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Synthesis, antifungal and insecticidal potential of Chitosan (CS)-g-poly (acrylic acid) (PAA) nanoparticles against some seed borne fungi and insects of soybean

Sahab, A. F.[1]; Waly, A.I.[2], Sabbour, M. M.[3] and Lubna S. Nawar[4]

[1]Plant Pathology Department, National Research Centre, Cairo Egypt
[2]Preparation and Finishing of Cellulosic Fabrics,NRC, Cairo Egypt
[3]Pests and Plant Prot. Department National Research Centre, Cairo Egypt
[4]Biology Dept., Fac. of Science, King Abd El-Aziz Univ., Jeddah, Saudi Arabia.

Abstract: Chitosan (CS)-g-poly (acrylic acid) (PAA) nanoparticles, which are well dispersed and stable in aqueous solution have been prepared by template polymerization of acrylic acid in chitosan solution. The prepared CS-PAA had a white powder shape and was insoluble in water and diluted acid. The mean particles size were found to be around 50nm. FTIR spectra of CS-PAA nanoparticles for CS, the intensities of the amide band were observed clearly. The board peak appeared at 2500cm^{-1}, which confirmed the presence of NH_3^+in the CS-PAA nanoparticles.

The percentage of fungal infection of soybean seeds was ranged from 78.57 and 92.00% for samples of clark and calland soybean cultivars respectively on PDA medium, while the number of fungi as cfu/100 seeds was 156.12 and 440 respectively. Isolation trails showed eleven fungi comprising seven genera namely, *Alternaria tenuis*, *Aspergillus flavus, A. niger, A. terreus , A. versicolor, Fusarium oxysporum, F. solani, Mucor mucedo, Penicillium spp., Rhizoctonia solani* and *Sclerotium rolfsi*i detected and identified on PDA medium. Nano-chitosan (CS-g-PAA) showed a remarkable antifungal effect against some fungi isolated from soybean seeds. The % inhibition of *F. oxysporum* for example was significantly high (41.04 mean % inhibition) than the other tested fungi, followed by *A. terreus* and *F. solani* (40.08 and 40.00 mean % inhibition respectively. Regarding zone of inhibition, nano-chitosan exhibited high inhibition against *Aspergillus niger*, followed by *Fusarium solani and F. oxysporum,* as the zone of inhibition were 20.67, 20.33 and 20.33 mm at 100 ppm respectively.

Concerning the insecticidal activity the nano-chitosan (CS-g-PAA) showed highest effect against the three insect of soybean. as the means number of eggs deposited /female were significantly decreased. Under laboratory and semifield condition, *Aphis gossypii* were significantly decreased to 20.9±9.1 and 28.9±9.2 eggs/female respectively as compared to 97.3±4.9 and 90.3±4.9 eggs/female in the control, respectively. The same trends were also observed against *Callosobruchus maculatus* and *Callosobruchus maculatus* insects.

Keywords: nanoparticles, chitosan, antifungal, insecticidal activity, soybean.

Introduction:

Soybean, (*Glycine max* (L) Merril) is a world wide economic crop and the most important cultivated legume with hundreds of food, feed and industrial uses. Among the serious diseases of soybean, most of them are seed transmitted. Fungi of *Cercospora kikuchii, Colletotrichum dematium var. truncatum, C. lindemuthianum, Fusarium equiseti, F. oxysporum, F. solani, Macrophomina phaseolina, Myrothecium roridum* and *Phomopsis sojae* were encountered on soybean seeds [1].

Plant pathogens induce decay on a large number of agricultural crops during the growing season and postharvest. Even though effective and efficient control of seed borne fungi of seeds can be achieved by the use of synthetic chemical fungicides, the same cannot be applied to grains for reasons of pesticide toxicity [2]. Hence, there is growing emphasis on environmentally friendly technologies in pest control, and evaluation of various alternatives to reduce dependency on harmful synthetic pesticides [3]. Consequently, several nonchemical treatments have been proposed for pest control.

Nanoparticles synthesis is currently intensively researched due to its wide variety of potential applications. As an alternative to chemical manufactured pesticides, use of nanoparticles as an antimicrobial agents has become more common as technological advances have made their production more economical [4]. Numerous studies on the antimicrobial activity of chitosan and its derivatives against most economic plant pathogens have been investigated [5,6, 7, 8]. Therefore, these compounds are considered as useful pesticides in the control of plant diseases.

Another important attribute of this natural compound is associated with its fungistatic or fungicidal properties against pathogens of postharvest fungi such as, *Alternaria alternata Colletotrichum gloeosporioides*, *F. oxysporum, Rhizopus stolonifer* and *Penicillium* spp. was inhibited on nutrient media amended with various concentrations of chitosan [9, 10, 11, 12].

The cotton aphid (*Aphis gossypii*) is a major pest of soybeans [13] which transmit the bean yellow mosaic virus on soybeans. Pests that attack soybean seeds during storage at the warehouse consists of several species including *Callosobruchus chinensis* and *C. maculatus*. This warehouse pests known as the beetle bruchus caused a lot of damage to stored products [14].

Chitosan has been found to show strong insecticidal activity in some plant pests [15]. They found that a chitin derivative (N-(2-chloro-6-fluorobenzyl-chitosan) caused 100% mortality of larvae of the cotton leafworm (*Spodoptera littoralis*) . While, chitosan treatments have been found to effective against herbivorous insect pests, it has actually been used successfully as an ingredient in the artificial diet fed to carnivorous insects being reared for use in the biological control of chitinous pests [16]. This finding suggests that chitin-based products could potentially be less harmful to non-target insects than conventional insecticides.

In the present work a study of preparation of the nano-chitosan formulation prepared via the application of the predominant radical graft polymerization of acrylic acid onto chitosan was investigated. The study also undertaken to detect the seed-borne fungi associated with soybean seeds and to evaluate the antifungal and insecticidal activity of nano-chitosan formulation against some selected soybean seed borne fungi and against *Callosobruchus maculatus*, *C. chinensis* and *Aphis gossypii* of soybean insects.

2 Material and Methods:

2.1. Materials. The raw materials used for ion exchange preparation are:

* The chitosan (CS) used in this study was purchased from HAS HMRZEL laboratories LTD (Netherlands)

* The acrylic acid was purchased from Sd.Finc.Chem. Limited (Laboratory grade for synthesis) and was freshly distilled under reduced pressure to eliminate any inhibitors.

* Both initiators (ammonium persulfate and sodium bisulfite) and other chemicals were of analytical reagent and used as received.

2.2. Experimental Procedure:

The graft copolymerization reaction was performed in a 250ml quick fit conical flask immersed into a thermostatic water bath preset at the operating temperature (30-60˚C). In a typical experement, a required amount of chitosan (2-20g/L) was dissolved in 50 ml acrylic acid solution of pre-determined concentration (10-50g/L) under shaking. A clear solution was obtained and after adjustment of temp. , the desired amount of initiators (redox system) was added to the solution (ammonium persulfate followed by sodium bisulfate). The reaction mixture was allowed to react under regular shaking (120 rpm) and during the polymerization an opalescent begins to appear. After the desired reaction time (5-90min), few drops of 250 ppm hydroquinone (in methanol) were immediately added to the reaction product ,while cooling in an ice bath for ceasing the polymerization reaction. Next, a 50 ml of ethanol was added to induce precipitation. The suspension was

centrifuged and the supernatant was discarded. The grafted product was washed and then oven dried at 80˚C at a constant weight. Finally , the copolymer Chit-g-PAA was immersed into a dimethyl formamide (DMF) solution under magnetic stirring over night to remove any traces of the homopolymer. The final copolymer was centrifuged and dried to constant weight [17]. The size of the solution of CS-PAA were determine by using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK).

2.3. Test fungi:

Seeds of soybean were collected from environmental farm of National Research Centre of Egypt . One hundred seeds were surface disinfected by soaking in 2% sodium hypochlorite for 3 min. followed by 70% ethanol for 2 min. and then thoroughly washed in sterile water. Drain excess water, dried between two layer of sterilized filter papers. The seeds were platted on potato dextrose agar (PDA) medium at rate of 4 seeds/dish [18]. The plates were incubated at 27±2˚C for 7 days. Fungi growing from the seeds were isolated, purified and identified according to [19, 20, 21].

3- Assessment of antifungal assay:

3.1: Mycelium growth inhibition: *in-vitro* assay was performed on growth medium treated with different concentrations (0.0, 1000, 2000, 4000and 8000 ppm) of chitosan grafted acrylic acid (CS-g-PAA) against some soybean seed borne fungi. The agar plates were inoculated by a 5mm disc of 7 day- old phytopathogenic fungi and then incubated at 27˚C until it fully grow of control plates. Radial inhibition was calculated when growth of mycelia in the control plate reached the edge of the petri-dish. The toxicity of the nanoformulation (CS-g-PAA) to growth of fungi in term of percentage inhibition of mycelial growth was calculated by using the formula:

% inhibition =dc- dt/dc x100 according to [22]

Where dc= av. increase in mycelial growth in control, dt= av. increase in mycelial growth in treatment.

3.2: Agar well diffusion method: nanochitosan formulation(CS-g-PAA) of different concentrations were screened for antifungal activity by agar well diffusion method with sterile cork borer of size 6.0 mm according to [23], 72 hours old cultures grown on PDA medium were used for inoculation of fungal species on PDA plates. An aliquot (0.2ml) of inoculums was introduced to molten PDA and poured in to a petri-dish. After solidification, the appropriate wells were made on agar plate and 500 µl of nanochitosan formulation solution, homogenized using an ultrasonic cleaner, filled in deep blocks. Incubation period of 4-5 days at 27±2˚C was maintained for observation of antifungal activity by measuring zone of inhibition of fungal growth surrounding the well. The zone of inhibition was measured in mm and the experiment was carried out in triplicates. Synthetic fungicide (8 hydroxy quinolin, 100µg/ml) was used as control.

4.Insecticidal activity:

A susceptible strain of the three target insect pests, *Callosobruchus maculatus* (F.)*, C. chinensis* (L.) and *Aphis gossypii* (Glover) were used for bioassay. *C. maculatus* and *C. chinensis* were reared on soybean at 28±2°C , 60±5% R.H. and a 16 h light photoperiod under laboratory conditions, while, *A. gossypii* reared on castor leaves [24]. These strains were reared for many years in a laboratory of Pests and Plant Protection, National Research Center without exposure to any pesticides. In a standardised screening toxicity test, third-instar larvae of target pests were selected from the laboratory colony.

One ml of nanochitosan formulation (CS-g-PAA) incorporated with 12.5 part of an artificial diet according to [25], the untreated diet was used to serve as controls. Treated diet was divided and placed in Petri dishes. Three replicates were used for each particular treatment and ten larvae were introduced onto each replicate. The experiments were kept in a growth chamber, at 25 ± 2°C, 70 ± 5% RH and a 16 h light photoperiod. After 7 days of continuous feeding, larval mortality was scored; if no movement was observed, larvae were considered as dead. Larval growth inhibition was assayed relative to the control based on larval weight gain through 7 days of feeding. The growth inhibition was calculated from the following equation:

Growth inhibition (%) = (CL – TL) × 100 where:
CL – larval weight gained in the control
TL – larval weight gained in the treatment
The percentage of feeding inhibition was determined after 7 days by the formula of [26]:

Antifeedant (%) = (C – T) × 100 where:
C = weight of diet consumed in untreated control. T = weight of diet consumed

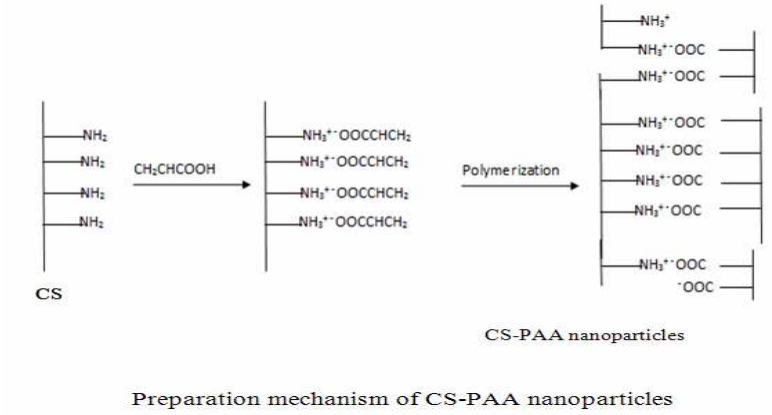
4. Statistical Analysis:

Data obtained was statistical analysed using Duncan's multiple range test according to [27].

3. Results and Discussions

3.1. Characteristic of Chitosan-Polyacrylic Acid Nanoparticles:

The preparation of CS-PAA nanoparticles was based on an electrostatic induced interaction between the positively charged CS and negatively charged PAA (Fig. 1). The prepared CS-PAA had a white powder shape and was insoluble in water and diluted acid. The surfaces of the CS-PAA nanoparticles had positive charges, as the amount of CS was absorbed onto the surface.



Preparation mechanism of CS-PAA nanoparticles

The obtained CS-PAA nanoparticles were tested for their size which shows the particle size and particles size distribution, since the mean particles size were found to be around 50nm. FTIR spectra of CS-PAA nanoparticles are shown in Figs. (2, 3), for CS the intensities of the amide band were observed clearly. The basic characteristics of CS at 3469.31 cm^{-1} (OH stretching and N-H stretching) was observed. However the two characteristic peaks of CS decreased dramatically and a new absorption band at 1649 cm^{-1} which could be assigned to the absorption peak of the carboxylic peak of PAA was observed. The board peak appeared at 2500cm^{-1}, which confirmed the presence of NH_3^+in the CS-PAA nanoparticles. In addition the absorption peak at 1640 and 1420 cm^{-1}were assigned to asymmetric and symmetric stretching vibration of COO^-anions groups and NH_3^+cations groups respectively , indicate ionic interaction between PAA and CS associated with the formation of nanoparticles. These results indicate that the COOH groups of PAA were dissociated into COO^- groups, which complexes with protonated to form the polyelectrolyte complex during mixing procedure. The experimental results are presented , discussed and compared with pertinent results and information available in the literature [17].

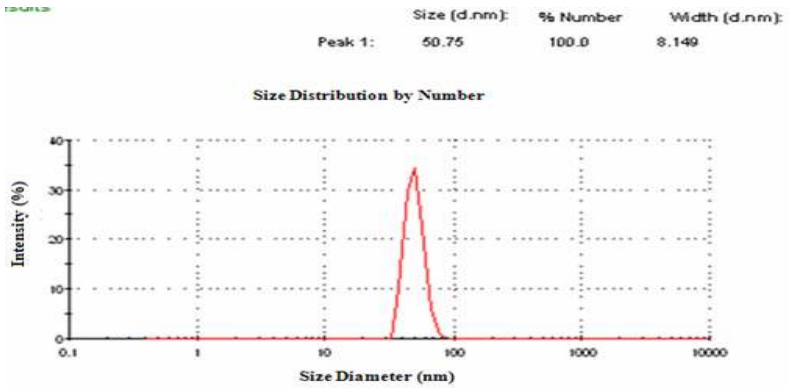


Fig.(2): Size distribution by intensity of CS-PAA nanoparticles using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK).

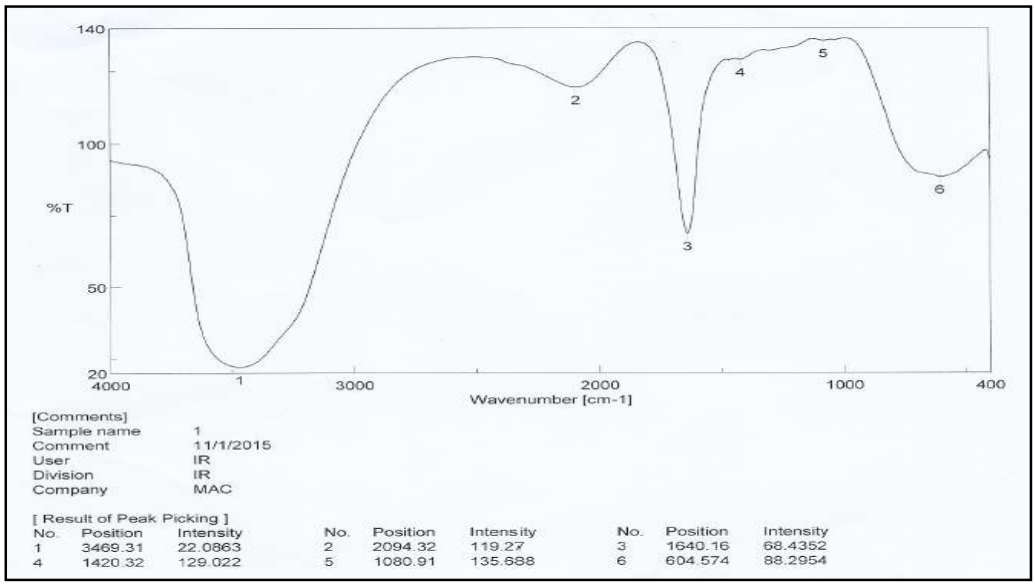


Fig.(3): FTIR of CS-PAA Nanoparticles Solution

3.2. Seed borne fungi of soybean cultivars:

Data presented in Table (1) showed that the percentage of soybean contamination ranged from 78.57 and 92.00% for samples of the two soybean cultivars on PDA medium. These findings are similar to those reported previously by [28, 29] who reported 65% field incidence of pod and stem blight diseases in USA.

It is clear also from Table (1) that the number of fungi as cfu/100 seeds was 156.12 and 440 for clark and calland cvs respectively. Moreover, seeds of clark cv. detected low fungal densities than the other cultivar.

3.3. Prevalence of seed borne fungi of soybean

Altogether eleven fungi comprising seven genera namely, *Alternaria tenuis*, *Aspergillus flavus, A. niger, A. terreus , A. versicolor, Fusarium oxysporum, F. solani, Mucor mucedo, Penicillium spp., Rhizoctonia solani* and *Sclerotium rolfsi*i were detected and identified on PDA medium (Table,1). These fungi were reported as seed borne in soybean by a number of other workers [, 1, 30, 31, 32]. Depending upon their frequent occurrence the genera and species were grouped as major component include: *Alternaria tenuis* (15.47%), *Fusarium solani* (28.57%), *Rhizoctonia solani* (11.90%) and *S. rolfsii* (11.90%) on the seeds of clark cultivar, while, the most frequent isolated fungi on calland cultivar were *Aspergillus flavus* (36.36%) and *A. terreus* (25.45%).

Table (1): Percentage of fungal infection, total fungal count and frequency occurrence (%) of fungi associated with clark and calland soybean cultivars collected from environmental farm of National Research Centre of Egypt on PDA medium.

Fungal genera	Soy bean cultivar	
	Clark	Calland
Alternaria tenuis	**15.47**	**3.64**
Aspergillus flavus	**2.38**	**36.36**
Aspergillus niger	**4.76**	**3.64**
Aspergillus sydowi	**-**	**3.64**
Aspergillus terreus	**2.38**	**25.45**
Fusarium oxysporum	**4.76**	**-**
Fusarium solani	**28.57**	**5.45**
Mucor mucedo	**9.52**	**5.45**
Penicillium spp.	**7.14**	**3.64**
Rhizoctonia solani	**11.90**	**7.27**
Sclerotium rolfsii	**11.90**	**7.27**

% infection	**78.57%**	**92%**
TFC (CFU/100seeds)	**156.12**	**440**

***Tests were rune in quadruplicate**
*** Counts represent the number of fungi /100 seeds incubated at 28±2ºC for 7 days on PDA medium.**

3.4. Effect of nano-chitosan (CS-g-PAA) formulation on fungal growth:

a. Fungal linear growth:

Table (2) represented the effect of chitosan **(CS-g-PAA)** formulation on the colony growth (mm) against some phytopathogenic fungi. A positive correlation was reported between the concentrations and colony growth, with the increasing of nano-chitosan concentration a significant increase was found for all fungi. The sensitivity of different fungal species to chitosan formulation was varied. For example, the % inhibition of *F. oxysporum* was significantly high (41.04 mean % inhibition) than the other tested fungi, followed by *A. terreus* and *F. solani* without significant difference (40.08 and 40.00 mean % inhibition respectively). Data also showed that the percentage of mycelium growth inhibition at higher concentration of 8000 ppm was varied from 36.67 % on *S. rolfsii* to 75.42% on *Aspergillus flavus*.

Nano chitosan also proved to be effective and gave the most promising antifungal activity at lower concentration of 1000 ppm against *F. solani* responsible for 41.48% inhibition followed by *F. oxysporum* (30.37% inhibition) with significant difference. Moreover, nano-chitosan was quite effective against *S. rolfsii* (11.63 mean % inhibition). According to [12] the degree of fungicidal activity has direct relationship with concentration of chitosan. In earlier reports workers found chitosan derivatives to have inhibitory effects on several plant pathogenic fungi . [33] reported that addition of chitosan to agar medium led to sever inhibition of *F. oxysporum*. Findings of the present study indicate that there are variation in tolerance to chitosan between different fungi. The same variation in tolerance was reported by [5, 6, 7, 8].

Table (2): Antimicrobial activity (% inhibition) of nano-chitosane (CS-g-PAA) formulation on the linear growth (mm) of some phytopathogenic fungi on PDA medium noted after 7 days of incubation at 27˚C.

Fungi	**Concentration (ppm)**					**Mean**
	0	1000	2000	4000	8000	
Alternaria tenuis	0.00 Q	18.89 P	54.44 I	59.26 GH	66.67 D	**39.85 B**
Aspergillus flavus	0.00Q	72.90 C	74.17 BC	76.67 A	75.42 AB	**59.83 A**
Aspergillus niger	0.00 Q	20.83 OP	47.92 J	53.33 I	63.75E	**37.17 C**
Aspergillus terreus	0.00 Q	22.08 O	47.50 J	57.50 H	73.33 C	**40.08 B**
Fusarium solani	0.00 Q	41.48 L	45.19 K	52.69 I	60.36 FG	**40.00 B**
Fusarium oxysporum	0.00 Q	30.37 N	47.04 JK	61.85 EF	65.93 D	**41.04 B**
Sclerotium rolfsii	0.00 Q	0.00 Q	1.85 Q	19.63 M	36.67 M	**11.63 D**
Mean	**0.00 E**	**29.51 D**	**40.65 C**	**50.71 B**	**61.11 A**	

-Three replicates were used for each treatment
-Values followed by the same letter are not significantly different at P ≥ 0.05 according to Duncan's multiple range test. Means followed by the same letters are not significantly differed

b). On zone of inhibition:

As regard to nano-chitosan **(CS-g-PAA)** for different fungi was a tendency towards increase in the zone of inhibition rates proportional to the increase in the tested concentrations (Table, 3). There were significant differences among all the analyzed concentrations. Nano-chitosan formulation exhibited high inhibition against *Aspergillus niger*, followed by *Fusarium solani and F. oxysporum,* as the zone of inhibition were 20.67, 20.33 and 20.33 mm at 100 ppm respectively.

Table 2: Testing of antifungal activity of of nano-chitosan (CS-g-PAA) formulation against some different phytopathogenic fungi (zone of inhibition values in mm).

Tested organisms	Concentration %					*Fungicide 100µg/ml
	0	25	50	75	100	
Alternaria tenuis	0.00 L	0.00 L	8.33 JK	14.67 HI	16.00 F	39.33 B
Aspergillus flavus	0.00 L	8.67 JK	10.33 IJ	12.67 GH	15.33 F	34.00 C
Aspergillus niger	0.00 L	9.67 JK	14.33 FG	18.33 E	20.67 D	42.00 A
Aspergillus terreus	0.00 L	8.67 JK	10.33 IJ	12.67 GH	15.33 F	34.00 C
Fusarium oxysporum	0.00 L	0.00 L	8.33 K	14.33 FG	20.33 D	42.33 A
Fusarium solani	0.00 L	0.00 L	8.33 K	14.33 FG	20.33 D	42.33 A
Sclerotium rolfsii	0.00 L	0.00 L	9.33 JK	11.67 HI	16.00 F	39.33 B
Mean	0.00 F	3.85 E	9.90 D	14.38 C	17.71 B	

-Positive control for fungi= 8 hydroxy quinoline sulphate
-Three replicates were used for each treatment
-Values followed by the same letter are not significantly different at P ≥ 0.05 according to Duncan's multiple range test. Means followed by the same letters are not significantly differed

In spite of these inhibition values were not equal to the tested fungicide (positive control) the nano-chitosan formulation at low concentration of 25%, showed activity reached 9.67 mm for *Aspergillus niger* and 8.67mm for *A.flavus* and *A. terreus* compared to 0.0mm in the control. As reported by [34, 35,36] the polycationic nature of chitosan interferes with negatively charged residues of macromolecules exposed on the fungal cell surface that causes leakage of intracellular electrolytes and proteinaceous constituents.

3.5. Insecticidal activity of of nano-chitosan (CS-g-PAA) formulation on insects:

a). On *Aphis gossypii :*

The effect of nano-chitosan on *Aphis gossypii* of soybean insect pest showed that, mean number of eggs/female ±SE of *A. gossypi* were significantly decreased to 20.9±9.1 and 28.9±9.2 eggs/female as compared to 97.3±4.9 and 90.3±4.9 eggs/female of the non-treated controls, under laboratory and under semifield conditions, respectively (Table, 4). The percentage of insect growth were significantly decreased from 99% in semifield control to 22% (77.8% decrease) in treated insects under semifield condition.

Table (4): Effect of nano-chitosan (CS-g-PAA) on *Aphis gossypii* under laboratory and semifield conditions.

Growth%	%Emergence(F1)	Mean number of eggs/female ±SE of *A. gossypii*	Treatments
100	100	97.3±4.9	**Control (laboratory)**
25	27	20.9±9.1	**Under laboratory**
22	23	28.9±9.2	**Under semifield**
99	98	90.3±4.9	**Control (semifield)**
		23	**F value**
		14	**Lsd 5%**

b). On *Callosobruchus maculatus:*

The effect of nano-chitosan (CS-g-PAA) on soybean insect pest showed that mean number of eggs/female ±SE of *C. maculatus* were significantly decreased to 10.9±9.9 and 19.9±9.9 eggs/female as compared to 95.3±4.9 and 94.3±4.9 eggs/female in the control of non- treated under laboratory and under storage condition , respectively (Table, 5). The percentage of insect growth were significantly decreased from 99% to 28% (71.7% decrease) in treated insects under store condition.

Table (5). Effect of nano-chitosan (CS-g-PAA) against *Callosobruchus maculatus* under laboratory and store conditions

Growth%	% Emergence (F1)	Mean number of eggs/female ±SE of *Callosobruchus maculatus*	Treatments
100	100	95.3±4.9	**Control (laboratory)**
35	27	.9±9.910	**Under laboratory**
28	23	19.9±9.9	**Under store**
99	98	94.3±4.9	**Control (store)**
		19	**F value**
		11	**Lsd 5%**

c). On *C. chinensis*:

The effect of nano chitosan on the *C. chinensis* of soybean insect pest showed that the mean number of eggs/female were significantly decreased to 21.9±1.5 and 21.1±6.9 eggs/female in treated insect under laboratory and store condition respectively as compared to 96.3±4.7 and 91.3±4.5 eggs/female in the control under laboratory and under storage condition respectively (Table, 6). The percentage of insect growth were significantly decreased from 100% to 27% (73.0% decrease) in treated insects under store condition.

Table (6). Effect of nano-chitosan (CS-g-PAA) formulation against *Callosobruchus chinensis* under laboratory and store conditions

Growth%	% Emergence (F1)	Mean number of eggs/female ±SE of *C. chinensis*	Treatments
100	100	96.3±4.7	**Control (laboratory)**
29	28	21.9±1.5	**Under laboratory**
27	22	21.1±6.9	**Under store**
100	99	91.3±4.5	**Control (store)**
		20	**F value**
		12	**Lsd 5%**

The same results also obtained by [37, 38,39,40]. As reported by [41], that chitosan exhibits insecticidal activity against various aphids at a range of concentrations from 600 to 6000 mg/l. In addition, chitosan showed a 70–80% insecticidal activity against *Rhopalosiphum padi, Metopolophium dirhodum*, and *Aphis gossypii* pests, while *Sitobion avenae* and *M. persicae* showed a lower susceptibility to chitosan [42]. The aphicidal activities of chitosan diethyl phosphate and chitosan ethyl carbamate, at different concentrations against the green peach aphid (*Myzus persicae*) and compared with imidacloprid were evaluated by [43]. They showed that chitosan at 0.5% showed the highest lethal activity compared to imidacloprid *in-vitro*, while in pot assays, chitosan diethyl phosphate and imidacloprid insecticide reported a systemic effect on sugar beet plants.

4. Conclusion

CS-g-PAA nanoparticles can be prepared by polymerizing acrylic acid into chitosan template. In the present work it was demonstrated that CS-g-PAA nanoparticles has significant antifungal and insecticidal activities against fungi and insects. Thus, it can be effectively used various crops against phytopathogenic and insect instead of using commercially available synthetic fungicides and or insecticides which show higher toxicity to humans.

5. References

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