

A chemical-biological control approach for nematodes on cotton

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ABSTRACT

Over three seasons various single applications or combinations of applications of nematicides, bionematicides or organic amendments were compared for their efficacy in the management of nematodes on cotton and to determine their effect on yield with the main aim of increasing the use of environmental friendly agents or organic additives and decreasing or eliminating the use of nematicides. Nematode population densities were assessed before application and at six and 12 weeks after planting. The main nematode species found in the soil prior to planting were *Meloidogyne incognita* race 4 and *Pratylenchus teres*. In the 1999/2000 season a respective 39% increase in yield was realized by the EDB (ethylene dibromide) fumigation followed by an aldicarb application and 28% with aldicarb followed by an oxamyl application when compared with the untreated control. The yield of some of the Biostart 2000® treatments was even less than that of the untreated control. The yield of the single oxamyl application six weeks after planting was higher than that of the untreated control. This effect could also be observed in the Biostart Treatment-A followed by oxamyl and PL-Plus® followed by oxamyl treatments. The reproduction of *P. teres* was once again suppressed by the high root-knot nematode incidence. In the 2001/2000 season the aldicarb followed by Biostart 2000® (six applications) resulted in the best root-knot nematode control at 12 weeks after planting and this treatment yielded a 40% increase in yield when compared with the untreated control. In the 2001/2002 season, aldicarb followed by RUM® resulted in the best root-knot nematode control with a yield increase of 35% when compared with the untreated control. The aldicarb + Biostart 2000® treatment yielded a 31% yield increase while the single aldicarb and oxamyl treatments respectively gave a 27 and 30% increase in yield.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are among the most damaging and economically important pests of subtropical and tropical crops throughout the world. *Meloidogyne incognita* is the only species that is both widespread and causes severe yield losses of cotton in South Africa (Louw, 1982). Within this species, different biological races have been identified and two of these, races 3 and 4, are known to attack cotton. Only race 4 has been found in South Africa.

Meloidogyne species became a limiting factor in cotton production in one of the irrigation schemes in South Africa as early as the nineteen seventies. A lesion nematode species, *Pratylenchus teres* Khan & Singh 1974 (Carta *et al.*, 2002), was first reported in that area in the 1995/96 season. Although the pathogenicity of this nematode species is not known, it is suspected that some of the nematode damage in the area can be attributed to this nematode species. High initial densities of *P. brachyurus* can also be a restrictive factor (Starr and Mathieson, 1985).

Since the profitability of cotton is marginal, reducing the occurrence of damaging nematode species is imperative. Aldicarb has been used on a wide scale for the control of nematodes, but due to the misuse of this chemical, certain constraints have been placed on its use. The study compared the efficacy of various chemical/fumigant nematicides, bio-nematicides and organic additives alone or in combination in increasing the use of environmental friendly agents or organic additives and decreasing or eliminating the use of nematicides in the control of nematodes on cotton.

Experimental procedure

During the 1999/2000, 2000/2001 and 2001/2002 seasons, field experiments were conducted at a trial site at Jan Kempdorp in the Northern Cape Province of South Africa, to evaluate the efficacy of nematicides and bionematicides in the management of nematodes on cotton and to determine their effect on the yield.

The fields were ploughed and disked to obtain a fine tilth before commencing with the trials. The soil at the trial site was a light sandy soil (<10% clay). The cotton cultivar NuCOTN 37B was planted in the 1999-2000 and 2001-2002 seasons. NuCOTN 35B was used in 2000-2001.

The trials were flood-irrigated when rainfall was insufficient. Normal agronomic practices were used throughout the trials. All plants were treated identically with respect to cultivation, fertilization, insect control and harvesting.

The selection of nematicides depended largely on their non-phytotoxic effect to the cotton plant and their cost. Nematicides could also aid the bionematicides by reducing the nematode numbers. The choice of bionematicides was mainly dependant on their availability as a final product and to determine whether the use of nematicides could be decreased or eliminated. Chitin was included for the possible effect that it could have on the reproduction of the *Bacillus* species, while chicken manure was included due to its possible stimulating effect on the build-up of nematode destroying fungi in the soil.

The treatments consisted of the fumigant EDB (ethylene dibromide), and the nematicides aldicarb and oxamyl, the bionematicides PL-Plus® and Biostart 2000(R)® and organic additives such as RUM®, chicken manure and chitin, which can enhance the effectiveness of the bionematicides. These treatments were compared with an untreated control. PL-Plus® contains an isolate of the fungus *Paecilomyces lilacinus*. Biostart 2000® contains three *Bacillus* spp. namely *B. chitinosporus*, *B. laterosporus*, *B. licheniformis*. These biological control products are pathological to nematode eggs. RUM® is an earthworm derivative and a typical RUM® analysis is as follows: N - 15%; P - 1.1%; K - 1.4%; S - 96 mg/R; Ca - 44 mg/R; Mg - 110 mg/R; Na - 110 mg/R; Cu - 0.1 mg/R; Zn - 0.23 mg/R; Mn - 0.49 mg/R; Fe - 2.6 mg/R; Bo - 0.16 mg/R. RUM® also contains some biological agents.

The different treatments, dosages and time of application for the three seasons are indicated in Tables 1, 3 and 5.

The EDB plots were fumigated 14 days prior to planting by means of a soil fumigant injector gun. The applications were 30 cm apart and 30 cm deep. The injection holes were sealed immediately afterwards. The correct amounts of chitin and aldicarb were measured out for each 5 m row and manually applied with a hand shaker. They were applied in a 40 cm band over the row after planting but prior to closing the planting holes. The oxamyl EC was applied with a knapsack sprayer fitted with a hollow-cone nozzle. The mixture needed for each plot was mixed in the spray tank and applied as a banded application. All the Biostart® treatments were also applied with a knapsack sprayer as a banded application next to the stems. The chicken manure was broadcasted and incorporated into the soil before planting. Seeds were coated with RUM® liquid formulation before planting. The remainder of the RUM® treatments was applied with a knapsack sprayer. The PL-Plus® applications took place as follows. For each plot the appropriate concentration of the fungus, *P. lilacinus*, with the growth medium was prepared according to the application levels. The spore concentration was kept in suspension during application. The mixture was applied evenly over the row with a watering can.

The experiments comprised a randomized complete block design with 10 treatments, replicated five times. The plots consisted of six rows, each row being five meters long. Seeds were spaced at 0.20 meter intervals in the row. The four center rows were used as the data rows to eliminate any side effects.

Soil was sampled for nematodes by taking 20 cores, 30 cm deep from the four data rows of each plot. The soil samples were combined and thoroughly mixed. Nematode assessments were done on a representative sample from each plot. The nematode population densities were assessed before applying the

chemicals and at six and 12 weeks after planting. Nematodes were extracted from 250 ml of soil from each plot according to the procedure of Jenkins (1964).

To determine nematode root infestation, four randomly selected plants from each plot were sampled at six and 12 weeks after planting. Ten grams were excised from the roots in each plot for counting of nematodes. Nematodes were extracted from the excised roots by centrifugal sugar flotation (Coolen and D'Herde, 1972).

The nematode data were \log_e (number of nematodes+1) transformed for analysis. Data were analyzed using Genstat 5 and were subjected to an analysis of variance. Means were compared by Tukey's Multiple Range test ($P \leq 0.05$).

Results

The main nematode species found in the soil prior to planting were the root-knot nematode, *M. incognita* race 4, and the lesion nematode, *P. teres*. Various ectoparasitic nematode species were also present at this trial site. The root-knot and lesion nematodes were evenly distributed in the soil of the various plots before any of the treatments were applied (Tables 2, 4 and 6).

1999/2000 season

There was a significant decrease in the total *M. incognita* race 4 population in the roots six weeks after planting where plots were fumigated with EDB + aldicarb (EA) two weeks prior to planting. This treatment had significantly lower root-knot nematode numbers when compared with the untreated control (C) and the Biostart® treatment-B (B2) (Table 2). Twelve weeks after planting the total root-knot nematode population was significantly lower in the EDB + aldicarb treatment (EA) compared with the single oxamyl treatment-A applied six weeks after planting. At that stage the total root-knot nematode population was approximately 60% less in the EDB + aldicarb (EA) treatment when compared with the rest of the treatments with the exception of the aldicarb + oxamyl treatment (AO). The lowest number of lesion nematodes was found in roots of the aldicarb + oxamyl treatment. Reproduction of the lesion nematode species was suppressed by the high root-knot nematode incidence (Table 2). The EDB + aldicarb (EA), PL-Plus® + oxamyl (PO) and aldicarb + oxamyl (AO) treatments realized a significant higher yield when compared with the untreated control (C), the Biostart Treatment-A (B1) and the Biostart® Treatment-B + oxamyl (B2O) treatments. The yields of the Biostart® (B1) and Biostart® + oxamyl (B2O) treatments were even lower than that of the untreated control (C). The single oxamyl application (O) 6 weeks after planting was not able to control the root-knot nematodes. The yield of this treatment, however, was higher than that of the untreated control. This effect could also be observed in the Biostart® Treatment-A followed by oxamyl

(B1O) and the PL-Plus® followed by oxamyl (PO) treatments (Table 2).

2000/2001 season

At the 6-week sampling, there was a significant decrease in the total *M. incognita* race 4 population in the roots of plants on plots where aldicarb had been applied at planting (AB1 and AP). The *P. teres* population in the roots was also significantly lower in some of the aldicarb treated plots (AB2, AP) (Table 4). At twelve weeks the total root-knot nematode population was significantly lower in the aldicarb + Biostart® (AB2) treatment. At that stage, the total root-knot nematode population in the EDB + aldicarb (EA) and EDB + Biostart® (EB1) treatments was also 50% less compared with the chitin + Biostart® (CB1) and Biostart® (B2) treatments. There was also a significant decrease in the lesion nematode numbers in the EDB + aldicarb (EA) and aldicarb + PL-Plus® (AP) treatments compared to chitin + Biostart® Treatment-A (CB1) (Table 4). The aldicarb followed by Biostart® (AB2) treatment gave the best root-knot nematode control at the 12-week sampling period and this treatment gave a 39.7% increase in yield when compared with the untreated control (Table 4).

2001/2002 season

At 6 weeks, the aldicarb followed by RUM® (RA) and chicken manure followed by aldicarb (CmA) resulted in the best root-knot nematode control (Table 6). A significant amount of rain created havoc with the nematode population densities during the rest of the season. High root-knot numbers were present in the RUM® and chicken manure (Cm) treatments at the 12 week sampling period (Table 6). The aldicarb followed by RUM® (RA) resulted in the best root-knot nematode control with a yield increase of 35.3% when compared with the untreated control. The aldicarb + Biostart® (AB1) treatment yielded a 31.2% yield increase while the single aldicarb (A) and oxamyl (O) treatments respectively gave a 26.9 and 30% increase in yield. The use of RUM® added an additional 8.4% to the yield in the aldicarb + RUM® (RA) treatment (Table 6).

Discussion

1999/2000 season

Lesion nematodes normally have an effect on the feeding of a sedentary endoparasite such as the root-knot nematode depending on reproduction rate and time of penetration (Eisenback and Griffin, 1987). The root-knot nematode pressure, however, had an effect on the reproduction rate and time of penetration of the lesion nematode species at the start of the season.

Trials in Mississippi, USA resulted in a similar high reduction in root-knot nematode numbers in the aldicarb and oxamyl treatments (Lawrence *et al.*, 1997). The effectiveness of *B. chitinosporus* on the eggs of the

root-knot nematode, *M. javanica*, as demonstrated by means of electron microscope studies (SEM) by Dr. L.R. Tiedt of the Potchefstroom University for Christian Higher Education (Tiedt date, 1999) was not observed under natural conditions. It is also known that *B. laterosporus* can stimulate plant growth and root development. However, under natural conditions, where the above-mentioned bacteria have to compete with other organisms in the soil, it is necessary to modify the rhizosphere to make it more accessible for the establishment of the added micro-organisms. Although the single oxamyl treatment could not control the nematodes, the yield was higher than that of the untreated control and this could possibly be attributed to the stimulation effect of this nematicide on plant growth. Both aldicarb and oxamyl stimulate plant growth in the presence or absence of nematodes (Barker and Powell, 1988; Ragab, 1981; Reddy *et al.*, 1990a, b).

2000/2001 season

The previous season, the results of the Biostart 2000® treatment were disappointing and a it was decided to investigate methods to modify the rhizosphere to make it more accessible for the establishment of added micro-organisms (Stirling, 1988). Such methods included the application of chemicals, organic additives or other soil treatments. Where Biostart® (Treatment-A) was preceded by an organic additive, such as chitin, there was no advantage. The chitin was not applied for its effectiveness against the nematodes, but to determine whether it could aid the reproduction of *B. chitinosporus* after it had been added to the soil. A chemical such as aldicarb can possibly be advantageous to a biological control agent such as Biostart® 2000®. A yield increase of between 18.5 and 31.7% was realized when Biostart treatment (A or B) or PL-Plus® was preceded by an aldicarb treatment. In the 99/2000 season, the initial *M. incognita* race 4 population was more than 60% higher than the *P. teres* population. This season, however, more than 90% of the plots had a higher *P. teres* population. This is probably the reason why the aldicarb-treated plots performed better than the EDB-treated plots. The low root-knot nematode numbers at the beginning of the season were advantageous to the reproduction rate and time of penetration of the lesion nematode, *P. teres*.

2001/2002 season

PL-Plus® contains an isolate of the fungus *P. lilacinus*. This fungus disrupts layers of the nematode eggshell and the eggshell can no longer protect the developing nematode juveniles. The fungus eventually destroys the egg content. *B. chitinosporus* is one of the bacteria present in Biostart 2000®. Secretions of this *Bacillus* sp. cause the egg wall structure to change and it becomes opaque, which prohibits the hatching of nematodes, resulting in their death. The eggs then disintegrate and are broken down by the bacteria. During this time, the bacterium, *B. chitinosporus*, breaks down the chitin in the egg wall fragments.

None of the biological control agents or organic additives performed effectively on their own and can, only constitute part of an integrated management program for nematodes. They can, therefore, not act as substitutes for aldicarb. Some of the biological control products will also be difficult to apply. When biological control agents are applied to the soil, there is competition for substrate or nutrient supply and an interaction between microbial communities. The indigenous micro flora must, therefore, be decreased for the biological control agent to get a head start. The nematode population will also have to be decreased by using a nematicide. Most of the aldicarb combinations are currently those that give the best nematode control and highest yields. A substitute must, however, be found for aldicarb in order to develop a more environmentally friendly approach to nematode control.

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Table 1. Experimental treatments for 1999/2000.

Treatment***	Abbreviation	Dosage	Area treated	Time of application
1 Untreated control	C	-	-	-
2 EDB + Aldicarb	EA	30 liter/ha 10 kg/ha	Broadcast Row	2 weeks before planting 6 weeks after planting
3 Aldicarb + Oxamyl EC	AO	10 kg/ha 3,75 liter/ha	Row Row	At planting 6 weeks after planting
4 Oxamyl EC (O)	O	3,75 liter/ha	Row	6 weeks after planting
5 Biostart 2000 ¹ [Tr. A]	B1	15 ml Biostart + 15 ml activator	Row	*
6 Biostart 2000 (Tr. A) + Oxamyl EC	B1O	15 ml Biostart + 15 ml activator 3,75 liter/ha	Row Row	* 6 weeks after planting
7 Biostart 2000 [Tr. B]	B2	15 ml Biostart + 15 ml activator	Row	**
8 Biostart 2000 [Tr. B] + Oxamyl EC	B2O	15 ml Biostart + 15 ml activator 3,75 liter/ha	Row Row	** 6 weeks after planting
9 PL-Plus ²	P	3 kg/ha	Row	At planting
10 PL-Plus + Oxamyl EC	PO	3 kg/ha 3,75 liter/ha	Row Row	At planting 6 weeks after planting

* Biostart 2000 (Treatment-A) was applied after plant emergence, 2, 6 and 10 weeks after planting.

** Biostart 2000 (Treatment-B) was applied after plant emergence and 2, 6, 10, 14 and 18 weeks after planting.

*** Tr.A = Treatment-A; Tr. B = Treatment-B.

Table 2. Density of *Meloidogyne* and *Pratylenchus* populations in the soil before application and in the roots six and twelve weeks after planting as well as the yield (kg/ha) for the various treatments (1999/2000).

Nematode*	Treatments**										LSD _T
	C	EA	AO	O	B1	B1O	B2	B2O	P	PO	
2 weeks before planting											
Mel	1.079	0.738	0.358	0.416	0.139	0.497	0.904	0.513	0.139	0.000	NS
Prat	0.659	0.693	0.555	1.039	0.879	0.970	0.636	0.555	0.277	1.214	NS
6 weeks after planting											
TotMel	6.83b	2.97a	5.11ab	4.49ab	5.83ab	5.16ab	6.02b	5.60ab	5.11ab	5.24ab	3.05
Prat	0.86	1.83	0.28	0.97	1.24	1.41	0.57	1.98	1.68	2.02	NS
12 weeks after planting											
Totmel	3.75ab	1.40a	2.02ab	4.79b	3.60ab	4.11ab	3.90ab	3.88ab	4.10ab	4.01ab	3.04
Prat	2.35	1.63	1.21	1.73	2.32	1.50	2.34	2.56	1.68	2.49	NS
YIELD	2173ab	3589d	3024bcd	2635abcd	1761a	2432abcd	2318abcd	1892a	2286abc	3112cd	938

* Mel = *Meloidogyne*; Prat = *Pratylenchus*; TotMel = Total *Meloidogyne*.

** C = Untreated control; EA = EDB + Aldicarb; AO = Aldicarb + Oxamyl; O = Oxamyl; B1 = Biostart 2000® Treatment-A; B1O = Biostart 2000® Treatment-A + Oxamyl; B2 = Biostart 2000® Treatment-B; B2O = Biostart 2000® Treatment-B + Oxamyl; P = PL-Plus®; PO = PL-Plus® + Oxamyl.

Values within rows followed by the same letter are not significantly different according to Tukey's Multiple Range test

Table 3. Experimental treatments 2000/2001.

	Treatment***	Abbreviation	Dosage	Area treated	Time of application
1	Untreated control	C	-	-	-
2	EDB + Aldicarb	EA	30 liter/ha 10 kg/ha	Broadcast Row	2 weeks before planting 6 weeks after planting
3	Chitin + Biostart 2000® (Tr. A)	ChB1	100 kg/ha 15 ml Biostart + 15 ml activator	Row Row	At planting *
4	EDB + Biostart 2000® (Tr. A)	EB1	30 liter/ha 15 ml Biostart + 15 ml activator	Broadcast Row	2 weeks before planting *
5	Biostart 2000® (Tr. A)	B1	15 ml Biostart + 15 ml activator	Row	*
6	Aldicarb + Biostart 2000® (Tr. A)	AB1	10 kg/ha 15 ml Biostart + 15 ml activator	Row Row	At planting *
7	Biostart 2000® (Tr. B)	B2	15 ml Biostart + 15 ml activator	Row	**
8	Aldicarb + Biostart 2000® (Tr. B)	AB2	10 kg/ha 15 ml Biostart + 15 ml activator	Row Row	Planting **
9	PL-Plus®	P	3 kg/ha	Row	After plant emergence and 6 weeks after planting
10	Aldicarb + PL-Plus®	AP	10 kg/ha 3 kg/ha	Row Row	At planting At plant emergence and 6 weeks after planting

* Biostart 2000® (Treatment-A) was applied after plant emergence, 2, 6 and 10 weeks after planting.

** Biostart 2000® (Treatment-B) was applied after plant emergence and 2, 6, 10, 14 and 18 weeks after planting. ***Tr.A =Treatment-A; Tr. B = Treatment-B.

Table 4. The density of *Meloidogyne* and *Pratylenchus* populations in the soil before application and in the roots six and twelve weeks after planting as well as the yield (kg/ha) for the various treatments (2000/2001).

Nematode*	Treatments**										LSD _T
	C	EA	ChB1	EB1	B1	AB1	B2	AB2	P	AP	
2 weeks before planting											
Mel	1.39	1.25	0.96	1.55	0.42	0.42	0.68	0.75	1.51	1.23	NS
Prat	0.876	1.991	1.271	1.245	1.214	1.626	1.235	1.368	1.235	1.617	NS
6 weeks after planting											
Totmel	2.69b	2.13ab	1.61ab	1.57ab	2.39ab	0.00a	2.81b	1.22ab	2.95b	0.39ab	2.57
Prat	2.17abcd	3.2cd	3.35cd	1.01ab	3.12bcd	1.22abc	3.02bcd	0.51a	3.73d	0.77a	2.18
12 weeks after planting											
Totmel	3.36ab	1.65ab	4.12b	1.98ab	3.59ab	3.33ab	4.13b	0.98a	3.14ab	2.64ab	2.83
Prat	2.41abc	0.69a	3.58c	3.11bc	2.17abc	1.68abc	1.62abc	1.47abc	2.01abc	1.08ab	2.24
YIELD	2464a	3574bcd	2964abc	2712a	2968abc	3643bcd	2886ab	4089d	2559a	3746cd	847.88

* Mel = *Meloidogyne*; Prat = *Pratylenchus*; Totmel = Total *Meloidogyne*.

** C = Untreated control; EA = EDB + Aldicarb; ChB1 = Chitin + Biostart 2000® Treatment-A; EB1 = EDB + Biostart 2000® Treatment-A; B1 = Biostart 2000® Treatment-A; AB1 = Aldicarb + Biostart 2000® Treatment-A; AB2 = Aldicarb + Biostart 2000® Treatment-B; P = PL-Plus®; AP = Aldicarb + PL-Plus®.

Values within rows followed by the same letter are not significantly different according to Tukey's Multiple Range test

Table 5. Treatments 2001/2002.

	Treatment	Abbreviation	Dosage	Area treated	Time of application
1	Untreated control	C	-	-	-
2	Aldicarb	A	10 kg/ha	Row	At planting
3	Biostart 2000®	B1	15 ml Biostart + 15 ml Activator	Row	*
4	Oxamyl	O	3,75 liter/ha	Row	6 weeks after planting
5	RUM®	R		Row	**
6	Aldicarb + Biostart 2000®	AB1	10 kg/ha 15 ml Biostart + 15 ml Activator	Row Row	At planting *
7	Aldicarb + Oxamyl	AO	10 kg/ha 3,75 liter/ha	Row Row	At planting
8	RUM® + Aldicarb	RA	10 kg/ha	Row	** At planting
9	Chicken manure	Cm	2000 kg/ha	Broadcast	At planting
10	Aldicarb + Chicken manure	CmA	10 kg/ha 2000 kg/ha	Row Broadcast	At planting At planting

* Biostart 2000® was applied after plant emergence and 2, 6 and 10 weeks after planting

** Seed treatment – 4 ml/kg

3 l/ha - At planting; 4 l/ha - 4 weeks after planting; 4 l/ha - 8 weeks after planting; 4 l/ha - 12 weeks after planting

Table 6. The density of the *Meloidogyne* and *Pratylenchus* population in the soil before application and roots six and twelve weeks after planting as well as the yield (kg/ha) for the various treatments (2001/2002).

Nematode*	Treatments**										
	C	A	B1	O	R	AB1	AO	RA	Cm	CmA	LSD _T
2 weeks before planting											
Ecto	2.57	2.75	2.12	2.42	2.93	1.9	2.18	2.59	2.74	2.34	NS
Mel	0.863	0.774	0.599	0.636	0.886	0.497	0.921	0.578	1.133	0.599	NS
Prat	1.19	1.813	1.524	1.397	1.38	1.485	2.127	1.316	1.619	1.669	NS
6 weeks after planting											
Totmel	2.02	0.5	0.86	2.15	1.52	0.29	0.55	0.00	0.72	0.00	NS
Prat	2.94b	1.64ab	1.27ab	2.66ab	1.70ab	1.36ab	0.00a	0.5ab	0.39ab	0.62ab	2.80
12 weeks after planting											
Totmel	1.12abc	1.47abc	2.96abc	1.90abc	3.56c	2.51abc	0.91ab	0.68a	3.5bc	2.18abc	2.59
Prat	1.06	0.55	0.22	0.00	1.33	0.58	0.44	0.58	2.06	1.18	NS
YIELD	1691a	2315abc	1989abc	2414bc	1766ab	2459bc	2295abc	2612c	1997abc	2411bc	715.52

* Mel = *Meloidogyne*; Prat = *Pratylenchus*; TotMel = Total *Meloidogyne*.

** C = Untreated control; A = Aldicarb; B1 = Biostart 2000® Treatment-A; O = Oxamyl; R = RUM®; AB1 = Aldicarb + Biostart 2000® Treatment-A; AO = Aldicarb + Oxamyl; RA = RUM® + Aldicarb; Cm = Chicken manure; CmA = Chicken manure + aldicarb.

Values within rows followed by the same letter are not significantly different according to Tukey's Multiple Range test.