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IOP Conf. Series: Journal of Physics: Conf. Series **1323** (2019) 012017 doi:10.1088/1742-6596/1323/1/012017

Ecotoxicity evaluation: preparation of poly-Ɛ-caprolactone and chitosan nanoparticles as carriers of thiamethoxam pesticide

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Abstract. The transmission of Huanglongbing (HLB) disease on citrus plants is through dissemination of the bacteria *Candidatus* Liberibacter ssp, by *Diaphorina citri* psyllid, its insect vector. Chemical control of the psyllids, and thiamethoxam (neonicotinoid insecticide) is one of the active ingredients used in the control of HLB. This insecticide is water soluble, unstable and rapidly degraded by photolysis. Pesticide nanoformulation is one of the strategies to control release of active compound as well as protection for premature degradation. Thus, studies of the effectiveness of encapsulated pesticide formulations are extremely important for enabling its use in agriculture. This study reports the encapsulation of the insecticide thiamethoxam in polymeric particles from poly-ε-caprolactone (PCL) and chitosan by double emulsion and solvent evaporation method using different concentrations of chitosan and two Pluronic (poloxamer) copolymers, F 127 and F68. These nanoparticles were characterized in terms of size, zeta potential, polydispersity, and encapsulation efficiency. The encapsulation efficiency, measured by liquid chromatography was 34%. The nanoparticles obtained from optimized conditions resulted in homogeneous and monodisperse particles with a positive superficial charge. The microalgae *Raphidocelis subcapitata* (bioindicator chloroficea) and microcrustacean *Artemia salina*, were used to evaluate the ecotoxicity of nanopesticide in comparison to pesticide already in the market. The ecotoxicity study demonstrated that nanopesticide was less toxic that commercial formulations in the studied conditions.

1.Introduction

Huanglongbing (HLB) is probably the most serious disease of citrus plants. It's directly related to significant economic losses affecting the worldwide citrus industry. Affect all commercial cultivars, resulting in major reductions in fruit quality, yield and lifespan of infected trees [1,2].

There are quite a few factors that contribute to rapid dissemination of this disease, but the most important are the ways of transmission and HLB detection on field. The transmission of disease can occurred during the grafting process through the use of infected buds or through dissemination of the bacteria *Candidatus* Liberibacter ssp, by *Diaphorina citri* psyllid, its insect vector. The absence of genetic resistance in citrus and besides that there is no treatment, HLB has became the most destructive citrus disease [3-6]. In Brazil, according to a survey by Fundecitrus (2018), the incidence of HLB in orange trees, in São Paulo and Minas Gerais states is of 18.15%, that corresponds to 35.3 million trees. The increase over the incidence disease from 2017 is cause for concern Brazilian agroindustry.

The control of this disease has been done from two different ways: removal all contaminated plants and the chemical control of the psyllid vector. Unfortunately both of them are not enough to avoid the dissemination of this disease and minimize the economic impact [7,8].

Nowadays, the HLB detection on field is mainly done by visual inspection of the late stages symptoms, and afterwards, the infection is then confirmed by polymerase chain reaction method (PCR) [9]. As the disease is detected in late stages, a lot of plants can be contaminated, due to it is in the asymptomatic stages of the infection, resulting in significant economic losses [10]. On the other hand, the vector control by insecticides is associated to increase of environmental impact, development of vectors biological resistance, due to its excessive use [11,12].

After the HLB introduction in citrus orchads, the use de pesticides increased a lot. It arises from increasing the production costs, environmental impact and could rise to serious risks for the health and safety of consumers, due to the concentration of pesticide residues over the limit set by legislation.

The use of nanotechnology in agriculture has created a great interest, offering the potential for significantly enhanced agricultural productivity and efficiency with lower cost and less waste [13,14]. It has been used for the development of sensors to detect diseases, as well as in controlled releasing systems for nutrients and agrochemicals. The reduced size and high surface area of nanoparticles, can improve the effectiveness, solubility, permeability and stability of agrochemical [15,16,17] Thiamethoxam is a neonicotinoid insecticide, extensively used for crop protection against a broad spectrum of chewing and sucking pests and for a variety of other purposes such as: seed and pet treatment [18,19].

This study reports the encapsulation of the insecticide thiamethoxam in polymeric particles from poly-ε-caprolactone (PCL) and chitosan by double emulsion and solvent evaporation method using different concentrations of chitosan and two different triblock surfactant poloxamer, P123 and F68, in order to optimize the preparation conditions [20]. PCL is a cheaper biodegradable polymer, GRAS, slow degradation rates and non-toxic [21]. Chitosan is a biopolymer used for the encapsulation of bioactive compound, due to different properties [22]. These nanoparticles were characterized and their ecotoxicity were studied.

2. Material and methods

2.1. Preparation of polymeric nanoparticles: PCL-chitosan loaded thiamethoxam pesticide

The polymeric nanoparticles were prepared using the modified double-emulsion-solvent evaporation method [20]. The oil phase was formed by 100 mg of poly-ε-caprolactone, (PCL 10.000 g/moL, Aldrich), dissolved in 10 mL ethyl acetate. An aqueous solution of thiamethoxam (2 mL, 4 mg/mL), and the same volume of Pluronic (F68 or P123) aqueous solution (1.5% w/v) were first emulsified with the oil phase under ultrasonication for 3 min. The resultant emulsion was transferred to a vessel containing 40 mL of Pluronic aqueous solution (1.5% w/v) and 10 mL of chitosan solution (1 to 2 mg/mL)(low molecular weight, ~ 81% acetylation, Polymar-Brazil), and mixed by sonication during 10 min (w/o/w). The solvent was eliminated by evaporation at 40°C in a route evaporator (120 rpm), and the volume was reduced to 10 mL.

2.2. Characterization of nanoparticles

The size distribution of the bulk nanoparticles, polydispersity index values and zeta potential were determined by the Dynamic Light Scattering (DLS) in a ZetaSizer Nano Series (Malvern Instruments). Samples were diluted in MilliQ ® Water (1:10 v/v) and analyzed in triplicate, at 25 °C.

2.3. Evaluation of encapsulation efficiency

Pesticide encapsulation efficiency was expressed as the percent of the total pesticide added that is encapsulated in nanoparticles. The amount of pesticide in nanoparticles was calculated by the difference between the total amount of pesticide added and the amount of unbound pesticide remaining in the aqueous supernatant[23]. The concentration of thiamethoxan in the supernatant

was obtained by the suspension ultrafiltration/centrifugation procedure, using 500 µl of nanoparticles through a Microcon ®Ultra centrifugal filters (Millipore), regenerated cellulose, 10kDa. The system was centrifuge at 14.000 rpm, at 25 °C, during 40 min, and the filtrate was analyzed using a high liquid chromatograph and UV-vis detector, Shimadzu LC10 AD.

The chromatographic separation was performed on a reversed-phase analytical column Lichrosorb RP- 18 (250 mm x 4.6 mm, 5µ), supplied by Phenomenex mobile phase (A) MilliQ® Water and (B) Acetonitrile, gradient elution mode, started 70 % (A), increased to 80% (A) in 20 min, maintained for 2 min, and back to the initial equilibrium. The detector wavelength (λ) was set at 254 nm and flow rate of 0.6 mL/ min. The chromatographic peak at 6,7 minutes was attributed to thiamethoxam. External standards were utilized in its quantification[24].

2.4. Test organism and toxicity assessment

For evaluation of toxicity assessment, two bioindicator organisms were used, the microalgae *Raphidocelis subcapitata.*. Microcustacean *Artemia salina*, which effectively responds to the insecticidal action of several compounds [25].

2.4.1. Microalgae (Raphidocelis subcapitata) bioassay

The algae culture were maintained according to OECD (1984), under controlled conditions of temperature (20 ± 2ºC), light intensity (~ 1300 lux) and continuous agitation (100 rpm). The algal suspension were distributes in 96-well plates, initial concentration ~10^5 cells/mL and total test suspension volume of 300 µL per well. A total of 10 replicates were prepared for each test condition. Algae suspensions were exposed to the concentrations of the active ingredient equals to 0.06;0.6;6;60;600 and 1200 mg/L, prepared in the same medium of algae culture. Algal growth was monitored by absorbance readings (λ 750 nm, microplate reader, Sunrise Tecan Group Ltd.) of the suspensions every 24 h for 72 h. The specific growth rate and the concentration that inhibited 50% of it (EC50-72h) were determined.
[26].

2.4.2. Microcrustacean (Artenia salina) bioassay

Approximately 24 h before the test, 900 ml of synthetic seawater were placed in a 1 L Erlenmeyer. This water was prepared by adding 30 g of salt “Sera Premium®” (Sera GmbH, Heinsberg) in 1000mL of water (pH = 7.2; conductivity = 110_Scm−1) from an artesian well.
Approximately 50 mg of Artemia cysts (INVE Aquaculture Inc., Ogden) in synthetic seawater, were kept for 48h under intense aeration through a porous stone at a temperature of 25±1 ◦C and ~6300 lux brightness Brine shrimp nauplii were obtained. A total of 10 nauplii were exposed to the test-solution at concentrations 10.00, 16.00, 25.60, 40.96, 65.53 and 104.85 mg/L prepared in the same saline solution, during 48 h at 20±2 ◦C. At the end exposure time, the number of organisms was recorded and the and the concentration that affects mobility in 50% of the population (EC50-48 h) along with its 95% confidence interval were determined [27,28].

3. Results and Discussion

In this study, nano-encapsulation of thiamethoxam insecticide by modified double emulsion method (water in oil in water) and solvent evaporation was obtained. Through the nanotechnology approach, were obtained positive and bioadhesive nanoparticles. A major difficulty encountered was the encapsulation efficiency of this insecticide. Thiamethoxam has a hydrophilic nature (water solubility 4 mg/L, log P = -0.13). Its solubility in water leads to a great diffusion of the molecule to the external phase before the precipitation of the polymer, thus reducing encapsulation efficiency. According Prado et al. [20], the natural polymer chitosan improves the encapsulation of hydrophilic molecules, in polymeric nanoparticles. On the other hand, the results obtained from Mazzarino et al. [29] in the curcumin encapsulation by nanoprecipitation method, show that the size and surface charge of the nanoparticle suspensions were dependent of chitosan concentration. In previous studies performed by Quemeneur et al. [30] have demonstrated that the molar mass of this natural polymer have no influence on zeta

potential of nanoparticle. In order to improve the encapsulation efficiency of thiamethoxam, two different concentrations of chitosan were available, with a same poloxamer (F68). Chitosan concentration of 2 mg/L resulted in greater encapsulation efficiency (36.7%) and it was selected to further studies about the effect of poloxamer.

The poloxamer used in this study were Pluronic -P123 (PEO_{20}-PPO_{65}-PEO_{20}) and Pluronic F68, actually, Kolliphor P188 (PEO_{80}-PPO_{27}-PEO_{80}). Basically, the copolymer were composed by two hydrophilic poly(ethyleneoxide) PEO e one hydrophobic poly(propyleneoxide) PPO regions. The triblock copolymer chains bound the nanoparticle surface through hydrophobic interactions with PPO block. The table 1, shows the mean particle size, polydispersity index, zeta potential and encapsulation efficiency for nanoparticles obtained from both poloxamer studied.

Table 1. Effect of Pluronic P123 and F 68 on particle size, zeta potential, polydispersity index and Encapsulation Efficiency

Properties	F68	P123
PDI	0.278±0.004	0.529±0.040
Size (nm)	313.5 ± 7	492.7 ± 18
Zeta (mV)	+38.1± 1.9	+33.5±0.9
EE (%)	36.6±0.2	32.6±0.7

The F68 – nanoparticles was found better than P123 nanoparticles, considering homogeneity of system, size and encapsulation efficiency, as showed in Table 1. The longer hydrophobic blocks in P123 copolymer and smaller hydrophilic block can be decreased the interaction between chitosan, and possibly, with thiamethoxam insecticide [29].

One important detail of the preparation approach is related to the pH of nanoparticle suspension since, due to the pKa of Chitosan (pKa =6.5), is expected that pH variation can be result in instability of the system. Berni et al. [31] demonstrated in their studies, that higher amounts of chitosan cause agglomeration above pH 8, but they were stable up to pH 6. On the other hand, low pH and high inorganic salt concentration may cause irreversible damage to plant. Thus, chitosan was prepared using acetic acid (0.2%) and after magnetic agitation (24 h), the pH was adjusted to 5.5, and thus used to prepare the nanoparticles.

The values of the zeta potential were positive due to the presence of the cationic chitosan polymer. The positive charge droplets might be expected to attract to their surface for electrostatic interaction between plant leaf and nanoformulations, since the surface of organisms is typically negatively charged besides that provide information concerning the stability of systems, zeta potential greater than ± 30 mv are considered to be stable in suspension considering only the electrostatic interactions [32,33].

The better result found to encapsulation efficiency was of the Pluronic F68 and chitosan solution of 2 mg/L approach (+36.6±0.2 mV). Similar results were obtained by Grillo et al. [34] for polymeric nanoparticle loaded ametryn pesticide and polyhydroxybutyrate (PHB) polymer. Grillo et al. [35] showed that the encapsulation efficiency of the herbicide atrazine in hydroxyvalerate copolymer (PHBV) was higher than 30%. The fraction of the inseticide unassociated with the polymeric nanoparticles, can be provide the initial eradication pest and subsequent control by slower release from the nanoparticles, that is desirable for agricultural applications [32].

The ecotoxicity was evaluated only with the F68 nanoparticles and chitosan solution of 2 mg /mL using microalgae, bioindicator chloroficea *Raphidocelis subcapitata* (cell growth rate 72 h) and microcrustacean *Artemia salina* (mobility 48 h) in comparison with pesticide already marketed. The table 2 shows the EC50 values for each organism and solution -test pest evaluated.

Table 2. EC50 values for each organism and solution -test pest evaluated.

Pesticide (solution test)	**Organism Test**	**EC 50 (mg/L)**
Nanoparticles (without thiamethoxam)	*R. subcapitata*	94.26 (22.42 – 166.10)
Nanoparticles (with thiamethoxam)		56.15 (-18.91 – 131.21)
Pesticide marketed (thiamethoxam)		42.67 (76.28 – 94.65)
Nanoparticles (without thiamethoxam)	*A. salina*	>100
Nanoparticles (with thiamethoxam)		>100
Pesticide marketed (thiamethoxam		>100

Table 2 shows the EC50 values for each organism and parameter evaluated. Considering the values obtained for EC50 to *R. subcapitata* the pesticide already marketed is more toxic than nanopesticide. For *A. salina* no toxicity was founded (EC 50>100 mg/L). For these organisms the EC50 was indicated as > 100 mg/ L, the highest concentration tested and recommended by the OECD protocol [36]. These data categorize the test material as practically without toxic effects for these [37].

4. Conclusion

The nanopesticide obtained from optimized preparation conditions resulted in homogeneous and monodisperse particles with a positive superficial charge. The Pluronic F68 is more adequated than P123, considering size, PDI and encapsulation efficiency. The high chitosan concentration used in the nanoparticle preparation resulted in increasing inseticide encapsulation efficiency. The toxicity results demonstrated that nanopesticide was less toxic that commercial formulations for *R. subcapitata* and no toxic for *A. salina* in the studied conditions.

5. References

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Acknowledgement
Thanks to FAPESP for supporting.