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Efficiency of neem oil nanoformulations to *Bemisia tabaci* (GENN.) Biotype B (Hemiptera: Aleyrodidae)

Eficiência de nanoformulações a base de óleo de nim sobre *Bemisia tabaci* (GENN.) Biótipo B (Hemiptera: Aleyrodidae)

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Abstract

The nanotechnology, through encapsulation of active ingredients, has showed an important way to avoid problems with quickly degradation of the pesticide molecules. Thus, neem (*Azadirachta indica*) oil nanoformulations containing β-ciclodextrin and poli-ε-caprolactone (PCL) were tested as to their control efficiency against eggs and nymphs of *Bemisia tabaci* (Genn.) biotype B reared in soybean. The Lethal Concentration (LC_{50}) was estimated using a commercial neem oil (Organic Neem®) on first-instar nymphs to establish the adequate volume of the nanoformulations per treatment. After that, they were sprayed on eggs and first-instar nymphs in laboratory and greenhouse and on third-instar nymphs in greenhouse. The commercial neem oil and distilled water were used as controls. Egg viability was not affected by any treatment. Among six nanoformulations, only one was efficient against the first-instar nymphs in laboratory conditions. However, its effective period was not increased as expected. In greenhouse, first-instar nymphs were more affected by two nanoformulations which were significantly different of the commercial neem oil – the most effective one. No mortality differences among the formulations in the third-instar test were observed. The nanoformulations were less efficient to control the *B. tabaci* biotype B nymphs than the commercial neem oil.
Key words: Whitefly, botanical pesticide, nanotechnology, β-ciclodextrin, poli-ε-caprolactone

Resumo

A nanotecnologia, através do encapsulamento de ingredientes ativos, tem-se revelado uma importante estratégia para evitar problemas com a rápida degradação de moléculas inseticidas. Assim, nanoformulações à base de óleo de nim (*Azadirachta indica*) utilizando os polímeros β-ciclodextrina e poli-ε-caprolactona (PCL) foram testadas quanto a sua eficiência de controle de ovos e ninfas de *Bemisia tabaci* (Genn.) biótipo B mantidas em soja. Foi estimada a CL_{50} utilizando uma formulação comecial de óleo de nim (Organic Neem®) sobre ninfas em 1º ínstar da qual se estipulou o volume das nanoformulações a serem utilizadas por tratamento. Depois disso, os tratamentos foram aplicados sobre ovos e ninfas de 1º ínstar em laboratório e em casa de vegetação e sobre ninfas de 3º ínstar em casa de vegetação. O óleo comercial e água destilada foram utilizados como controles. A viabilidade dos ovos não foi afetada por qualquer dos tratamentos. Das seis formulações testadas, somente uma delas mostrou-se eficiente para ninfas de 1º ínstar em condições de laboratório, embora não tenha apresentado aumento

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do período residual como esperado. Em casa de vegetação, ninfas de 1º ínstar foram mais afetadas por duas das formulações, que diferenciaram do óleo de nim que causou maior controle. No teste com ninfas de 3º ínstar, não houve diferenciação de mortalidade entre as formulações. As nanoformulações foram menos eficientes para controlar ninfas de *B. tabaci* biótipo B do que o óleo de nim comercial.
Palavras-chave: mosca-branca, inseticida botânico, nanotecnologia, β-ciclodextrina, poli-ε-caprolactona

Introduction

Azadirachta indica A. Juss (Meliaceae), the most studied plant with insecticide properties due to its effects against several insects, contains azadirachtin, the most efficient substance, which provokes feeding inhibition, disruption of immature development, lower fecundity and fertility of adults, behavior alterations, cell anomalies and eggs, larvae and adults mortality (SCHMUTTERER, 1990; MORDUE; BLACKWELL, 1993; MARTINEZ, 2002; BOEKE et al., 2004). However, the its exact mechanism of action remains unknown or uncertain (GENTZ; MURDOCH; KING, 2010).

Nonetheless, that molecule shows low stability in field conditions due mainly to photodegradation (STOKES; REDFERN, 1982); as a result, repeated sprays are necessary in a short period (SCHMUTTERER, 1990). Thus, new neem formulations with higher photostability, increasing period between sprays, are necessary in order to increase their use in agriculture.

New techniques aiming to improve the performance of pesticides are being used by chemical industries supported by the knowledge in nanotechnology to encapsulate the molecules of active ingredient. Mattoso, Medeiros and Martin Neto (2005) report some applications of nanotechnology in agriculture such as enhancing the efficiency of chemical and natural pesticides, decreasing their problems with photodegradation, more security on handling those products for reducing toxicity risks to mammals as well as a controlled release of active ingredients. However, it is important to consider the possible toxicity on animals, humans or plants of nanoparticles and nanocapsuls before using them extensively, but this depends on factors as the materials used and the size and the enhancements added (PEREZ-DE-LUQUE; RUBIALES, 2009).

The encapsulant agent, β-cyclodextrin, is used in pharmaceutical (BARROS; STRINGHETA, 2006), food (LINDNER; SZENTE; SZEJTLI, 1981; BOBBIO; BOBBIO, 2001; DRUNKLER; FETT; LUIZ, 2001) and pesticide industry (SZEJTLI, 1998; ROMI et al., 2005). It is a cyclic polymer which contains seven unities of glucose joined in α-1,4 obtained through enzymatic degradation of the amylum (BOBBIO; BOBBIO, 2001). Another biodegradable polymer, poly-ε-caprolactone (PCL), has been studied by researchers not only in releasing systems of pharmaceutical drugs (SCHAFFAZICK et al., 2006) but also in civil building industry (ALMEIDA; FERREIRA, 2006). PCL is an aliphatic polyester obtained by polymerization of ε-caprolactone in the presence of aluminum isopropoxide (CHIELLINI; SOLARO, 1996).

Among the insects that seeks to control with application of botanical extracts, is the whitefly *Bemisia tabaci* (Genn.) biotype B, it has caused great socio-economic losses to many crops due to its aggressiveness. Virus can be transmitted to plants by the feeding of infected adults and cause damages like productivity reduction and / or commercialization annulment.

Therefore, the aim of this work was to evaluate the efficiency of neem oil nanoformulations using as encapsulant agents the polymers β-cyclodextrine and poly-ε-caprolactone to control *B. tabaci* biotype B.

Material and Methods

The population of whiteflies was acquired from the Campinas Institute Research (Instituto Agronômico de Campinas, BR) which were confirmed as biotype B by Dr. Judith K. Brown, University of Arizona, EUA. The insects were reared on soybean plants, cultivar IAC 18, in a greenhouse.

Letal concentration 50 (LC_{50}) of commercial neem oil

The assay was conducted on 'IAC 18' soybean when the first trifoliate leaf was completely developed. Soybean was grown in greenhouse in plastic pots (0.5 L) which were infested in order to obtain eggs for the assay. Twenty adults of *B. tabaci* per plant were kept 24 hours for oviposition being the first trifoliate leaf wrapped in a cylindrical leaf cages (15 cm in length and 6 cm in diameter) made of voile.

Eleven days after the infestation, ten first-instar nymphs were selected in a trifoliate leaf and a spot close to them was marked with a permanent pen. Afterwards, the treatments were sprayed on leaves using different concentrations of commercial neem oil (Organic Neem® Dalquim Indústria e Comércio). The level of azadirachtin concentration in the commercial oil, 384.3 mg/L, was determined using the HPLC-MS/MS method according Forim et al. (2010).

The concentrations tested were: 0.48, 0.96, 1.92, 2.73, 3.84 and 5 mg of azadirachtin/L in 50 ml of solution plus a control treatment (distillate water). These concentrations were based on preliminary tests. The sprays were applied with a small hand held pump directed at the underside of the infested leaves with a flow of 3.96 L/h and work pressure of 2.85 psi. Each concentration was replicated ten times, and the experiment was carried out in laboratory conditions (23±2°C, 65±10% relative humidity and 14h photophase).

Mortality was evaluated at two, four, six and ten days after the treatments using a stereoscope microscope to count the dead insects. Nymphs were considered dead when they showed a small size (approximately 0.25 x 0.15 mm), elliptic shape similar to first-instar nymphs according to Eichelkraut and Cardona (1989) and Patel et al. (1992), and dark coloration (personal observation). The total mortality was considered to estimate the LC_{50} through Probit analysis.

Efficiency of nanoformulations on nymphs

The azadirachtin quantity equivalent to LC_{50} was used as a base to calculate the volume of each nanoformulation. Six nanoformulations (Table 1) were tested, and distillate water and commercial neem oil were used as controls. The phenological stage of the plants, infestation technique, selection of nymphs and spraying technique were all made as described on LC_{50} assay.

Two assays with first-instar nymphs, the first in laboratory and the second in greenhouse with plastic cover, were done with the purpose of comparing whether solar radiation may interfere on releasing efficiency of insecticide molecule by the polymers.

A third assay was made using third-instar nymphs in greenhouse for studying the control efficiency in the same concentrations but using older individuals.

In the laboratory assay (25±2°C, 60±10% relative humidity and 14h photophase), the treatments were applied onto leaves with nymphs ten days after the infestation. Each treatment was replicated 10 times being each experimental unit a leaf with 10 nymphs. The evaluations were made at two, four, eight and ten days after the spraying.

The greenhouse assay with first-instar nymphs had a wide range of temperature and humidity (from 15°C to 45°C and from 10% to 50% relative humidity) and 13h natural photophase. Treatments were applied at ninth day after infestation, but the nanoformulation 6 was not tested due to its

similarity to nanoformulation 5 which contained only *a* β-cyclodextrin unlike nanoformulation 6 which contained *a* and *b* β-cyclodextrin. Another reason was its low efficiency on the first-instar nymphs in the laboratory assay. Number of repetitions and evaluations were equal to assay described previously.

Table 1. Neem oil nanoformulations tested on eggs and on 1st and 3rd instar nymphs of *Bemisia tabaci* biotype B.

Nanoformulations	Composition
Nanoformulation 1	powder oil extract (0.1 mg) neem oil (0.5 g) β-Cyclodextrin solution (3.0 ml) (*a*) tensoactive Renex 40 (0.08 g)
Nanoformulation 2	powder oil extract (0.1 mg) neem oil (0.5 g) β-Cyclodextrin solution (3.0 ml) (*b*) tensoactive Renex 40 (0.08 g)
Nanoformulation 3	NC[a] – 59 (nanoprecipitation method)
Nanoformulation 4	NC – 60 (emulsion solvent diffusion-evaporation method)
Nanoformulation 5	NC – 60 (0.3 g) β-Cyclodextrin solution (5.0 ml) (*a*)
Nanoformulation 6	NC – 60 (0,3 g) β-Cyclodextrin solution (5.0 ml) (*ab*)

Characteristics	Azadirachtin level (mg/Kg)	Particles diameter (ηm) – solution	Polymer	Drying support
Powder oil extract	5,554.0			silicon dioxide
Neem oil	4,900.0			
NC – 59	2,809.0	Aprox. 4 µm (dried)	PCL[b]	silicon dioxide
NC – 60	2,569.6	Aprox. 4 µm (dried)	PCL	silicon dioxide
β-Cyclodextrin solution (*a*)		83.2	β-Cyclodextrin	
β-Cyclodextrin solution (*b*)		3.8	β-Cyclodextrin	

aNC: suspension colloidal of nanocapsules.
bPCL: poly-(ϵ-caprolactone)In the third-instar nymphs assay, the infestation was in laboratory conditions during eight hours with 30 adults of *B. tabaci* biotype B per plant. Afterwards, nymphs were kept in laboratory and observed every day to be identified when they reached the third instar to spray the treatments. After spraying, the plants were kept in greenhouse and the evaluations were at two, four, six and eight days after spraying. The environmental conditions were: 15°C to 51°C temperature, 10% to 30% relative humidity and 12h natural photophase. The assay had seven treatments replicated ten times and each repetition had 10 nymphs.
Source: Elaboration of the authors.

Efficiency of nanoformulations on eggs

To obtain the eggs for that assay, the infestation technique was the same as the assay with first-instar nymphs in greenhouse. After removing of the adults, ten eggs per leaf were selected and the others were crushed, and after that, the nanoformulations and controls were applied. The evaluations of egg hatching and nymphal mortality were made at 12, 15, 18 and 21 days after the spray. The assay as carried out in greenhouse and its environmental conditions were: 12°C to 48°C temperature, 10% to 30% relative humidity and 12h natural photophase. Each treatment was replicated ten times.

Statistics

All assays had a randomized design being used the nonparametric test Kruskal-Wallis (KRUSKAL; WALLIS, 1952) for the data because it not showed normal and homogeneous. For the LC_{50} test the data was submitted to Probit analysis (FINNEY, 1971). The software R (R DEVELOPMENT CORE TEAM, 2008) was used to Kruskal-Wallis analysis and to LC_{50} test was used the software POLO-PC (LEORA SOFTWARE, 1987).

Results

Lethal concentration 50 (LC_{50}) of commercial neem oil

The value of LC_{50} estimated through Probit analysis was 1.6mg/L of azadirachtin for first-instar whitefly nymphs (Slope ± SE: 2.892 ± 0.199; LC_{50} 1.6 mg/L (1.35-1.75); X^2: 1.764; GL: 3).

Efficiency of nanoformulations on nymphs

In the laboratory assay with first-instar nymphs (Table 2), the mortality on the control treatment with distilled water was low showing that assay conditions were adequate. Neem oil provoked the highest mortality not only in each evaluation but also on the total mortality (60%). The higher mortality was reached until the eighth day and decreased after that period.

Despite being statistically equal to nanoformulations 1, 3, 5 and 6, the nanoformulation 2 was the only one that equaled neem oil, but its efficiency was almost half of the neem oil. That nanoformulation also showed some efficiency throughout time and decreasing during the assay. On the other hand, the nanoformulation 5 in spite of showing low values of mortality had a trend of increasing its efficiency throughout assay.

The results obtained with first-instar nymphs in greenhouse (Table 3) were similar to laboratory assays. In this assay, the highest mortality per day and during the total period with occurred with neem oil spraying (60% total mortality).

Table 2. Mortality of first-instar nymphs of *B. tabaci* biotype B in response to application of neem oil and neem oil nanoformulations in laboratory conditions (25±2°C, 60±10% RH and 14h photophase).

	Nymphal mortality %									
Treatment	1-2 days[a,b]		3-4 days[a,b]		5-8 days[a,b]		9-10 days[a,b]		Total[a,b]	
Water	0.00	(325)a	0.00	(320)a	1.00	(305)a	1.00	(361.5)ab	2.00	(269)a
nano 1	1.00	(361)ab	5.00	(443)a	2.00	(340)a	1.00	(361.5)ab	9.00	(408.5)ab
nano 2	10.00	(596,5)bc	5.00	(439)a	7.00	(437)a	6.00	(486)ab	28.00	(561)bc
nano 3	0.00	(325)a	0.00	(320)a	6.00	(433)a	2.00	(369)ab	6.00	(344)ab
nano 4	0.00	(325)a	0.00	(320)a	2.00	(317)a	0.00	(325)a	2.00	(252,5)a
nano 5	1.00	(361)ab	1.00	(356)a	2.00	(340)a	4.00	(413)ab	8.00	(332)ab
nano 6	0.00	(325)a	1.00	(356)a	4.00	(387)a	0.00	(325)a	5.00	(333.5)ab
Oil	15.00	(621.5)c	16.00	(686)b	19.00	(682)b	10.00	(599)b	60.00	(739.5)c
X^2	42.39		39.24		27.85		23.66		41.85	
P	0.001		0.001		0.001		0.001		0.001	

[a]Values followed by the same letter in a column are not significantly different (Kruskal Wallis test, $p<0.05$).
[b]Values in parentheses are ranks sum.
Source: Elaboration of the authors.

Table 3. Mortality of first-instar nymphs of *B. tabaci* biotype B in response to application of neem oil and neem oil nanoformulations in greenhouse conditions (15 to 45°C, 10 to 50% RH and 13h natural photophase).

	Nymphal mortality %											
Treatment	1-2 days[a,b]		3-4 days[a,b]		5-6 days[a,b]		7-8 days[a,b]		9-10 days[a,b]		Total[a,b]	
Water	0.00	(295)a	1.10	(309)b	1.00	(207.5)a	0.00	(270)a	0,00	(280)a	2,10	(163,5)a
Nano 1	1.00	(325)b	0.00	(275)a	3.00	(262.5)b	4.44	(427)c	3,00	(373,5)a	12,30	(348,5)c
Nano 2	0.00	(295)a	1.90	(333)bcd	9.00	(381.5)bc	4.00	(368)b	4,00	(378,5)a	18,90	(401)d
Nano 3	1.10	(326,5)b	2.10	(339,5)cd	4.10	(301)c	0.00	(270)a	3,30	(352,5)a	10,60	(343)c
Nano 4	1.22	(332,5)b	0.00	(315)bc	4.44	(339)d	0.00	(340.5)b	0,00	(319)a	5,67	(271)b
Nano 5	0.00	(373)c	0.00	(357)d	7.63	(445.5)e	0.00	(353)b	1,25	(392)a	8,88	(380)d
Oil	17.78	(538)d	14.44	(557)e	15.56	(548)f	8.89	(456.5)d	3,33	(389,5)a	60,00	(578)e
X^2	31.16		29.22		20.64		17.07		6,44		26,68	
P	0.001		0.001		0.002		0.009		0,37		0,001	

[a]Values followed by the same letter in a column are not significantly different (Kruskal Wallis test, $p < 0.05$).
[b]Values in parentheses are ranks sum.
Source: Elaboration of the authors.

The nanoformulation 2 also provoked one of the highest mortalities. The nanoformulation 5 provoked the highest mortality on the sixth day after spraying; however, the progressive performance on the laboratory assay by this nanoformulation was not observed in the greenhouse assay.

The spraying on third-instar nymphs (Table 4) did not show difference of mortality among the treatments except on the fourth day after spraying when there was a high mortality by neem oil (26.10%). However, considering the total mortality, the most values of mortality occurred on sprayed plants with nanoformulation 1 and neem oil.

Table 4. Mortality of third-instar nymphs of *B. tabaci* biotype B in response to application of neem oil and neem oil nanoformulations in greenhouse conditions (15 to 51°C Temp., 10 to 30% RH and 12h natural photophase).

	Nymphal mortality %									
Treatment	1-2 days[a,b]		3-4 days[a,b]		5-6 days[a,b]		7-8 days[a,b]		Total[a,b]	
Water	0.00	(315)a	2.00	(290)a	3.50	(314.5)a	0.00	(341,5)a	5.00	(246.5)a
Nano 1	2.00	(385)a	4.60	(341)a	7.00	(392.5)a	2.10	(372)a	15.70	(456.5)bc
Nano 2	1.00	(350)a	5.10	(389)a	4.10	(347)a	0.00	(305)a	10.20	(304.5)ab
Nano 3	1.00	(350)a	1.00	(260)a	4.10	(347)a	4.10	(410)a	10.20	(304.5)ab
Nano 4	1.00	(350)a	2.00	(290)a	6.00	(417)a	0.00	(305)a	9.00	(311.5)ab
Nano 5	1.00	(350)a	3.00	(320)a	2.00	(281)a	1.00	(337)a	7.00	(269.5)a
Oil	2.00	(385)a	26.10	(595)b	7.00	(386)a	5.00	(414)a	40.10	(592)c
X^2	2.78		25.88		4.70		8.06		23.98	
P	0.84		0.001		0.58		0.23		0.001	

[a]Values followed by the same letter in a column are not significantly different (Kruskal Wallis test, $p < 0.05$).
[b]Values in parentheses are ranks sum.
Source: Elaboration of the authors.

Efficiency on eggs

The eggs viability was not affected by both nanoformulations and neem oil. The nanoformuation 5 differed both nanoformulations 2,3 and 6, and control treatments water and neem oil, provoking highest nymphal mortality at 18 days after spraying, but this difference among the treatments did not occur at 21 days after spraying. The total nymphal mortality caused by the nanoformulations was similar to that of neem oil, being the nanoformulation 2 and 5 as well as neem oil significantly differents from the control treatment water (Table 5).

Table 5. Eggs viability and nymphal mortality of *B. tabaci* biotype B in response to application of neem oil and neem oil nanoformulations in greenhouse conditions (12 to 48°C Temp., 10 to 30% RH and 12h natural photophase).

Treatment	Eggs viability (%)	Nymphal mortality %		
		1-18 days[a,b]	19-21 days[a,b]	Total[a,b]
Water	100.00	2.00 (284) a	1.00 (342) a	3 (234) a
Nano 1	98.00	4.00 (348) ab	3.11 (423.5) a	7.11 (355) ab
Nano 2	98.00	12.44 (515.5) a	1.00 (342) a	13.44 (474.5) b
Nano 3	94.00	6.11 (322) a	3.11 (423.5) a	9.22 (325) ab
Nano 4	98.00	8.00 (360) ab	5.00 (464) a	13.00 (432.5) ab
Nano 5	95.09	15.40 (581.5) b	0.00 (335) a	15.40 (505.5) b
Nano 6	99.00	5.86 (366) a	2.11 (386.5) a	7.97 (361.5) ab
Oil	99.09	9.82 (462.5) a	8.91 (553.5) a	18.73 (552) b
X^2	7.9	16.79	14.34	15.69
P	0.34	0.02	0.04	0.03
DMS	-	238.53	238.53	238.53

[a]Values followed by the same letter in a column are not significantly different (Kruskal Wallis test, $p < 0.05$).
[b]Values in parentheses are ranks sum.
Source: Elaboration of the authors.

Discussion

Usually the azadirachtin level in oils containing neem derivates is not showed on papers. Thus, to compare LC_{50} of different papers is not correct because azadirachin is known by its high instability (SCHMUTTERER, 1990; DUREJA; JOHNSON, 2000; BARREK; PAISSE; GRENIER-LOUSTALOT, 2004; CABONI et al., 2006).

About the efficiency on first-instar nymphs, we observed that laboratory and greenhouse assays had similar results. Thus, greenhouse conditions had not a high interference on the performance of the nanoformulations, even the plastic cover and the sides have allowed partial incidence of sunlight. However, both, solar radiation and the high temperature may accelerate the degradation of the polymers PCL and β-cyclodextrin releasing the active ingredient more quickly. It is known that ultraviolet radiation acts as degradation agent of polymeric materials due to its association with photodegradation mechanism (ALMEIDA; FERREIRA, 2006). Probably, the rupture of the polymer and the exposition of the active ingredient occurred gradually.

According to Chandra and Rustgi (1998), the temperature plays an important role on the polymers degradation which improves its rate when there is an increment on the temperature. Lotto et al. (2004) reported that temperature provoked the highest degradation of the PCL among the factors evaluated.

The lower mortality found in a few treatments on the third-instar nymphs assay, mainly in neem oil, compared with first-instar nymphs assays can be due to the fact that a higher quantity of active ingredient should be necessary to kill older nymphs. Pinheiro et al. (2009) proved that LC_{50} value of neem oil to *B. tabaci* nymphs was altered due to tested instar being that the concentration 0.5% (2 x the LC_{50} value to first-instar nymphs), caused only 33.8 % of mortality in third-instar nymphs.

In the egg assay, we noticed dead nymphs, soon after their hatching, stuck on the egg corium. Prabhaker, Toscano and Henneberry (1999) observed high mortality of neonate nymphs supposing that the contact of the nymphs with residuals toxic composts sprayed onto the corium could cause their mortality. Nonetheless, azadirachtin provokes phagodeterrence besides growth regulator and sterility promoter (BUTTERWORTH; MORGAN, 1968). Thus, when nymphs come in contact with toxic compound (azadirachtin) on the corium they will die during their ecdysis since the metamorphosis period is strongly affected since that molecule causes many morphogenetics defects as well as mortality suggesting that penetration of the azadirachtin into the corium could occur which would affect the nymphal viability (SCHMUTTERER, 1990).

Conclusion

In general, the nanoformulations assessed under the conditions presented in this work did not provoke higher mortality and residual effect than did neem oil. Thus, further studies are necessary to improve the releasing rate of de azadirachtin by the polymers.

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