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Influence of Fungal Chitosan to Control Root-Knot Nematode *Meloidogyne incognita* on Banana Plants

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Chitosan (poly-N-acetylglucosamine) is a natural and biodegradable biopolymer. Chitosan and its derivatives can be variously used as a permeability control agent and an antimicrobial compound. So, addition of three molecular weights of fungal chitosan ($1.9x10^5$; $1.4x10^5$ and $2.1x10^4$ Da) at soil as well as castor oil and Nemacur 10% G (chemical control) significantly reduced ($p \leq 0.05$) root-knot nematode infection caused by *Meloidogyne incognita* and improved growth of banana plants cv. Williams as compared with the control. Fungal chitosan type at Mw ($2.1x10^4$ Da) gave the most effective results in reducing the number of galls and the final population of nematode. On the other hand, fungal chitosan type at Mw ($1.9x10^5$ Da) surpassed the other tested materials and showed relatively maximum growth parameters as compared with the other treatments and the check.

Keywords: Banana, chitosan, *Meloidogyne incognita* and root-knot nematode.

Plant parasitic nematode are responsible for significant economic losses in agricultural activities, for diseases affecting public health, and for environmental contamination derived from the use of chemical products for their control (Malsam *et al*., 1997; Jansson *et al*., 1997; Chet and Inbar 1997 and Rich *et al*., 2004). This has revealed the need for developing a more efficient and effective system to control vegetal, animal, and/ or human pathogens. Chitin plays an important role in several aspects of nematode biology and may provide an excellent target for novel control methods directed against a variety of parasitic nematode. Chitin and its deacetylated derivative (chitosan) derived from the exoskeleton of shell of crustacean such as shrimp, lobster, crab, squall, krill, etc., have high economic value owing to their versatile biological activities and agrochemical applications (Spindler *et al*., 1990 and Badawy and Rabea, 2011). They were reported to be active against viruses, bacteria, fungi, nematode and other pests when applied to foliage or to soil (El Hadrami *et al*., 2010). Diverse organisms (containing chitin or not) produce a great variety of chitinolytic enzymes with different specificities and catalytic properties. Microorganisms, as the main environmental chitin degraders, constitute a very important natural source of chitinolytic enzymes. Interest in chitinolytic

enzymes in the field of biological control has arisen due to their possible involvement in antagonistic activity against pathogenic chitin-consisting organisms (Gortari and Hours, 2008). Chitinases in the soil are produced by some fungi (Main *et al*., 1982) and bacteria (Ordentlich *et al*., 1988 and Inbar and Chet, 1991), but chitinases are also released by many plants as part of their defence mechanism against various pathogens (Punja and Zhang, 1993) and plant parasitic nematodes (Roberts, 1992). Chitinases depolymerize the chitin polymer into N-acetylgyucosamine and chitobioses. Furthermore, microbial activity results in the deamination of the sugar and accumulation of ammonium ions and nitrates (Rodriguez-Kabana *et al*., 1983). So, addition of chitin to soil at 1% (w/w) eliminated plant parasitic nematodes in a first planting of cotton "cv. Rowden" and significantly reduced *Meloidogyne incognita* infestation in a second planting, confirming long-term *nematode* suppressiveness induced by this organic amendment (Hulimann *et al*., 1999). Nematicidal concentrations of ammonia in associated with a newly formed chitinolytic microflora are believed to cause nematode suppression (Main *et al*., 1982 and Godoy *et al*., 1983). Chitosan has been shown to increase the production of glucanohydrolases, phenolic compounds and synthesis of specific phytoalexins with antifungal activity, and also reduces macerating enzymes such as polygalacturonases, pectin methyl esterase etc. (Bautista-Baños *et al*., 2006). Recent advances in chitin synthesis is regulated and responds to cell wall stress sustains the attraction of this process as a potential antifungal drug target, possibly in combination with inhibitors of b(1,3)-glucan synthesis and/or the cell wall compensatory pathway (Lenardon *et al*., 2010). This study describes the results of different molecular weight of fungal chitosan against root-knot nematode which may be of great help to prevent the development of diseases and to decrease the effect of chemical nematicides on economic plants and environment.

Materials and Methods

Nematode population:

Eggs of *Meloidogyne incognita* were extracted from tomato roots (*Lycopersicon esculentum*), cv. Castle Rock, infected with the nematode using sodium hypochlorite solution (Hussey and Barker, 1973). Second-stage juveniles (J2) were collected daily from eggs and stored at 15°C. The juveniles used in the experiments were less than 5 days old.

Production of fungal chitosan:

The used fungal strain of *Aspergillius niger*, was grown on Potato Dextrose Broth (PDB; Merck, Germany) at 28°C for 72 h under shake incubation conditions. Mycelial growth was harvested by centrifugation, washed twice with distilled water and then homogenized with 1 mol Na OH at 100°C for 1h (Roberts, 1992). The alkali insoluble fraction was separated, washed and neutralized with 5% acetic acid. Three available chitosans were obtained from the previous methods like, DD-84.9%; DD-83% and DD-95%.The molecular weights of the previous types were recorded (1.9×10^9; 1.4×10^5 and 2.1×10^4 Da), respectively. All chitosans were acid soluble, while DD-95% is soluble in water, white coloured powder form (Table 1).

Table 1. Physio-chemical characteristics of produced fungal chitosan types

Chitosan type	Specification				
	Colour	Viscosity (cP) centipoises	Solubility (Dissolving solvent)	Degree of deacetylation (DD%)	Molecular weight (Da)
CTS No. A	White	5.8	Acetic acid 1% aqueous solution	84.9	1.9×10^5
CTS No. B	White	3.5	Acetic acid 1% aqueous solution	83	1.4×10^5
CTS No. C	White	3.1	Water	95	2.1×10^4

Characterization of the produced chitosans:

The physio-chemical characteristics of the produced chitosans, after different treatments in their preparations, were determined according to Anonymous, 1990 (colour, viscosity and solubility), whereas the molecular weights of prepared chitosans were determined by gel permeation chromatography (GPC) using refractive index detector (PN-1000, Postnova Analytics, Eresing, Germany).

Deacetylation of the produced chitosans:

The extent of chitosan deacetylation was determined by titration with 0.01 mol l^{-1} NaOH (Donald and Hayes, 1988). The method involved hydrolysing the acetyl groups in chitosan with a strong alkali and converting the salt to acetate, which was evaporated as an azeotrope with water and titrated. The acetyl percentage was determined from the equation:

$$\text{Acetyl (\%)} = V \times 0.04305/w$$

Whereas, V is the corrected volume of NaOH and w is the weight of the sample. The degree of deacetylation was calculated using the equation:

$$\text{Deacetylation (\%)} = 100 - \text{Acetyl (\%)}$$

Pot experiment:

Two months old, obtained from banana plants (cv. Williams) tissue culture (GEBRI*) were planted in 30 cm diameter plastic pots containing a mixture of 1:2 sterilized clay/sandy soil. Twenty four pots were infested by *Meloidogyne incognita* juveniles at the rate of 2,000 (J2s) per pot at the planting time. Six days later, 12 pots were treated with 10 ml 0.1% w/v of different molecular weights of chitosan ($1.9x10^9$; $1.4x10^5$ and $2.1x10^4$ Da), and also four pots were treated by 2 ml of Castor oil per pot. Four pots were treated with 3g "Nemacur 10%G" per pot. Finally, the remaining four inoculated pots served as untreated control without nematode. Emulsions and Castor oil were added to the soil by a pipette in a hole in soil then followed by water. The plants were treated by previous concentrations of the emulsion every month for three months. The treated plants were removing after treatment for three months. Weight and length of root, shoot and sucker were determined. Nematode populations in soil and immature stages, females and females with egg-masses in roots were counted. The data was statistically analyzed.

Results

Characteristics of chitosan products:

The properties of different molecular weights of chitosan derived from fungi are shown in Table (1). The viscosity of fungal chitosan was 3.1-5.8, centipoises (cP), considerably lower than the viscosity of chitosan. The degree of deacetylation of fungal chitosan was ranged between 83-95%. The Molecular weight was differed with products ranging from $2.1x10^4$ to $1.9x10^5$ Da.

Pot experiments:

The success of used materials in eliminating the development and reproduction of *Meloidogyne incognita* has reflected positively on both inhibiting and growth of banana cv. Williams. Data presented in Table (2) show that application all treatments of various molecular weights of fungal chitosan or a comparable nematicide (Nemacur 10%G) gave significant reduction in number of galls, immature stages and egg-masses in banana roots. The same finding was noticed on the count of nematode juveniles in soil and total population of nematodes as well as the rate of nematode build-up compared with those of control (untreated-infected plants).

Table 2. Effect of different molecular weights of fungal chitosan on banana plants infected with *Meloidogyne incognita*

Treatment (Mws of chitosan)	Number of galls	Number in soil	Number in root			Total*	Rate of** build-up
		Juveniles (J2s)	Immature stages	Females	Egg-masses		
CTS No. A DD-84.9% (1.9×10^5)	240.75 ab	9968 bc	112.18 a	262.23 b	217.3 ab	10559.7 cb	4.97 bc
CTS No. B DD-83% (1.4×10^5)	241.65 ab	7256 bcd	87.25 a	262.33 b	296.95 a	7902.53 bcd	3.95 bcd
CTS No. C DD-95% (2.1×10^4)	199.88 abc	6848 cd	86.95 a	169.48 b	166.5 ab	7270.93 cd	3.66 cd
Castor oil	224.23 bc	10952 b	85.25 a	200.5 b	203.05 ab	11440.8 b	5.72 b
Nemacur 10%G	120.15 abc	4564 d	31.35 b	134.43 b	118.68 b	4848.45 d	2.42 d
Nematode alone	308.65 a	22612 a	120.33 a	479.65 a	304.45 a	23516.43 a	11.76 a

Means followed by the same letter(s) within a column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

* Total population including immature stages + females + Females egg-masses + numbers of juveniles in soil.

** Rate of build-up = pf (final population /initial population) (Norton, 1978).

Generally, it could be concluded that the molecular weight of chitosan {DD-91%} was the most effective material used in reducing the population of root-knot nematode, *M. incognita* (68%) followed by the molecular weight {DD-82%} which recorded (66.41%), {DD-86%} (57.44), while castor oil (51.36%) and Nemacur 10%G (79.42) reduction (Fig. 1).

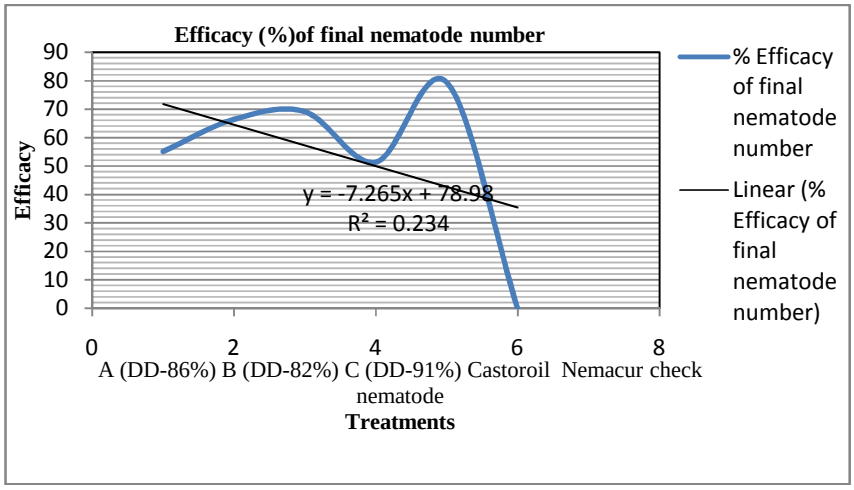

Fig. 1. Effect of various molecular weights of fungal chitosan on final nematode number of root-knot nematode infecting banana plants.

Data on growth parameters based on weights and lengths of both shoots and roots as well as weights of suckers are listed in Table (3). All treatments could improve the growth parameters of banana plants. For instance, shoot weight of those plants was recorded much higher than those of the untreated check. Consequently, the growth rates of shoots weights recorded increasing at all treatments which ranged between 101.8 and 47.02% as compared to check treated with nematode. Variable responses of root growth parameters were also detected.

Table 3. Efficacy of different molecular weights of fungal chitosan on growth of banana plants infected with root-knot nematode, *M. incognita*

Treatment (Mws of chitosan)	Shoot		Root		Sucker weight
	Weight	length	Weight	length	
CTS No. A DD-84.9% (1.9×10^5)	38.2 ab	32.25 a	22.6 b	33.5 b	14.2 ab
CTS No. B DD-83% (1.4×10^5)	32.9 ab	26.5 a	19.38 ab	24.2 ab	10.13 c
CTS No. C DD-95% (2.1×10^4)	37.68 ab	32.75 a	19.45ab	23.5 a	11.28 bc
Castor oil	32.9 ab	30.25 a	18.98 ab	25 ab	12.08 bc
Nemacur 10%G	27.9 bc	29.75 a	14.48 b	24.75 ab	10.23 bc
Nematode alone	18.93 c	25.75 a	12.8 b	21.75 a	9.25 c
Check	40.75 a	33.25 a	18.26 ab	25.25 ab	16.8 a

Means followed by the same letter(s) within a column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

In general, all treatments of the tested materials as "Nemacur 10%G" and castor oil caused remarkable increase in the plant growth criteria (Fig. 2).

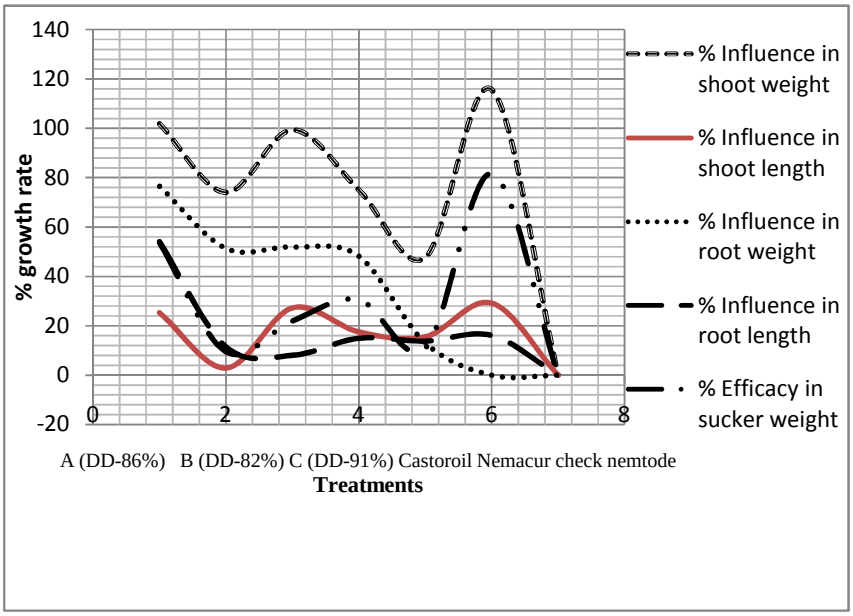

Fig. 2. Percentage of increase of growth rate of banana plants due to applications of different weights of fungal chitosan against *Meloidogyne incognita*.

D i s c u s s i o n

The physio-chemical characteristics of 3 molecular weights of chitosan ($1.9x10^9$; $1.4x10^5$ and $2.1x10^4$ Da) produced from *Aspergillus niger* were determined. The result was similar to those reported by Shimahara *et al*. (1989). Otherwise, the degree of deacetylation of different Molecular weights of fungal chitosan ranged between 83-95%. These results were slightly different from those reported percentage degree of deacetylation of chitosan from fungal mycelia of 65-95% (Shimahara *et al*., 1989 and Miyoshi *et al*., 1992). The degree of deacetylation an important parameter was affecting the physio-chemical properties of chitosan. Chitosan with a high degree of deacetylation has high positive charges and is more suitable for an microbial agent (Crestini *et al*., 1996). Also, the molecular weight was relatively lower than that of chitosan from crab shells. Thus, fungal chitosan could have potential medical and agricultural applications. In addition, chitosan with a low molecular weight was reported to reduce the tensile strength and elongation of the chitosan membrane but to increase its permeability (Rong and Horng, 1996).

Application of different molecular weights of chitosan derived from fungi for controlling the root-knot nematode (*Meloidogyne incognita*) and for improving the plant growth has been used as one of the most non harmful control methods to human, vertebrates as well as invertebrates. When, the treatments were applied with

three different molecular weights from fungal chitosan to control *M. incognita* in feeding banana plants, they exhibited antagonistic action and most of them affected development and reproduction of nematode. Total number of galls, immature stages, females, females with egg-masses, nematode count in soil, as well as the rate of nematode build-up was greatly reduced when compared with those of the untreated-infected plants. The majority of tested previously treatments have been known to possess nematicidal properties that affect on nematode life cycle. The obtained results indicated there are the suppressive effects of chitosan on root-knot nematode, *M. incognita* and consequently the increased banana plant growth. These results are in agreement with those reported by Spiegel (1986 and 1987) and Kokalis-Burelle (2002). Also, Hulimann *et al*. (1999) who found that the addition of chitin to soil at 1% (w/w) eliminated plant parasitic nematodes in a first planting of cotton "cv. Rowden" and significantly reduced *Meloidogyne incognita* infestation in a second planting, confirming long-term *nematode* suppressiveness induced by this organic amendment. In addition, Bautista-Baños *et al*. (2006), reported that chitosan, a non-toxic and biodegradable polymer of beta-1,4-glucosamine, can affect microorganisms due to its pesticide activity, but it can also induce a series of defence reactions in treated plants. So, the mode of action of chitin and chitosan in controlling plant-parasitic nematodes may be stimulated growth of bacteria, actinomycetes, and some fungal species with chitinolytic properties as antagonistic microorganisms by damage chitin-containing egg shell (Rodríguez-Kábana *et al*., 1987 and Spiegel *et al*., 1987). Also, the nematicidal activity of chitosan increased ammonia concentration due to chitin hydrolysis (Main *et al*., 1982). Our results showed that chitosan improved banana plant growth, i.e. shoot weight, shoot length and root weight were similar to those by Bell *et al*., (2000) who reported that low rates of chitin increased plant growth parameters in white clover. Chitin applied at rates of 2, 4, and 8 g/m² significantly ($p \leq 0.05$) reduced *M. incognita* reproduction and increased plant growth parameters of rapeseed, (Korayem *et al*., 2008).

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تاثير الكيتوزان الفطرى على مكافحة نيماتودا تعقد الجذور *Meloidogyne incognita* على نباتات الموز

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الكيتوزونات المستخدمة عبارة عن (عديد من الأستيل جلوكوزأمين) و تتميز بأنها مواد طبيعية مستخرجة من الفطريات. تتميز أيضا بأن لها إستخدامات عديدة من أهمها مكافحة الأفات. ولذلك فإنه تم إستخدام ثلاثة أنواع مختلفة من الأوزان الجزيئية للكيتوزونات وهى (2.1×10^4 و 1.4×10^5 و 1.9×10^5 دالتون) وتم إضافتها للتربة و مقارنتها ببعض المركبات الطبيعية مثل زيت الكافور وأيضا مقارنتها بالمبيد الكيميائى (النيماكور المحبب ١٠% جم). وقد وجد أنها حققت نسبة خفض معنوى عند نسبة 0% من نيماتودا تعقد الجذور من جنس (ميلودوجينا إنكوجنيتا) بالإضافة الى أنها تحسن من نمو نباتات الموز المستخدمة من صنف ويليامز مقارنة بالكنترول. يعتبر الوزن الجزيئى من النوع (2.1×10^4 دالتون) من الكيتوزونات المستخدمة هى أفضل الأوزان الجزيئية المستخدمة فى خفض عدد التورمات على الجذور المصابة و العدد الكلى النهائى للنيماتودا. من ناحية أخرى، فإن الكيتوزان المستخدم ذوالوزن الجزيئى (1.9×10^5 دالتون) كان أكثرهم تفوقا فى زيادة النمو الخضرى عن باقى المعاملات مقارنة بالكنترول.