeb @ 0.2%	10.39 (18.07)	72.11	0.00
3	Chitosan @ 0.02% spray 30 DAT	12.65 (20.50)	66.04	-21.75
4	Chitosan @ 0.04% spray 30 DAT	10.13 (18.54)	72.81	2.50
5	Chitosan @ 0.02% spray 30, 45 DAT	9.46 (17.82)	74.60	8.95
6	Chitosan @ 0.04% spray 30, 45 DAT	9.36 (17.75)	74.87	9.91
7	Chitosan @ 0.02% spray 30, 45, 60 DAT	9.21 (17.42)	75.28	11.36
8	Chitosan @ 0.04% spray 30, 45, 60 DAT	7.81 (16.07)	79.03	24.83
SE (m) ±		1.85		
CD (0.05)		5.66		
CV		15.63		

PDI: Per cent Disease Index; Values in parentheses are arc sin transformed.

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