Grafting of Vinyl Acetate onto Chitosan and Biocidal Activity of the Graft copolymers

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ABSTRACT: Modification of chitosan by grafting with vinyl acetate (VAc) was carried out using potassium persulfate and sodium bisulfite as redox initiators. The effect of monomer, initiator concentration, time, and temperature was studied. The grafted samples were subjected to alkaline hydrolysis and the polyvinyl acetate (PVAc) branches were consequently partially converted into polyvinyl alcohol (PVAl) graft, which showed enhanced swelling in water. The graft copolymers showed a better dye uptake for both acidic and basic dyes. Chitosan/VAc and chitosan/VAl copolymers were both subjected to reaction with dimethyl sulfate in alkaline medium to yield quaternized copolymers. The antifungal behavior of chitosan and its graft copolymers was investigated *in vitro* on the mycelial growth, sporulation, and germination of conidia or sclerotia of the following sugarbeet: *Beta vulgaris* pathogens isolated in Egypt, *Rhizoctonia solani* Kühn ($AG_{2\text{-}2}$) *Sclerotium rolfsii* Sacc. and *Fusarium solani* (Mart.) Sacc. These polymers were also screened against several fungi and it has been found that grafting with polyvinyl alcohol branches enhances the antifungal activity dramatically. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 1651–1663, 2007

Key words: chitosan; graft copolymer; vinyl acetate; hydrolysis; dye uptake; swelling; quaternization; antifungal activity

INTRODUCTION

Chitin is a polysaccharide having an acetamide group at C-2 and can be converted into chitosan with free amino groups at the C-2 position using strong alkali. It is the second most abundant natural polymer after cellulose or the third if we consider lignin. Although chitin is structurally similar to cellulose and is produced in huge amounts in nature, it is still almost an idle biomass owing to its inherent intractability and insolubility in most of the known solvents. After hydrolysis, chitosan is formed and it is considered as a random copolymer of β-(1,4)-2-acetoamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. Because of its special biological, chemical, and physical properties, chitosan and its derivatives have been applied in many industrial, agricultural, and biomedical fields.[1,2] Chemical modification of chitosan to impart new properties has attracted the attention of many researchers in recent years.[1–4] Chitosan is nontoxic and bioabsorbable with gel-forming ability at low pH.[5] It has antiacid antiulcer activities, which prevent or weaken drug irritation in the stomach.[6] All these interesting properties make this polymer and its derivatives ideal candidates for controlled drug release formulations. Graft copolymerization is expected to be one of the most promising methods leading to the design of novel tailored hybrid materials composed of natural polymers and synthetic ones. The resulting graft copolymers could be widely controlled by the characteristics of the side chains. Thus, chitosan was grafted with styrene[7,8] and acrylamide[9] using γ radiation. Acrylamide was also used to produce graft copolymer with crosslinked chitosan for controlled drug release.[10] Grafting of chitosan was performed with acrylonitrile,[11] vinyl acetate,[12,13] methyl methacrylate,[11,14,15] hydroxyelthyl methacrylate, HEMA[16,17]; HEMA was also plasma-induced grafted to chitosan to produce membranes,[18] vinylpyridine,[19,20] methyl acrylate.[19,20] Chitosan-*graft*-polystyrene, prepared by radiation induced method, showed higher adsorption of bromine than chitosan. Polystyrene-grafted chitosan films showed less swelling and better elongation in water than chitosan films.[21,22] Chitosan grafted with poly(acrylic acid) produced a hydrogel with enhanced water uptake.[23] Chitosan and its derivatives are becoming increasingly important because of their metal chelating ability.[24–30] Chitosan-Zn complex has been found to have an antimicrobial activity as well as a potential use as medicament or nutriment.[31]

In this work, we studied the modification of chitosan by grafting copolymerization with VAc to investigate eventual changes produced in the properties of the products and to compare this with that of the unmodified chitosan. Moreover, the combined effect of the

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principal reaction variables for the grafting process was systematically studied. The chitosan-*graft*-polyvinyl acetate copolymers were also hydrolyzed to the corresponding polyvinyl alcohol analogue. The swelling behavior of the graft copolymer before and after hydrolysis was investigated. A bioassay of the fungicidal activity of chitosan and the grafted chitosan copolymers and their complexes with several metals ions has been investigated.

EXPERIMENTAL

Samples

The chitosan was kindly supplied by Prof. Dr. Furuhata of Tokyo Institute of Technology (T.I.T) (with 83% extent of deacetylation[20]). Vinyl acetate, from ACROS organics, was distilled just before its use (bp 73°C). Initiators (potassium persulfate and sodium bisulfite) were analytical grade reagents from Merck chemicals and were used as received. All solvents from Aldrich were purified by distillation according to the conventional methods.

Grafting reactions

Heterogeneous grafting reaction

An exact amount of dry chitosan was mixed with water in stopper flask, with 1 : 50 liquor ratio, followed by the addition of monomer and initiator in this order. The flask was placed in a thermostated bath and the reaction mixture was shacked occasionally. After a suitable time, the product was filtered, washed with water to remove the unreacted monomer and dried at 60°C till constant weight. Exhaustive extraction of the product with methanol in a soxhlet allowed the separation of polyvinyl acetate homopolymer (PVAc) formed during the grafting reaction. The graft copolymer was thoroughly washed with water, followed by drying to constant weight. The PVAc homopolymer, separated by methanol extraction, was precipitated in water and its structure was confirmed by Fourier transform infrared (FTIR) spectroscopy.

Homogeneous grafting reaction

The same above-mentioned procedure was repeated except that the chitosan sample was dissolved in 5% diluted acetic acid and mixed with monomer and redox initiators in this order. At the end of the grafting reaction, the medium was neutralized with enough ammonia to precipitate the graft copolymer. The flask was filtered and the precipitate was extracted with methanol as described earlier. The graft copolymer was dried and subjected to elemental analysis (C, H, and N) and IR-spectroscopy.

The grafting parameters such as grafting percentage (%*G*), homopolymer percentage (%*H*), and grafting efficiency percentage (%*E*) was determined as follows:

$$\%G = \frac{W_2 - W_1}{W_1} \times 100$$

$$\%H = \frac{W_4 - W_2}{W_3} \times 100$$

$$\%E = \frac{W_2 - W_1}{W_3} \times 100$$

where, W_1, W_2, W_3, and W_4 denote the weight of initial chitosan, grafted chitosan after extraction with methanol, vinyl acetate monomer charged, and grafted chitosan before extraction with methanol, respectively.

FTIR analysis

FTIR spectra were taken using Bruker Vector 22 spectrometer in the range from 400 to 4000 cm^{-1}.

Thermal analysis

Thermogravimetric analysis was conducted using a Shimadzu TGA-50H at a heating rate of 10°C/min under nitrogen atmosphere.

Hydrolysis of the graft copolymers

Alkaline hydrolysis of vinyl acetate chitosan copolymer

About 1 g grafted chitosan sample is refluxed in 50 mL 10% NaOH in (1 : 1) methanol/water solution for 4 h. The hydrolyzed sample was washed with water and dried. The weight loss was taken as a measure of the degree of hydrolysis. FTIR and elemental analysis confirmed also the hydrolysis process.

Swelling measurements. The swelling degree was measured by the tea bag method at room temperature (25°C), where the polymer samples were placed in a tea bag and immersed in distilled water for the required time. The tea bag was then hung for 5 min to remove the unabsorbed water and weighed. The % swelling $= (W - W_0)/W_0 \times 100$, where W is the weight of the swollen sample, and W_0 is that of the dry sample.

Dye uptake measurements. In a 100-mL round-bottomed flask, 0.5 g of the grafted sample was charged together with 25 mL of 1% dye solution (molar ratio 1 : 50). The flask was placed in a water thermostat and the temperature was raised during 30 min to 95°C. The mixture was refluxed for 25 min at this temperature. The solution was left to cool to room temperature then filtered and the filtrate was transferred to a 25-mL measuring flask and completed to the mark. The UV–visible spectra of the samples were then measured.

A calibration curve for each dye was previously constructed and the amount of dye absorbed onto each grafted sample could be then determined from the difference of absorption of the solution before and after the reaction with the grafted samples. To investigate the dye stability onto the grafted copolymers, the dyed samples were boiled in water for 30 min, left to cool, and the liquor solution was transferred to a measuring flask and completed to the mark and finally the concentration of the leached dye was determined by UV spectroscopy using the calibration curve of each dye as described earlier. In another set of experiments, the dye uptake was determined at 37°C to simulate normal dye removal at room temperature.

Metal/polymer complexes

0.1*M* of metal ion (Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Cd^{2+}) solution and 0.5 g grafted chitosan sample were mixed well. The above mixture was subjected to stirring overnight. The chitosan/metal complexes were then filtered washed with distilled water and dried in oven at 60°C. The absorbed amount of metal ion was calculated by comparing the amount of metal ion before and after the treatment with the grafted polymers. The absorbed amount was expressed in mole of metal ion per mole of grafted sample.

Bioassay for fungicidal activity

Effect of chitosan and its graft copolymers on the soil-borne sugar-beet pathogens

Sources and culture of fungi. The fungi used in this work were isolated from diseased sugar beet roots.[32] These fungi were maintained in culture on modified Czapek-Dox agar,[33] which consisted of: sucrose, 20 g/L; KNO_3, 4 g/L; Na_2HPO_4, 2 g/L; $MgSO_4 \cdot 7H_2O$, 1 g/L; KCl, 0.5 g/L; yeast extract, 0.02 g/L; microelement mixture 1 mL, agar, 15, and distilled water, 1 L. Chitosan was dissolved in 0.04*M* HCl and the pH was adjusted to 5.6 with 2.0*N* KOH. The polymer was added to the nutrient solution to obtain chitosan concentration of 10–50 μg mL^{-1}.

Germination of macrcoconidia. Microscope slides were covered, each, with 1 mL of the microconidal suspension of *F. solani* in aqueous solution of the desired chitosan concentration in petri dishes and then incubated at 27°C for 9 h in complete darkness. The percentage of germination and the average length of the germ-tubes were assessed.[34] Five plates were prepared for each treatment and the means were compared.

Germination of sclerotia. Sclerotia of *R. solani* and *S. rolfsii*, produced on potato dextrose agar (PDA) and Czapek-Dox agar, respectively, were surface disinfected by soaking them for 5 min in 1 : 400 (w/v) bromine/water to kill hyphal extensions, washed thoroughly with distilled water, and dried. Ten sclerotia per petri dish for either pathogen were plated on the surface of tap water agar (1.5% w/v) supplemented with the relevant amounts of purified chitosan to produce concentrations in the range of 10–50 μg mL^{-1} in the medium. The dishes were incubated at 27°C for 12 h for *R. solani* and 30 h for *S. rolfsii*, and the percentage of germinated sclerotia and average length of hyphal extensions were determined. Five plates were prepared for each treatment and the means were compared.

Dry mass. Purified chitosan or chitosan derivatives were mixed aseptically with Czapek-Dox to produce medium concentrations of 10–50 μg mL^{-1} and dispensed in 50 mL aliquots into 250 mL Erlenmeyer flasks. A 6 mm agar disk bearing hyphae of either *R. solani* or *S. rolfsii* from 7-day-old colonies were incubated at 27°C for 9 days. The mycelium was harvested, dried to constant weight at 80°C, and the dry mass yield and final pH value were recorded. Five flasks were prepared for each treatment and the means were compared.

Production of sclerotia. PDA was used for *R. solani* and Czapek-Dox agar for *S. srolfsii.* Dried chitosan powder was mixed with the medium to produce the required concentration and poured into petri dishes. The fungi were transferred to the dishes and incubated at 27°C for 9 days. For *R. solani*, 1 mL of the hyphal suspension was added to each dish. This was prepared by transferring two 6-mm diameter PDA disks bearing hyphae into potato dextrose broth (PDB) in 250-mL Erlenmeyer flasks, each containing 50 mL of the medium. The flasks were incubated at 27°C for 3 days and filtered; the mycelial mats were washed with sterile distilled water. This mycelium was homogenized with 100 mL sterile water in a sterile microblender for 3 min to form a heavy suspension.[35] For *S. rolfsii*, one 6-mm diameter agar disk bearing hyphae of the fungus was transferred to each dish. The number of sclerotia produced per plate in each treatment was visually counted. Five plates were prepared for each treatment and the means were compared.

Production of macroconidia. Modified Czapek-Dox agar was mixed aseptically with purified chitosan powdered extracted in amounts calculated to produce the required concentration and poured into petri dishes. The dishes were incubated with a 6 mm disk of mycelium of *F. solani,* incubated for 9 days at 27°C, and the number of spores produced was calculated by a hemocytometer.[34]

Effect of chitosan and its graft copolymers on some soil fungi genera

The susceptibilities of the test fungal spore (*Aspergillus niger*, *Cladosporium herbarum*, *and Fusarium moniliforme*) as seeded in Dox's medium on filter paper discs (6 mm) soaked with 5 mg mL^{-1} of each compound, were determined.[36] The soaked and completely dried

filter paper discs were placed on the surface of the seeded Dox medium in triplicate tests for each compound. Plates were allowed to stand for 2 h to allow for diffusion. Later on, the plates were incubated for 48 h, after which the susceptibility of each organism to each compound was estimated by measuring the diameter of the zones of inhibition.

Determination of the minimum inhibitory concentration of the fungicidal compounds

The MICs of the isolated compounds on *Aspergillus niger*, *Cladosporium herbarum*, and *Fusarium moniliforme* were determined by the dilution method.[37] Known amounts of the purified isolated compounds were dissolved in sterile distilled water and serial dilutions prepared in the same solvent. The minimum inhibitory concentration was determined by incorporating a known volume (20 μL) of each dilution in a test tube with 2 mL of liquid Dox's medium containing 10,000 cfu (color forming units) of the test organisms. The tubes were incubated at 28°C and the results recorded after 2–5 days. Tubes prepared in an identical manner but devoid of the active substance served as controls. MIC was the lowest concentration required to completely inhibit the growth of the test organisms.

Statistics

The experiments were conducted in 3–5 replicates, and the results obtained were treated statistically with an analysis of variance and the significance was expressed at LSD 5 and 1%.

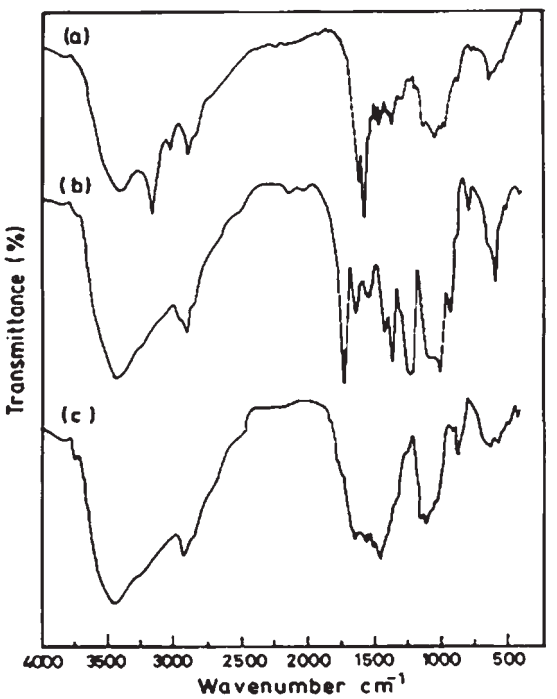

Figure 1 IR spectra of (a) chitosan, (b) VAc/chitosan graft copolymer with 105%*G*, (c) the same graft after hydrolysis to VAl/chitosan.

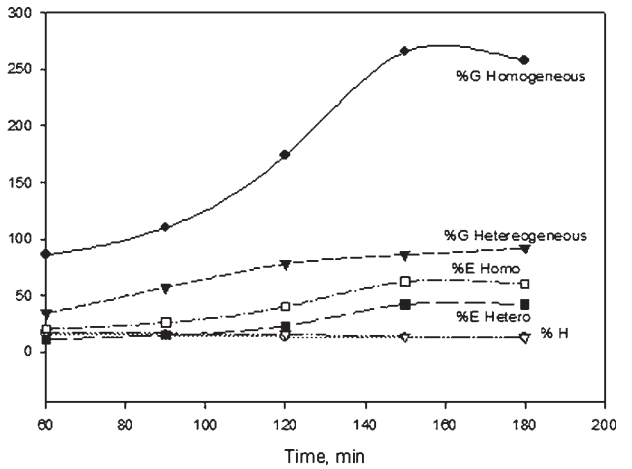

Figure 2 Effect of time on the extent of %*G*, %*H*, and %*E* of chitosan and PVAc both in homogeneous and heterogeneous conditions.

RESULTS AND DISCUSSION

The existence of grafting was established from the weight increase and by comparing the IR-spectra of pure chitosan and the graft copolymer as shown in Figure 1. A noticeable difference corresponds to the appearance of the carbonyl group absorption band at 1743.5 cm^{-1} in the graft copolymer. Moreover, a characteristic band for the CH_3 bending vibration of the vinyl acetate appears at 1376–1437 cm^{-1}. It is interesting to note that those two characteristic bands disappear from the graft spectra when the copolymer is subjected to hydrolysis and conversion of the acetate groups of the poly(vinyl acetate) into hydroxyl groups as shown also in Figure 1(c). The expected O—H stretching vibration band at 3446 and 3191 cm^{-1} due to

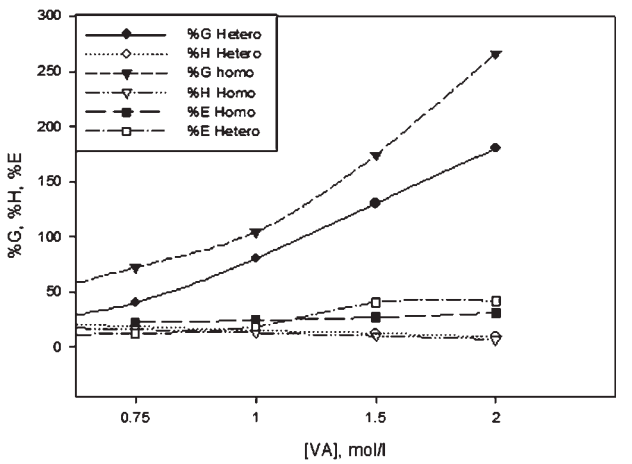

Figure 3 Effect of vinyl acetate concentration on %*G*, %*H*, and %*E* under homogeneous and heterogeneous conditions.

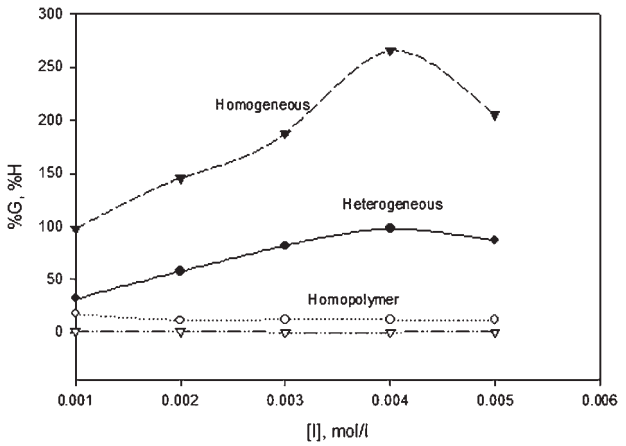

Figure 4 Effect of initiator concentration on %G and %H in homogenous and heterogeneous conditions, 1*M* VA at 55°C.

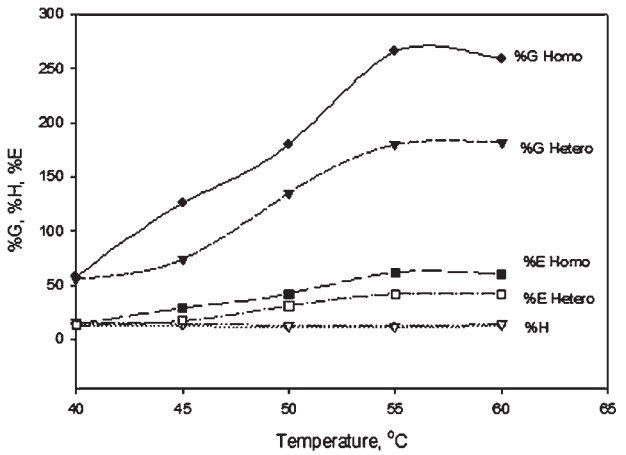

Figure 6 Effect of temperature on the extent of grafting and homopolymer formation of vinyl acetate under homogeneous and heterogeneous conditions, 2*M* vinyl acetate 1:0.75 $K_2S_2O_8$:Na_2SO_3.

H-bonding are overlapped with the N—H stretching in this region. The N—H bending vibration at around 1641 and 1602 cm^{-1} are both sharp and quite intense.

Elemental analysis could be used as a second evidence for the occurrence of grafting through the noticeable decrease in the nitrogen content with the grafting percentage.

Influence of the reaction conditions on the grafting extent

The effect of the reaction variables on the extent of grafting (%*G*), the grafting efficiency (%*E*), and the homopolymer percentage (%*H*) are shown in Figures 2–6. The effect of time of grafting, and monomer concentration was studied by using a set of predetermined reaction conditions based on preliminary tests. Figure 2 describes the effect of time and shows that %*H* is almost constant during the investigated time interval, while the %*G* increases and reaches almost a constant value after 140 min. Figure 3 illustrates that the percentage of grafting increases with increasing the vinyl acetate concentration, and reaches 180% and 266%, at 1.5*M* vinyl acetate, for the heterogeneous and homogeneous conditions, respectively.

Another reaction parameter has been investigated in the present work; the grafting was conducted in homogeneous as well as heterogeneous conditions and it was found that homogeneous medium showed a higher extent of grafting.

The effect of initiator concentration and the ratio of its components (Figs. 4 and 5) on the %*G* were studied at 55°C with 1*M* vinyl acetate for 3 h. Here, the maximum %*G* was obtained at approximately $4 \times 10^{-4}M$ of the redox initiators with ratio 1 : 0.75 potassium persulfate/sodium bisulfite.

In a single experiment, the combined effects led to a %*G* of 360%. Figure 6 describes the effect of tempera-

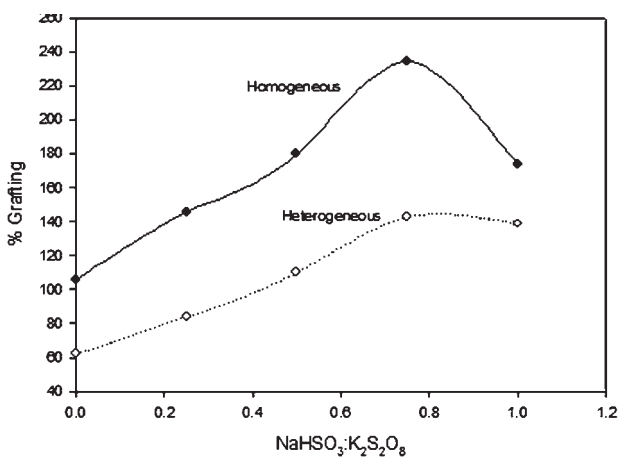

Figure 5 Effect of the ratio of the redox initiator on the extent of grafting of VA onto Chitosan, 1*M* VA at 55°C for 3 h.

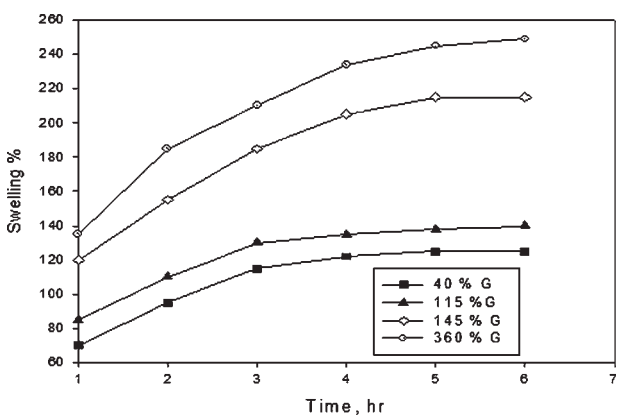

Figure 7 Swelling behavior of the grafted chitosan/VAc copolymers at different extent of grafting.

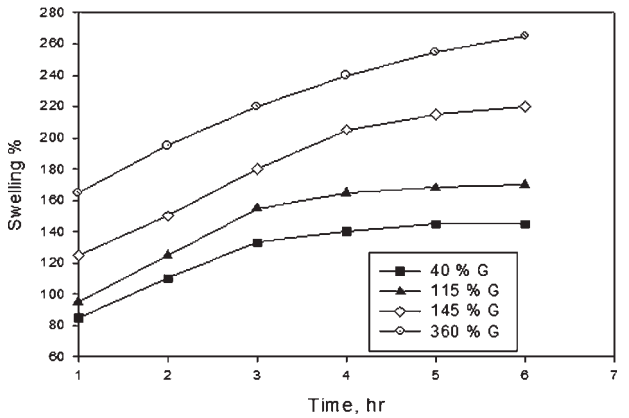

Figure 8 Swelling behavior of the chitosan/VAl copolymers.

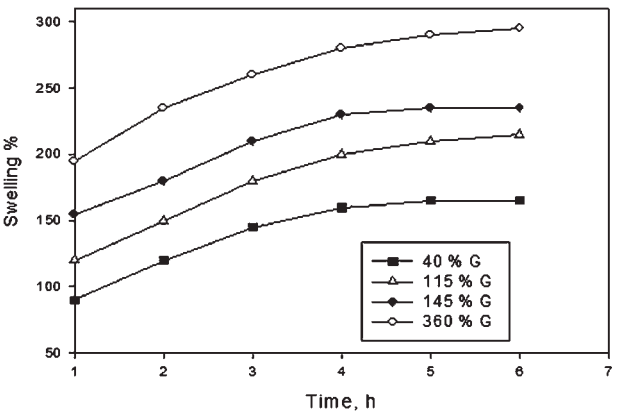

Figure 10 Swelling behavior of quaternized chitosan/VAl graft copolymer.

ture on the extent of grafting a maximum was observed at around 55°C.

From the results obtained in this work, it was clear that the homogeneous grafting method showed a marked enhancement in the grafting percentage (%*G*) up to 266% over the heterogeneous method. The optimum conditions for the grafting were obtained at 2*M* vinyl acetate, $4 \times 10^{-4}M$ of redox initiators with ratio of 1 : 0.75 potassium persulfate/sodium bisulfite, at 55°C and for about 3 h.

Hydrolysis of polyvinyl acetate/chitosan copolymer

To prepare polyvinyl alcohol/chitosan copolymer (chitosan/VAl), basic hydrolysis for the graft copolymer of vinyl acetate was carried out in 10% NaOH solution and refluxed for about 4 h, then the product was filtered, dried, and purified. The structure of the produced polyvinyl alcohol/chitosan copolymer was confirmed by IR-spectroscopy (Fig. 1).

Swelling behavior of chitosan/VAc grafted copolymers and their hydrolyzed and quaternized analogues

From Figures 7 and 8, it is clear that the extent of swelling increases with increasing the %*G*; this finding is in contrast to the work by Don et al.,[20] where the swelling extent decreased after grafting with vinyl acetate; they however reported an enormous swelling extent for pure chitosan, which was not confirmed in our work, increasing swelling with grafting should be a logic results since grafting should increase intermolecular spacing allowing more water to be trapped. The introduction of hydroxyl groups in the graft copolymer, as a result of hydrolysis, further increases the extent of swelling, which could have an important impact on the practical biomedical applications of this copolymer. Quaternization of the chitosan/VAc copolymers led also to an improvement in the swelling degree. However, when the hydrolyzed chitosan/VAl copolymer was subjected to quaternization reaction, no

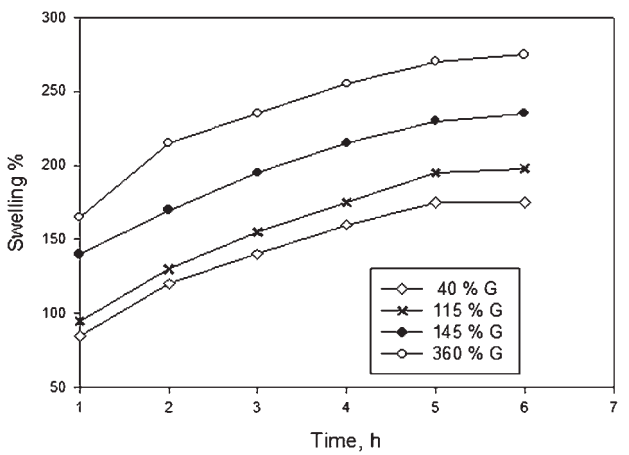

Figure 9 Swelling behavior of the quaternized graft chitosan/VAc copolymers. % Grafting is the same as given in Figure 7 and 8.

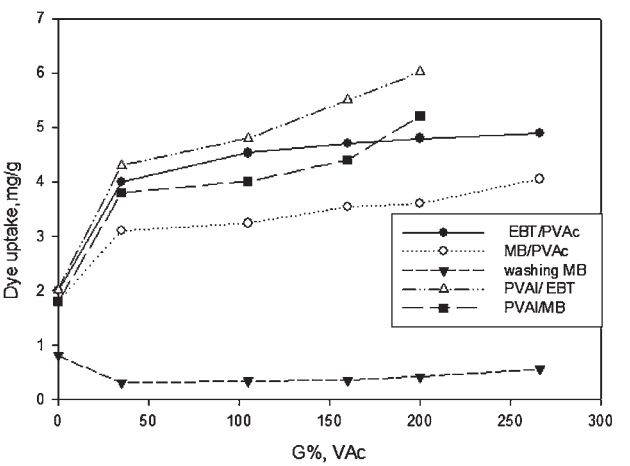

Figure 11 Dye uptake and fastness of EBT and MB by chitosan/VAc and chitosan/VAl graft copolymers as a function of the % grafting, pH = 6.5, and temperature 95°C.

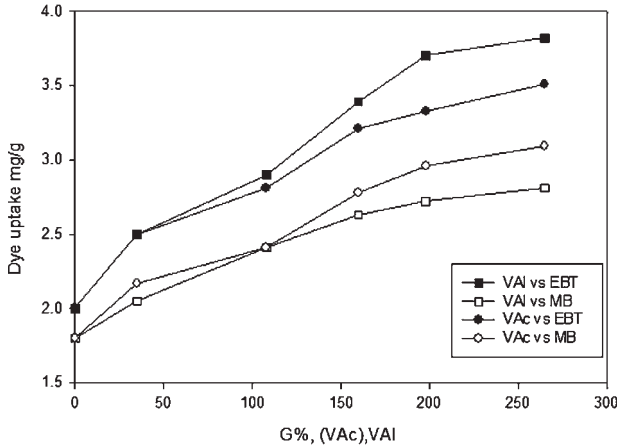

Figure 12 Combined dye uptake of chitosan/VAc, and chitosan/VAl graft copolymers, $T = 37°C$ and at pH = 6.5.

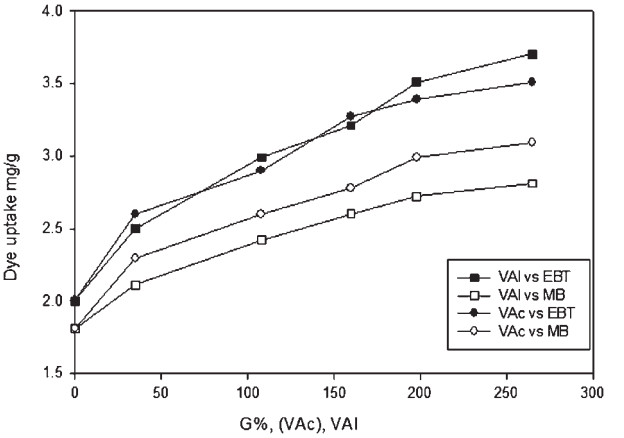

Figure 14 Combined dye uptake of chitosan/VAc, and chitosan/VAl graft copolymers, $T = 37°C$ and at pH = 10.

improvement of the swelling degree was observed as can be noticed from Figures 9 and 10.

Dye uptake of the grafted chitosan/VAc copolymers

Samples of copolymers with different degree of grafting have been investigated with respect to their ability to absorb dyes. Two types of dyes have been used: acidic erochrome black T (EBT), and basic one methylene blue (MB). The dye uptake was expressed as milligram of dye per gram of graft copolymer. To determine the dye fastness, the dyed samples were boiled in certain amount of water for 30 min, and the liquor was filtered, cooled, and completed to the mark of a measuring flask, then the UV absorbance was measured. From the calibration curve, the amount of the washed dye was estimated. Figure 11 shows the dye uptake of chitosan, chitosan/VAc and chitosan/VAl graft copolymers. The dye uptake, which was measured by raising the temperature to 95°C, increases with increasing the grafting percentage with a higher uptake for the acidic dye. Samples treated with EBT (acidic dye) did not show any trace of leached dye while the samples treated with MB showed small leaching of the dye as shown in Figure 11. The acidic dye was absorbed to higher extent (chemisorption) as expected since the chitosan has a cationic character attracting thus the acidic molecules of the EBT.

The dye uptake should be highly sensitive to the variation of the pH therefore the dye uptake was investi-

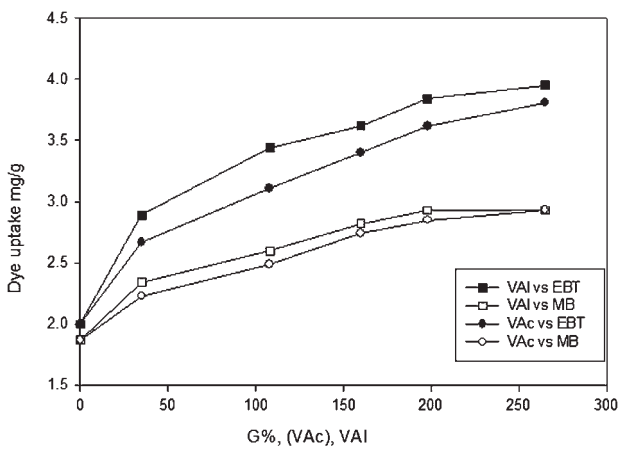

Figure 13 Combined dye uptake of chitosan/VAc and chitosan/VAl graft copolymers, $T = 37°C$ and at pH = 3.4.

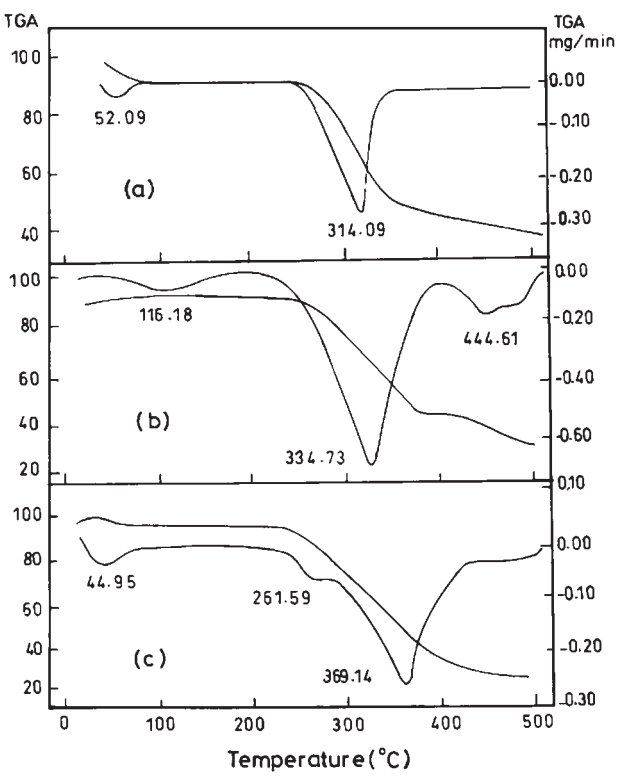

Figure 15 Thermogravimetric analysis of (a) chitosan and (b) polyvinyl acetate homopolymer (c) chitosan/VAc, 200% *G*.

TABLE I
Effect of Chitosan Concentrations on the Percent Germination (%*G*), Average Length of Hyphal Extension (L_h), Dry Mass Yield (D_m), Production of Sclerotia of *Rhizoctonia solani, Sclerotium rolfsii*, and Macroconidia of *Fusarium solani* at 27°C

μg mL^{-1}	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R. solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
10	50.2	800.1	960.7	5.7	7.4	59
20	48.2	709.2	870.3	5.7	7.4	48
30	38.1	601.2	800.4	5.7	7.4	28
40	20.3	403.2	609.3	5.6	7.2	19
50	6.5	109.3	220.4	5.6	7.2	8
LSD						
1%	3.9	13.7	17.5	–	–	6.5
5%	1.5	8.9	9.8	–	–	2.7
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
10	51.3	600.2	570.2	5.7	3.7	591
20	42.1	503.1	471.1	5.6	3.7	500
30	27.1	373.2	379.4	5.6	3.8	341
40	19.4	219.2	228.2	5.5	4.0	219
50	4.5	68.3	100.1	5.5	4.0	114
LSD						
1%	4.2	14.1	17.6	–	–	6.3
5%	1.6	8.7	9.7	–	–	2.7
F. solani	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia (10^4 mL^{-1})
Control	63.2	12.4	357.2	5.8	4.3	314.7
10	60.1	10.6	341.1	5.7	4.2	281.2
20	45.3	9.3	301.2	5.7	4.2	261.2
30	39.6	7.3	219.4	5.6	4.1	182.1
40	17.4	5.6	188.3	5.6	4.1	164.3
50	8.6	3.6	126.2	5.6	4.0	118.2
LSD						
1%	8.4	2.6	11.8	–	–	18.2
5%	5.5	1.2	4.9	–	–	7.8

gated at three pH namely acidic, neutral, and basic conditions and at temperature much lower than in Figure 11 namely 37°C. Figures 12–14 represent the effect of pH on the dyeabilty of chitosan/VAc and chitosan/VAl graft copolymers.

From these figures, one may observe that the dye uptake increases with decreasing the pH the same observation as was found in the example of chitosan/PVP graft copolymers.[38] The hydrolysis of the polyvinyl acetate branches led to a very small enhancement of the dye uptake, which is somehow unexpected; it should have been much higher effect due to the presence of the much hydrophilic OH groups as opposed to the acetate groups. Figure 14 shows the dye uptake at pH 10 where the trend is some what reversed as VAc graft absorbed the dye almost as the VAl graft or even slightly higher. The dye uptake increases with increasing the temperature as can be seen by comparing Figure 11 and Figure 12, at the same pH but different temperatures.

Thermal analysis

The thermogravimetric analysis of chitosan and one graft copolymer is shown in Figure 15. The unmodified chitosan shows a small weight loss before 100°C due to the loss of water, followed by one major decomposition band with a maximum temperature at 314°C. The figure also shows the thermal decomposition of polyvinyl acetate homopolymer, which shows major decomposition at 334°C. On the other hand, the grafted sample (98%*G*) showed the same water loss than a small decomposition at around 310°C probably due to the loss of the PVAc side chain, followed by the major decomposition band at 388°C. Figure 15 shows also a slight improvement in thermal characteristic of the chitosan/VAc graft copolymer over pure chitosan since the peak of maximum decomposition of the graft shifts to higher value (388°C).

Bioassay for the fungicidal activity

Effect of chitosan and its graft copolymers on the soil-borne sugar-beet pathogens

Chitosan is known to prevent the growth activities of several microorganisms *in vitro*[39]; it also exhibits antimicrobial activity against some strains of filamentous fungi,[40] yeast,[41] and bacteria.[42–47] Many chitosan derivatives, for example, *N*,*O*-acyl, *N*-alkyl, and *N*-aryl chi-

TABLE II
Effect of the Chitosan/VAc Graft Copolymers (*G*% 120) Concentrations on the Percent Germination (*G*), Average Length of Hyphal Extension (L_h), Dry Mass Yield (D_m), Production of Sclerotia of *Rhizoctonia solani, Sclerotium rolfsii*, and Macroconidia of *Fusarium solani* at 27°C

μg mL^{-1}	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R. solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
10	24.3	371.1	331.2	5.7	7.4	52
20	17.2	310.1	231.2	5.7	7.4	28
30	6.7	70.3	91.8	5.6	7.3	12
40	0	0	0	5.6	7.0	0
LSD						
1%	4.0	14.8	17.0	–	–	6.5
5%	1.6	8.9	9.1	–	–	2.7
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
10	22.5	321.4	251.6	5.7	3.7	329
20	16.6	149.2	121.2	5.7	3.7	91
30	3.9	51.2	90.3	5.6	3.8	56
40	0	0	0	5.6	4.2	0
LSD						
1%	3.8	12.8	17.3	–	–	6.3
5%	1.4	7.3	9.4	–	–	2.9
F. solani	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia (10^4 mL^{-1})
Control	63.2	12.4	357.2	5.8	4.3	314.7
10	36.3	7.8	202.1	5.7	4.2	212.3
20	24.8	5.9	121.4	5.7	4.2	198.1
30	15.4	3.6	70.1	5.6	4.1	103.1
40	0	0	0	5.6	4.0	0
LSD						
1%	8.2	2.5	12.4	–	–	18.2
5%	5.5	1.6	3.7	–	–	7.1

tosan derivatives were found to have insecticidal and fungicidal activity[48,49]

Sugar beet (*Beta vulgaris* L., Chenopodiaceae) is one of the most important cash crops grown mainly in the areas of temperate climatic conditions for sugar production. It has great economic importance for Egypt[32] since it is the second crop plant for the sugar production after sugarcane. Sugar beet is attacked by several root-rot diseases, the most serious of which are those caused by *R. solani* and *S. rolfsii*[50] and also a wilt disease caused by *Fusarium* species.[34,51] Taking this economic importance into consideration, the present work was designed to investigate the *in vitro* effect of chitosan and its graft copolymers on the growth activities of the sugar beet pathogens: *R. solani*, *S. rolfsii*, and *F. solani*. The fungicidal activity of chitosan toward three soil-borne sugar beet pathogens was investigated *in vitro* and the results are depicted in Table I.

From Tables I–III, one can see that the percent germination (%*G*) of sclerotia of *R. solani* and *S. rolfsii* decreased with increasing the chitosan and the chitosan graft copolymers concentration. The average length of hyphal extension (L_h μm) and dry mass yield (D_m mg) was affected similarly, decreasing proportionally to the polymers concentration. The pH of the growth medium shifted toward alkalinity for the *R. solani.* The pH increase in the culture medium during fungal growth may have been caused by differential uptake of cations and anions. Transport of anions such as phosphates may act as the hydroxide exchange system with the medium becoming more basic.[52] The rapid decline in the initial pH of the culture was probably due to the production of organic acids (oxalic acid) through the oxidation of carbon source.[53]

The number of sclerotia produced/plate by *R. solani* and *S. rolfsii* (column 7 in the Tables) at chitosan concentration ranging from 10 to 50 μg mL^{-1} were reduced proportionally to the chitosan concentration. No sclerotia were produced by the two graft copolymers at concentration of 40 μg mL^{-1}, while small number of sclerotial production was observed for pure chitosan at concentration of 40–50 μg mL^{-1}. The macroconidia of *F. solani* germinated in a wide range of polymer concentration 10–50 μg mL^{-1}, decreasing steadily with an increase in the chitosan and its graft copolymers concentration. Maximum inhibition for pure chitosan was recorded at a concentration of 50 μg mL^{-1}, while no macroconidia was observed at a concentration of 40 μg mL^{-1} for the graft copolymers. Similar results were obtained when measuring the length of the germ-tube after 9 h incubation. Maximum reduction in the germ-tube length was obtained at pure chitosan concentration of 50 μg mL^{-1} (from 12.4 to 3.6 μm) reaching 0 μm

TABLE III
Effect of the Chitosan/VAl Graft Copolymers (*G*% 120) Concentrations on the Percent Germination (*G*), Average Length of Hyphal Extension (L_h), Dry Mass Yield (D_m), Production of Sclerotia of *Rhizoctonia solani, Sclerotium rolfsii*, and macroconidia of *Fusarium solani* at 27°C

μg mL^{-1}	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R. solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
10	20.3	362.1	321.2	5.7	7.4	41
20	14.2	302.1	222.4	5.7	7.4	22
30	6.5	66.4	89.3	5.6	7.3	9
40	0	0	0	5.6	7.0	0
LSD						
1%	3.9	14.7	17.2	–	–	6.4
5%	1.5	8.3	9.0	–	–	2.6
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
10	19.4	313.4	246.4	5.7	3.7	321
20	10.2	142.3	111.1	5.7	3.7	89
30	3.4	46.1	88.2	5.6	3.8	52
40	0	0	0	5.6	4.2	0
LSD						
1%	3.7	12.9	17.5	–	–	6.5
5%	1.3	7.5	9.7	–	–	2.8
F. solani	*G* (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia (10^4 mL^{-1})
Control	63.2	12.4	357.2	5.8	4.3	314.7
10	34.2	7.0	199.3	5.7	4.2	210.2
20	24.6	5.6	110.3	5.7	4.2	192.2
30	14.3	3.5	69.2	5.6	4.1	100.1
40	0	0	0	5.6	4.0	0
LSD						
1%	8.1	2.6	12.4	–	–	18.3
5%	5.4	1.7	3.7	–	–	7.2

(number of germination) for the graft copolymers at a concentration of 40 μg mL^{-1}.

Dry mass yield estimations showed that the mycelial tolerance to chitosan concentration was highest for *F. solani* and lowest for *R. solani* and *S. rolfsii*. For *F. solani*, the results recorded at chitosan concentrations 10–50 μg mL^{-1} were significantly different from the control. The same trend was found for the investigated copolymers.

Effect of chitosan and its graft copolymers on some soil fungi genera

Chitosan and the prepared graft copolymers with 4-vinyl pyridine (prepared in a previous work and is given here for comparison[38]), and vinyl acetate, were screened for their fungicidal activity against *A. niger*, *C. herbarum*, and *F. moniliforme.*

Table IV contains data on the effect of these grafted copolymers on the tested fungi. The table shows that

TABLE IV
Effect of Different Chitosan/VAc, and Chitosan/VAl Graft Copolymers on *A. niger, C. herbarum, and F. moniliforme* (at 5 mg/mL) After 48 h of Incubation at 28°C by the Disc Plate Method (Mean Values of the Diameters of the Incubation Zones in millimeter)

Type of compound	%G	*A. niger*	*C. herbarum*	*F. moniliforme*	LSD 5%	LSD 1%
Chitosan	0	5.1	5.3	5.5	0.24	0.6
Chitosan/VAc	80	5.4	5.6	5.8	0.41	0.9
	120	6.3	6.6	6.9	0.3	1.0
	160	7.1	7.3	7.6	0.21	1.6
	220	7.4	8.0	8.2	0.42	1.2
Chitosan/VAl	80	6.5	6.8	7.1	0.22	0.52
	120	7.4	7.6	8.2	0.40	0.90
	160	7.9	8.1	8.9	0.32	1.10
	220	9.0	9.2	9.6	0.22	0.65
Chitosan/vinyl pyridine	120	5.8	6.1	6.8	0.30	0.90

TABLE V
Minimal Inhibitory Concentration (MIC) at μg mL^{-1} of the Grafted Chitosan Copolymers upon *A. niger*, *C. herbarum*, and *F. moniliforme*

Type of compound	%G	*A. niger*	*C. herbarum*	*F. moniliforme*	LSD 5%	LSD 1%
Chitosan	0	925	800	750	7.1	15.6
Chitosan/VAc	80	425	425	350	9.3	14.5
	120	400	375	300	7.5	16.1
	160	350	350	275	6.3	10.4
	220	300	325	250	6.5	11.1
Chitosan/VAl	80	375	375	350	8.5	14.3
	120	350	350	300	8.3	14.1
	160	300	325	225	7.5	13.6
	220	250	300	200	7.3	13.3
Chitosan/vinyl pyridine	120	300	325	325	3.6	7.8

the effectiveness depends on the nature of the compound and the tested fungus. The effect was observed by measuring the diameter of inhibition zones. In general, it has been found that the diameter of the inhibition zones increases with increasing the percentage of grafting. It was also observed that *A. niger* is the fungus least affected, while the *F. moniliforme* is the fungus most affected by the used materials. It seems that the chitosan/VAl is superior over the chitosan/vinyl acetate and chitosan/PVP analogues.

At the same time, the MICs of these graft copolymers were measured and are given in Table V, and again a supporting results are observed regarding the superiority of chitosan/VAl copolymer over the others.

From the table, it can be seen that grafting affect strongly both the inhibition zones and the MIC, the latter decreases almost by a factor of four by treatment with chitosan/VAl graft copolymer.

The graft copolymers with chitosan have the ability of forming metal complexes, which could have antifungal properties. The effect of several metal complexes of the grafted copolymers on the three chosen fungi was investigated and the preliminary data are depicted in Table VI. It has been found that the effectiveness depends on the nature of the metal ion as well as the tested fungus. The results obtained revealed also that the trend of growth inhibition of the complexes follows the order: Cd(II) > Ni(II)> Co(II)> Cu(II) > Mn(II).

Comparing the data in Tables II and III and Table VI indicates that the metal complexes have a tremendous effect on the growth inhibition of the tested fungi.

Large amounts of heavy metals are released into the environment by the human technological activities. The impact of these metals in aquatic systems and their accretion throughout the food chain can cause serious threat to animals and humans.[54] However, certain heavy metals, such as copper, nickel and zinc, are essential trace elements for normal growth and metabolism of microorganisms.[55] Copper inhibits glycolytic flux (above 1 μ*M*), inhibits growth (above 10 μ*M*), and induce yeast cell death in the logarithmic growth phase (at 0.80 m*M*).[56] Lethal concentrations of copper (higher than 100 μ*M* Cu^{2+}) induce the leakage of organic compounds from the fungi.[57] Nickel causes a partial inhibition of fermentation,[58] inhibition of glucose uptake and presumably other substrates,[56] and inhibition of macromolecular synthesis such as RNA and proteins[59] all

TABLE VI
Effect of Different Chitosan/VAc Graft Copolymers/Metal Complexes on *A. niger*, *C. herbarum, and F. moniliforme* (5 mg/mL) After 48 h of Incubation at 28°C by the Disc Plate Method (mean values of the diameters of the incubation zones in millimeter)

Type of metal complexes	Complex[a] composition (mol/mol)	*A. niger*	*C. herbarum*	*F. moniliforme*	LSD 5%	LSD 1%
Cu(II)	3.8	9.8	12.3	17.7	0.21	0.6
Ni(II)	2.45	10.2	13.6	18.4	0.25	0.70
Co(II)	2.35	10.1	13.4	18.0	0.31	0.75
Mn(II)	1.29	9.9	12.2	17.4	0.40	0.90
Cd(II)	0.4	17.1	18.2	32.2	0.43	1.1
LSD						
5%		0.30	0.25	0.18		
1%		0.70	0.63	0.40		

[a] Metal/chitosan molar ratio.

TABLE VII
Minimal Inhibitory Concentration (MIC) at μg mL^{-1} of the Grafted Chitosan Copolymers/Metal Complexes upon *A. niger*, *C. herbarum*, and *F. moniliforme*

Type of metal complexes	*A. niger*	*C. herbarum*	*F. moniliforme*	LSD 5%	LSD 1%
Cu(II)	37.5	37.5	25.0	1.9	5.5
Ni(II)	37.5	37.5	12.5	1.6	3.6
Co(II)	37.5	25.0	25.0	1.8	3.8
Mn(II)	50.0	50.0	25.0	1.7	3.4
Cd(II)	12.5	12.5	12.5	1.3	3.0
LSD					
5%	3.1	2.9	2.7		
1%	6.5	6.3	6.0		

resulting in the disturbance of physiological and biochemical processes.[60] Cadmium and mercury are strong inhibitors of microbial metabolism, even at low concentrations.[61]

Many workers have investigated the use of metal complexes as antifungal and antibacterial inhibitors, for example transition metal complexes with N-substituted acid hydrazides were tested for *in vitro* growth inhibitory activity against phytopathogenic fungi, viz. *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani*. An increase in the biocidal activity of the ligands as a consequence of coordination with metal ions was observed in terms of minimum inhibitory concentration (MIC) values (Table VII). The found trend of growth inhibition in these complexes was in the order Cu > Ni > Co.[62] Copper complexes with bioactive ligands were tested as antifungal agents against various strains of filamentous fungi.[63] Chohan et al.[64] synthesized thiocarbohydrazone, carbohydrazone, thiosemicarbazone, and semicarbazone ligands. These ligands were used for the preparation of Cu(II), Ni(II), Co(II), and Zn(II) metal complexes. All these ligands and their metal complexes showed good antibacterial and antifungal activities using the agar-well diffusion method.

From the data in Tables V–VII, it can be seen that chitosan, the grafted chitosan, and the complexes of chitosan graft copolymers are strong fungicidal agents and can be used either directly in the soil or as in seed treatment. The seed treatment with fungicides is essential because a large number of disease causing fungi are carried on and/or in the seed, and when the seed germinates, these fungi become active and cause either seed or seedling mortality or produce diseases at a later stage.[65] Fungicidal seed treatment usually involves the use of more than one fungicide since there is no one fungicide that may be recommended as a universal preventive for all seed pathogens. From a previous work using chitosan and some thiourea derivatives[66] and from this work, it seems that chitosan has a broad potential application against soil-borne pathogens, therefore it is justifiable to investigate this system and it is highly recommended to further use these materials for soil and seeds treatment, which will be a future plan of work.

CONCLUSIONS

Polyvinyl acetate could be grafted onto chitosan up to 360% under homogeneous conditions, and up to 180% in heterogeneous conditions, thus the homogeneous conditions are more favorable. It was possible to control the extent of grafting by varying the reaction conditions namely, monomer concentration, initiator concentration, temperature, and time. The grafted samples swelled in ethanol–acetic acid mixture and in water better than the unmodified chitosan. Acid hydrolysis for polyvinyl acetate/chitosan copolymer led to the preparation of polyvinyl alcohol/chitosan copolymer with a better swelling character than the chitosan/VAc copolymers. An enhancement of the dye uptake of the grafted copolymers was observed, especially for the acidic dyes due to the cationic character of chitosan. The thermal characteristic of the graft copolymer did not suffer from the grafting process; on the contrary, there was a slight improvement of the thermal resistance. The antifungal investigation of chitosan and its grafted copolymers revealed that chitosan and its derivatives could be excellent antifungal candidates, which warrant further study of these systems.

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