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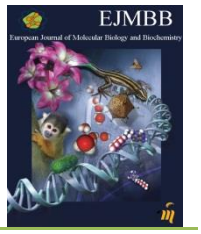

AGRICULTURAL APPLICATIONS OF CHITOSAN WITH LOW DEGREE OF DEACETYLATION

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ABSTRACT

Fungal biomass waste obtained from industrial fermentation units can form an alternate source for harvest of chitosan The spent biomass waste procured from fermentation units handling *Mucor, Rhizopus, Aspergillus* and *Tricoderma* spps gave chitosan yield ranging from 90 to 110 mg% and a low degree of de-acetylation ranging between 40 to 61%. Such chitosan molecules showed statistically significant difference in the yield of groundnut and wheat plants when used as a plant growth elicitator molecules through seed imbibition technique.

INTRODUCTION

The deleterious effect of the chemical fertilizers and pesticides in agriculture has evoked the use of biologically active & environmentally friendly substances for plant growth & protection from diseases & pest [1].

Chitosan is one such FDA approved natural polymer whose chemical & biochemical properties of non toxicity, degradability & non recalcitrance has stimulated its use in agriculture, as an auxiliary preparative for improving liquid fertilizer and as seed incrusting prep rate [1]. Hirano and Hiyashi [2] reported that coating rice, soyabeans & back pine seeds with chitosan or its derivatives induces chitinase activity which creates resistance against attack of pathogens & insects. Though Chitin /Chitosan along with abscissic acid, ethylene, jasmonic acid and other oligosaccharide molecules have been proposed as local and systemic signals of induced disease resistance [3] very few reports have dealt with its role in growth enhancement. Additionally most of the cited properties of Chitosan depends on its degree of de-acetylation (DD).

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Thus a cut off limit with regards to its acetyl content for agricultural applications needs to be formulated during the selection of the molecule for plant studies since the degradation and de-acetylation also occurs during its harvest. Legumes like groundnut are sown on an estimated 18.9 million hector land in 82 countries for use as food, oil & a high protein meal. In India it accounts for 45% area & 55% of the production of total oilseed in the country. However average production per unit area is very low as compared with other countries. Hence systematic approach to improve the production of this crop needs to be developed.

This study thus deals with the potential use of chitosan with lower degree of de-acetylation obtained from fungal biomass for growth enhancement of the commercially important plant like groundnut & wheat.

MATERIALS & METHODS

Biomass waste

Spent fungal mycelia biomass waste of *Tricoderma, Mucor, Rhizopus* and *Aspergillus* spps was collected from the effluents of the fermentation units of bulk pharmaceutical industries involved in the manufacture of cellulase, amylase and soya sauce. The discard solid biomass was obtained as one lot of compressed waste from the industry and preserved at-4ºC.

Extraction and estimation of chitosan

Chitosan was extracted as Alkaline Insoluble Fraction (AIF) using methods of Kobayashi et al [4], Arcidiacona et al [5] Miyoshi et al [6] and White et al [7]. The chitosan yield per g of AIF was estimated by Lehman & White method [8] using standard graph of glucosamine plotted within concentrations of 10 to 50 mcg/ml (Fig 1).

Degree of De-acetylation using FT-IR Spectroscopy

5 mg of chitosan was mechanically blended with KBr in the ratio of 1:100 and compressed under pressure to form KBr disc. The KBr disc was placed in a sample holder and transmittance of the IR radiation recorded between the ranges of 4000-450 cm^{-1} using the Shimadzu IR 408 spectrophotometer. The peak values in Absorbance mode at 1653 nm and 3410 nm was estimated and the % degree of de-acetylation (%DD) calculated as

$$\% \text{ degree of de-acetylation} = \frac{100 - [\log (1653)^0 A]}{[\log (3410)^0 A]} \times 115$$

Plant Studies

Chitosan harvested with varied degree of de-acetylation (DD), (28%, 33% 41%, 48% and 61%) were selected, solubilized in minimum quantity of acidified saline and then diluted to concentration of 5 mcg/ml, 25 mcg/ml, 50 mcg/ml in sterile saline. Standard 80% de-acetylated Chitosan (Sigma) was prepared similarly and used as a positive control while sterile acidified saline was used as a negative control

Seed Treatment

A set of 10 groundnut seeds & 50 wheat seeds of commercial variety were imbibed for 4 hrs in 10 ml each of the chitosan concentrations set up in triplets under gentle agitation. The seeds were transferred to 20 cms diameter pots containing 3.5 kg of garden soil. After 15-20 days of growth, the plantlets were transferred into a field plot of 49 sq ft. set up in randomized block design arrangement pattern. After 3 months the plants were uprooted and plant growth and its biometric parameters recorded. 6 groundnut and 30 wheat plants were randomly selected from each of the treatments triplicates for data analysis, using repeated measure ANOVA with primer software.

RESULTS

Induced Systemic Resistance (ISR) in plants triggered by biotic or abiotic agents in nature has gained importance, as presence of ISR in more than 30 crops has been recorded [9]. Fungal chitosan occurs in its cellwall due to the tandem action of chitin synthase and chitin deacetylase such that chitosan with an inherent degree of de-acetylation predominates in each fungal species.

Extraction procedures of de-proteinization and acidification can further deacetylate &/or depolymerise it such that variation in its degree of de-acetylation occurs. Thus evaluation of the different methods of extraction was assessed as the activity of chitosan molecule depends on its % Degree of De-acetylation (%DD), the results of which are shown in Fig 2. The methods of extraction yielded an alkaline insoluble fraction (AIF) from all the waste fungal biomass sources, except that of *Aspergillus* spps from where a negligible amount was recovered while an AIF harvest of 18 % & 16 % by Miyoshi method and Arcidiacono method of extraction respectively was obtained from *Mucor* spps.

Similarly *Rhizopus* spps yielded a maximum of 14 % of AIF by Miyoshi method of extraction followed in this case by Kobayashi method. Miyoshi method minimizes degradation through use of 0.5 N NaOH and 2% acetic acid when used at RT, in contrast to the other methods which use higher temperatures and thus may be one of the reasons for increased yield of AIF.

Additionally the AIF obtained from *Mucor* and *Rhizopus* biomass through Miyoshi method yielded the maximum yield of chitosan of 110 mcg & 100 mcg per mg of AIF. Arcidiacono's alkali extraction at 115^0C with IN NaOH showed comparable results with that of Miyoshi's method for Mucor & Rhizopus each yielding about 100 mcg/mg & 90 mcg/mg of chitosan indicating that though increased temperature affected the AIF yield, chitosan harvest from it is unaffected. This is similar to the results of Rane and Hoover [10] who reported that though no statistical difference was observed in the chitosan yield obtained by alkali treatment at different temperatures, variation in time period did show a significant difference.

Chitosan yield from the AIF of Rhizopus, & Tricoderma biomass gave more than 2 fold decrease when extracted by White method over that obtained by Miyoshi & Arcidacono's method of extraction. This may be attributed to the fact that the extraction procedure was carried out for 90 mins with the addition of ethanol. Park et al [11] reported that chitosan forms hydrogen bonds between hydroxyl groups and its amine group. Such bonding may be one of the reason by which chitosan molecules may have be removed along with the other ethanol precipitated substances leading to an decreased chitosan yield. A lower chitosan yield from all the fungal isolates was obtained using extraction procedures of Kobayashi in comparison with that obtained using the methods of Miyoshi and Arcidiacona.

This could be attributed to the fact that AIF in Kobayashi method was treated with 20% acetic acid at RT for 30 mins instead of refluxing or an overnight incubation in 2% acetic acid as used in the other two cases. This is similar to the findings of Park et al [11] who proposed that the intermolecular arrangement, its spatial configuration and its consequent precipitation is effected by the ionic strength and the degree of dissociation of the acids used.

Determination of Degree of Deacetylation (DD)

FTIR analysis was used to obtain the degree of de-acetylation and as expected, the degree of de-acetylation of chitosan obtained varied with respect to the

fungal strain and the method of extraction used. % DD of chitosan extracted by Arcidiacono modified method ranged between 33 to 42% while that obtained from Miyoshi method ranged between 28 to 44% DD. Kobayashi method comparatively was harsh as it caused DD variation ranging from 15 to 37%. (Fig 2). Though chitosan with the maximum Degree of De-acetylation of 61% was obtained from *Tricoderma* biomass waste using White method of extraction, its yield was quite low. This could be due to the fact that it was obtained from an industrial waste which had been subjected to prior treatment before its release. Additionally *Tricoderma* spps is not known to have high chitosan yield in its cell wall. Since no ester peaks at $1745cm^{-1}$ was detected in any of the fungal sample it could be inferred that triolein and related glycerides are absent and has been removed during the extraction treatment with alkali

Plant studies

Since Arcidionoca and Miyoshi methods gave reasonably high yields of chitosan from *Rhizopus* spps biomass chitosan with % DD of 41 and 28 was selected while that from *Mucor* biomass chitosan with %DD values of 33 and 44 was selected for its study as plant elicitator. Since the highest %DD of 61 was obtained it was also incorporated in the plant studies.

On groundnut plants

Chitosan at low degree of De-acetylation of 28 % did not favourably effect the growth or the yield of ground nut plant at all concentration tested as no statistically significant difference observed against the negative control **(Table 1)** In contrast, increase in its degree of de-acetylation to 33% significantly increased the harvest index of groundnut at 25 and 5 mcg/ml concentration while % shelling was significant at 25 mcg/ml concentration only. A further increase in %DD to 41 and 44% respectively significantly affected the growth and yield of the plant as detected through visual and statistical expression. At 41% DD total biomass of the plant increased by 59.5% and 28.17% due to imbibitions of chitosan at concentration of 50 & 25 mcg/ml respectively The increase at 50mcg/ml concentration was truly significant especially as increase in shoot weight was 23.35% more than that observed against the 80 % de-acetylated positive control used in this study (Fig 3). Increase in the pod weight with 41%DD chitosan was statistically observed only at 50mcg/ml (66.2%) and 25 mcg/ml (9.7%) concentration, while its seed numbers was significantly better than the results of 80% de-acetylated control plants at 50mcg/ml with an increase by 45.6%. 44% DD chitosan was found to effectively increase the biomass of groundnut plant only at 50 mcg/ml by 20.8% though statistical significant increase in the pod weight by 37.02% and 13.5 % was observed at. 50 and 25 mcg/ml concentration (Fig 4).

Thus it could be correlated that for effective increase in the yield of groundnut plant an increase in the %DD of Chitosan up to 41%-44% is required. Additionally at 44% DD, lower concentration of 5 mcg/ml can be used as it increased its % shelling and HKW while 41%DD is required at higher concentration of 50 to 25 mcg/ml for pod and seed weight increase.

On Wheat plants

In contrast to its effect on groundnut plant Chitosan, at low degree of de-acetylation of 28% did not favorably affect the yield of the plant at all concentration tested but significantly affected only its root system wrt to both its weight and length at 25 mcg/ml (Table 2).

33%DD chitosan did significantly increase the biomass of the wheat plant at 50 mcg/ml &25 mcg/ml such that lower concentration affected both the root and shoot system while 50 mcg/ml only showed a significant difference in the root length and weight.

Similar to the effect observed on groundnut plant increase in its degree of de-acetylation to 41% & 44% respectively significantly affected the growth and yield of the wheat plant. (Fig 5) Seedlings treated with 44% DD chitosan showed significant increase in the weight of the plant biomass and length of the plant when its seeds were imbibed at 50 and 25 mcg/ml concentrations of chitosan. Additionally, though HKW was not statistically significant both the concentrations significantly increased the weight and number of wheat seeds harvested. In addition to this fact, increase in the length and weight of the root system with 50 mcg/ml of 44% DD of chitosan imbibitions was truly significant as it was found to be more than the positive control. Increase in the number and weight of seeds from 44% DD chitosan treated plants was statistically significant at 50 mcg/ml and 25mcg/ml concentration which was also found to be better than the results of 80% de-acetylated control plants unlike 41% DD chitosan treated plants which were found to be significantly different only against negative control mainly at 50 mcg/ml. 61% DD chitosan treated plants at 0.7 mcg/ml concentration caused an increase in all the biometric parameters in comparison to the positive and negative control while 6% & 10%DD caused no statistically significant improvement in the wheat plant similar to that obtain with groundnut plants.

Thus it could be correlated that DD between 25-35% of chitosan affected the root system & shoot system of the wheat plant while treatment with 41%-44% DD chitosan showed an overall increase in the growth and yield of the wheat plant at concentration of 50 mcg/ml and 25 mcg/ml where as the lower concentration of 5 mcg/ml did not affect any growth parameters. Similarly biometric parameters of groundnut plants is best stimulated by imbibitions of 41% Chitosan at 50, 25, or 5 mcg/ml concentration or by using 44%DD Chitosan at 50 mcg/ml.

Table 1. Effect of chitosan with varying %DD on groundnut growth and yield studies

Chitosan Treatment at % DD/ conc mcg/ml	Shoot length/ plant	Shoot weight plant	Root length/ plant	Root weight/ plant	Total weight/ plant	Num of pods/ plant	Weight of pods/ plant	Numb of seeds/ plant	Weight of seeds/ plant	Har vest Index	% Shelling	HKW
33% 50	22.49	7.35	14.15	0.685	10.95	10.0	2.915	8.95	.715	.705	24.52	8.00
25	21.23	6.11	18.25	0.850	8.58	6.0	1.615	7.95	.655	***.811**	**40.26**	8.17
5	25.6	6.85	16.55	0.626	9.32	7.95	1.85	7.00	.475	***.802**	25.69	6.985
41%50	***42.28**	***12.15**	**22.5**	**1.39**	**19.70**	**13.95**	***6.15**	***19.95**	**1.79**	.687	29.15	8.95
25	**37.15**	**10.43**	**22.0**	**1.33**	**15.83**	10.0	**4.06**	**13.95**	**1.25**	***.741**	**30.88**	8.97
5	**32.25**	8.63	18.25	**1.645**	12.40	6.1	2.12	8.95	0.81	***.828**	**38.22**	9.14
44%50	**33.2**	8.42	20.45	**1.43**	**14.93**	**12.0**	***5.07**	**12.00**	**1.43**	.659	28.18	9.30
25	26.92	5.11	17.35	0.924	10.24	7.80	**4.20**	10.0	1.45	.589	24.85	10.91
5	22.75	4.63	16.25	0.785	9.39	6.0	3.97	7.95	1.06	.577	**41.18**	**15.66**
28%50	24.5	5.72	15.45	0.616	10.11	8.95	3.77	9.00	0.75	.626	19.99	8.57
25	27.3	4.95	14.85	0.519	8.27	6.0	2.81	7.95	0.59	.660	21.17	7.54
5	23.15	4.46	20.45	0.91	7.53	4.9	2.15	7.00	0.62	.713	28.77	8.85
61%	30.5	**9.41**	**25.48**	**1.195**	**15.26**	9.95	**4.65**	**16.0**	**1.64**	.694	35.26	10.27
+ve Control	35.4	9.85	24.55	1.55	16.35	10.55	3.75	13.7	2.3	.694	48.75	17.05
-ve Control	28.46	7.65	19.72	0.845	12.35	7.7	3.7	7.45	0.945	.693	25.27	4.79
CD(5%)	*3.2*	*1.06*	*2.18*	*0.177*	*1.107*	*2.40*	*0.28*	*3.6*	*0.187*	*.036*	*5.51*	*7.85*

KEY Bold ---Statistically significant difference over negative control
CD. ---Critical Difference at p=0.05 calculated using Primer software
*** ---- Statistically significant difference over positive control**

Table 2. Effect of chitosan with varying % DD on growth and yields of wheat plants

Treatment at % DD	Conc of chitosan	Root length/plant	Root weight/plant	Shoot length/plant	Shoot weight/plant	No.of seeds	Weight ofseeds	Hundred weight Kernel
	mcg/ml	cms	gms	cms	gms		gms	%
33	50	**13.49***	**0.14**	28.29	5.793	17.99	0.9	4.987
	25	**10.8**	**0.12**	**30.5**	**6.49**	16.99	0.85	4.933
	5	10.2	0.09	27.1	5.393	14.99	0.746	4.9
41	50	**10.61**	**0.10**	**35.42***	**7.193**	**24.99**	**1.247**	**5.033**
	25	**12.45**	**0.148***	**31.53**	**6.933**	22	1.067	4.833
	5	8.503	0.09	28.29	5.89	19.92	0.99	4.933
28	50	8.297	0.089	23.5	4.89	20.01	0.996	4.967
	25	**10.99**	**0.12**	22.1	5.087	15.99	0.796	4.9
	5	9.707	0.08	19.71	4.607	18.37	0.89	4.9
44	50	**15.4***	**0.18***	**34.61***	**7.087**	**33.32***	**1.5***	4.653
	25	**10.61**	**0.11**	**38.61***	**6.717**	**31.99***	**1.6***	4.8
	5	8.093	0.08	22.5	4.6	19.00	0.926	4.967
61	0.7	**14.48***	**0.16***	**33.49***	**7.196**	**27.98***	**1.4***	**5.1**
Negative control		9.923	0.096	28.48	5.827	20.5	1.023	4.933
Positive control		12.31	0.13	32.42	7.41	24.96	1.247	4.935
C.D (5%)		*0.33*	*0.011*	*0.44*	*0.16*	*1.08*	*0.102*	0.2

KEY Bold ---Statistically significant difference over negative control
CD. ---Critical Difference at p=0.05 calculated using Primer software
*** ---- Statistically significant difference over positive control**

DISCUSSION

Fungal chitosan offer an attractive alternate source for harvest of this polymer with unique physicochemical properties due to the alteration in its %DD that occurs during its synthesis as well as its recovery [12]. Such chitosan obtained have been reported to be more effective in inducing plant growth than the chitosan originating from shellfish chitin [12,13]. White et al [7] reported that chitosan isolated from *M. rouxii* cell wall can have %DD ranging from 80 to 50%, while Fukamizo et al [14] found cell wall components of *Fusarium oxysporum* to have 25 –35% acetylated chitosan.

Arcidiacono et al [5] reported that yields and molecular weight variation was influenced by extraction procedures, culture type, and length of incubation with reagents. This was similar to our results wherein various %DD chitosan ranging from 15 to 49% was harvested.

Tsugita [15] studied the effect of de-acetylated chitosan at different concentrations of 0.01%, 0.05%, 0.1% 0.5%, and 1.0% on growth of radish and observed that seed treatment by chitosan induced growth acceleration wherein the lowest concentration of chitosan (0.01%) showed the highest growth of root with an increase by 51.4%, an increase in the total plant weight by 72.4% & leaf length by 11.1%. Tsugita [15] also reported that 80 % de-acetylated chitosan (0.2%) has a growth accelerating effect, increasing the number of potatoes developed by 46.8% and the weight of the potatoes by 58.3% as compared to the untreated control. In contrast, our studies showed that lower degree of de-acetylation ranging between 33 to 44% can have a plant stimulating effect and it is not necessary to invest resources in obtaining higher de-acetylation ratio. Additionally, the chitosan effect is maximum at low concentration of 50 and 25 mcg/ml indicating that lowering de-acetylation ratios did not affect the concentrations required for it to act as an elicitator molecule Imbibitions of seeds affects the seed envelope permeability, causing microscopic ruptures through which chitosan oligosaccharides can diffuse. Hadwiger [16] reported that H^3 radiolabelled chitosan applied to wheat seeds was detectable even in leaves of seedling plants. This implies that interaction with the chitosan molecule might be initiated upon germination causing interactions between the emerging seedling and the seed coat, which could led to transfer of chitosan from seed coat to both the emerging plumule & radicle.

Ghaouth & Arul [17] reported that chitosan enhances elicitation of free phenolic compounds in peanut to activate disease resistance response genes while Sanford (18) reported increase in resistance response associated proteins, and increase in the enzymes chitinase, glucanase and phenylalanine ammonia lyase along with accumulation of pisatin, callose, and lignifications in response to chitosan intake. Such combined responses following chitosan application may aid to increase the vitality of host cells and hence plant growth [19]. Lignification was found to be enhanced when partially de-acetylated chitosan were used as compared to fully de-acetylated polymers (20). Reddy [19] reported that partially de-acetylated oligomers of chitosan inhibited light induced stoma opening with an accelerated stoma closing and this may be means through which invasion of pathogens is inhibited.

The results obtained indicate that agriculture is a very promising field for the application of chitin derivatives & in future the field of application needs to be broadened by studying the effect of chitin derivatives on various commercially important plants in horticulture & agriculture.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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